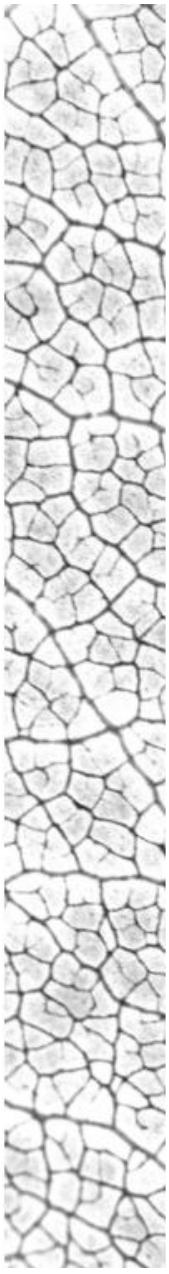

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Investigating monoclonal antibodies against the recombinant extracellular domain of the human PD-1 receptor

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Abstract
Monoclonal antibodies are used to block control points of the tumor development of many oncological pathologies. One of the critical control points of tumor development of several oncological pathologies is the receptor for programmed cell death (PD-1) and its ligands. Monoclonal antibodies against the PD-1 receptor are laboratory-derived humanized antibodies. An essential step in the humanization of antibodies is the production of murine hybrid cells producing monoclonal antibodies. This article describes studies of mice monoclonal antibodies against a recombinant human PD-1 receptor (rPD-1) expressed in *Escherichia coli*. To obtain strains of hybrid cells producing monoclonal antibodies, BALB/c mice were immunized with rPD-1 protein. B-lymphocytes of immunized mice were hybridized with the myeloma cell line of mice. Clones of hybrid cells were identified for productive activity and cloned by limiting dilution method. The properties of monoclonal antibodies were studied by enzyme-linked immunosorbent assay, Western blot and agar gel immunodiffusion. As a result, a hybrid cells producing monoclonal antibodies to a fragment of rPD-1 were obtained. Strains of hybrid cells have high productive activity in vitro and in vivo. Monoclonal antibodies react with rPD-1 protein, belong to the class of IgG1, and have a high binding constant. They efficiently bind to the PD-1 receptor and block the interaction of rPD-1 with the ligand. These monoclonal antibodies to rPD-1 can be used to obtain recombinant humanized monoclonal antibodies to the human PD-1 receptor.

Keywords: monoclonal antibodies, PD-1 receptor, oncology, recombinant protein

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INTRODUCTION

Currently, monoclonal antibodies (mAbs) against the programmed cell death receptor 1 (PD-1) are of great importance for immunotherapy aimed at blocking control points of tumor development. This receptor expresses thymocytes, peripheral CD4 + and CD8 + T-lymphocytes, B-lymphocytes, monocytes, T-killers (NK T-cells), and some dendritic cells (DC). The constant expression of PD-1 leads to decreasing of T cells and the inability of cells to secrete cytokines such as interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (Kim et al. 2015). The receptor is a negative regulator of the immune system and plays an essential role in the balance of protective immunity and immunopathology, homeostasis and tolerance. However, in chronic infections and malignancies, the expression of the PD1 receptor limits the protective immunity against these pathologies. In this regard, mAbs against PD1 receptor are the most effective tools in treating various tumors (Mamalis et al. 2014, Khoja et al. 2015, Zou et al. 2016, Sharpe and Pauken 2018). Since 2011, mAbs Ipilyumab has taken a stable position in the pharmaceutical market of the world. In 2014, two more drugs based on mAbs against PD-1 Pembrolizumab and Nivolumab became commercially available.

Studies have shown that the use of mAbs in 20% of patients with skin melanoma increased their immune response by several years. Chemotherapy for this disease, unlike mAbs, was significantly less effective (Leach et al. 1996, Hodi et al. 2010, Robert et al. 2015, Postow et al. 2015). At the first stage of clinical studies, the effect of pembrolizumab on patients with extensive metastases was studied. Authors did not find dose-

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dependent toxic effects of mAbs. Pembrolizumab has shown promising results for the fight against melanoma and lung cancer (Shimizu et al. 2016, Yamazaki et al. 2017).

All described mAbs are laboratory-derived humanized antibodies against human rPD1 (Nelson et al. 2010, Adler et al. 2017). In its structure, PD-1 is a glycoprotein whose extracellular domain contains four N-linked glycosylation sites. PD-1 analysis showed that it has two glycosylation sites N49 and N74, which play an essential role in stable expression of PD-1 on the cell surface and ligand binding (Okada et al. 2017). Several reports describe the independence of mAbs binding on the nature of PD-1 glycosylation (Tan et al. 2017, Chen et al. 2019, Liu et al. 2019). However, studies demonstrating the binding of nivolumab to glycosylation PD-1 imply the effect of glycosylation changes on the interaction with mAbs (Tan et al. 2017). Therefore, the study of the effect of PD-1 receptor glycosylation on antibody binding in the preparation of new mAbs variants is highly relevant.

The use of mAbs for cancer immunotherapy control points is an essential study for the Republic of Kazakhstan in connection with an increase in malignant melanoma among the population. Here we report a study of murine mAbs against a recombinant non-glycosylated fragment of PD-1 receptor expressed in *Escherichia coli*. The process of obtaining and characterizing the recombinant extracellular domain of the human PD-1 receptor has been described in previous studies.

MATERIALS AND METHODS

Animals, cells and antigens

A total of twenty BALB/c mice (6-8-week old) and one hundred and fifty outbred mice were used in this study. All laboratory mice were housed in cages with free access to water and to standard food chow and subjected to a 12 h light/dark cycle with a constant temperature (22-24°C). X-63 myeloma cell line was growing in RPMI 1640 (Sigma-Aldrich, USA) medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM Glutamax (Sigma-Aldrich, USA). Anti-Mouse IgG1 and IgG2a Subclass Specific Antibodies (Jackson ImmunoResearch Inc). Recombinant human PD1 protein (Abcam) (ab174035), anti-Mouse IgG (whole molecule) – Peroxidase antibody produced in goat (Sigma-Aldrich, A4416) and 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for enzyme-linked immunosorbent assay (ELISA) (Sigma-Aldrich, T0440) were used in this study.

Production of mAbs

BALB/c mice were injected intraperitoneally with 100 mg of recombinant PD1 (rPD-1) antigen with 0.1 mL of incomplete Freund's adjuvant (Gibco, USA) in phosphate-buffered saline (PBS), pH 7.2-7.4. The following immunizations were performed on days 7, 11, 12, 13, and 14. The antibody titer was evaluated using an enzyme-linked immunosorbent assay (ELISA) in a series of two dilutions of the serum of each animal, starting at 1:200. Cell hybridization was carried out according to the method of Oi and Herzenberg (1980). Cloning of hybridoma cultured cells was performed by the limiting dilution method described by Coding (1980).

To produce a preparative amount of antibodies, hybridoma cells were injected intraperitoneally into BALB/c mice. After the formation of an ascites tumor, ascites fluid was collected and separated from the hybrid cells by centrifugation at 150 × g for 10 min. MAbs were purified from ascites fluid by salting out with ammonium sulfate to 50% saturation. The formed precipitate was centrifuged at 3,000 × g for 30 min at 4°C. The antibody pellet was resuspended in a minimal volume of PBS (pH 7.2) and dialyzed against PBS during the day. Antibodies from the resulting solution were purified using HiTrap Protein A HP (GE Healthcare Life Sciences).

Binding affinity of mAbs

The method of Beatty et al. (1987) determined the binding affinity of mAbs. The recombinant protein was immobilized in three rows of a 96-well plate with the optimal concentration (10 µg/mL) and three rows of wells with a protein concentration two times lower than the optimal concentration (5 µg/mL) in 0.05 M bicarbonate buffer, pH of 9.6 and incubated at 4°C for 12 h. After washing the plate and blocking with 1% bovine serum albumin (BSA), double dilutions of mAbs were introduced into the wells, starting from 10 µg/mL, and incubated at 37°C for 1 h. After washing the plate, an antispecific conjugate was added at a dilution of 1:1000 and incubated at 37°C for 1 h. To develop the reaction, a TMB substrate was used, followed by the addition of a stop reagent. The optical density was measured at a wavelength of 450 nm. Based on the obtained data, graphs were constructed and the binding constant was determined.

The value of Kaff was calculated using equation (1).

$$Kaff = \frac{1}{4Ab' - 2Ab} \quad \text{..... (1)}$$

Where

Ab' – concentration of antibodies at 50% optical density of the reaction with rPD-1 in concentration 5 µg/mL;

Ab - concentration of antibodies at 50% optical density of the reaction with rPD-1 in concentration 10 µg/L.

Agar gel immunodiffusion

The class and subclass of mAbs were determined using agar gel immunodiffusion according to the Ouchterlony (1958) method. Melted 1% agar (Difco, USA) was poured onto the surface of defatted glass slides located on a strictly horizontal surface until a layer 1.5 mm thick was formed. After solidification of the agar using a unique stencil and punch, holes with a diameter

of 2-3 mm were cut out. mAbs in amount of 0.05 mL were introduced into the central well, and 0.05 mL of anti-Mouse IgG1 and anti-Mouse IgG2a Subclass Specific Antibodies (Jackson ImmunoResearch Inc) were added to the rest. Petri dish with slides was incubated in a humid chamber for at least 24 h at room temperature (RT).

The reaction results were taken into account by the presence of precipitation bands between the wells with the tested mAbs and monospecific sera. To contrast the precipitation bands, the preparation was stained with Coomassie G-250 solution for 10-15 min. A dye solution was prepared as follows: 5 g of Coomassie was dissolved in 1000 mL of a solvent consisting of 450 mL of distilled water, 450 mL of ethanol and 100 mL of glacial acetic acid. After staining the gel, the background was bleached using a distaining buffer.

ELISA

Solutions of recombinant human PD-1 protein (Abcam) (5μg/mL) in bicarbonate buffer (pH 9.6) were immobilized at 4°C for 16 h. The plate was washed by 0.15 M PBS with Tween 20 (PBS-Tw) (pH 7.4). After washing the plate, 1% BSA was added and incubated for 1 h at 37°C. Then, serial dilutions of mAbs beginning with 1:100 were introduced into wells. The plates were incubated as above and Anti-Mouse IgG (whole molecule)-Peroxidase antibodies produced in goat (Sigma-Aldrich) diluted 1:40000 were added to the wells. The incubation was performed as previously. To develop the reaction, a substrate of 3.3′,5.5′-tetramethylbenzidine (TMB) was added and incubated at RT. The reaction was then stopped by the addition of 2 M sulfuric acid. The intensity of the reaction product was measured at 450 nm.

Western blot

Recombinant protein was analyzed on a 12% SDS-PAGE by Laemmli (1970). Immunochemical properties of the rPD-1 were analyzed in Western blot by Towbin (1979) method.

The rPD-1 was transferred to a nitrocellulose membrane which was incubated in a 1% BSA at 4°C, 16 h. Then the membrane was incubated with 1:1000 mAbs for 1.5 h at RT. After a washing step with PBS-Tw the membrane was incubated with antispecies peroxidase-conjugate for 1 h at 37°C. After washing procedure protein detection was visualized by adding 4-chloro-naphthol substrate.

RESULTS

Obtaining of hybridoma cells producing mAbs to the human PD-1 receptor

In the enzyme immunoassay, the titer of antibodies against rPD-1 in the serum of immunized mice was 1:12800. B-lymphocytes of immunized mice were used to obtain strains of hybridoma cells producing mAbs. As a result of hybridization, out of 384 seeded wells, 100 showed the growth of clones of hybridoma cells, which is 26% of clone formation. After cloning, the hybridoma cells did not change their properties for 16 passages (observation time). The productivity of hybridoma cell lines was determined using ELISA for 8 days. Hybridoma cells were seeded in 8 wells of a 24-well plate in an amount of 2×10 cells per well. From day 1 to day 8, the culture medium was selected from one well per day to determine the antibody-producing activity of hybridomas by ELISA. To determine the hybridoma productivity, a comparison of the reaction intensity in the tested row of wells with a positive control, in which the concentration of murine antibodies is known, was used. The results of the analysis of the productivity of hybridoma cells are shown in (**Fig. 1**).

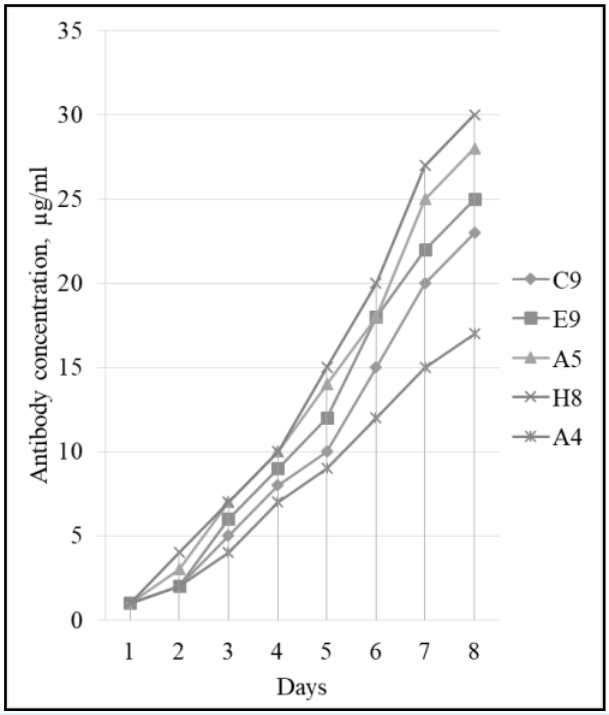

Fig. 1. Productivity curve of different hybridoma cell lines producing mAbs to rPD-1 protein in vitro. C9, E5, A5, H8, A4 – single clones

Fig. 1 shows that the concentration of antibodies in the culture fluid on the 8th day of cultivation reaches 17 - 30 μg/mL. To obtain a preparative amount of mAbs, hybrid cells were intraperitoneally administered to BALB/c mice. Antibody concentration obtained was 4 - 8 mg/mL from the total volume of ascites fluid 8 - 10 mL.

Immunochemical properties of monoclonal anti-PD-1 antibodies

Indirect ELISA was used to determine the constant affinity (Kaff) of the mAbs. Serial dilution of mAbs was loaded onto rows on a microtiter plate with rPD-1 at two concentrations - 5 and 10 μg/mL. The dilution results of five mAbs for two concentrations of PD-1 coating are shown in (**Fig. 2**).

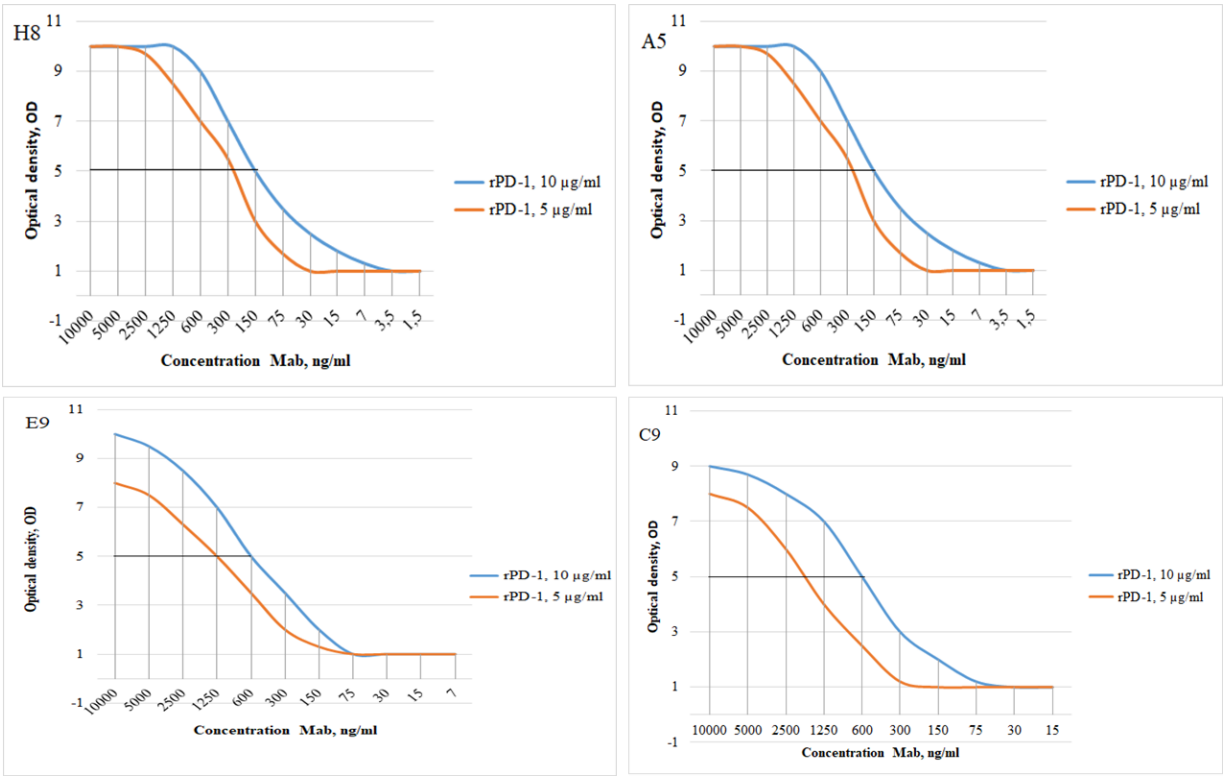

Fig. 2. Experimental ELISA curve for anti-rPD-1 mAbs at two concentrations of protein

The calculated antibody concentrations (ng/mL) at OD-50 were H8 - 300 and 150; A5 - 220 and 150; A9 - 1250 and 600; C9 - 2000 and 600. The binding constant of mAbs was determined according to formula (1). This formula determines the level of interaction of the mAbs with the antigen and is based only on the total concentration of antibodies at 50% optical density of the reaction for 96 well plates immobilized with two concentrations of antigen, 5 and 10 µg/mL. To determine the subclass of mAbs in the agar gel immunodiffusion used anti-mouse IgG1 and IgG2a subclass-specific antibodies (Jackson ImmunoResearch Inc). mAbs also were examined by ELISA with commercial recombinant human PD1 protein (Abcam) (ab174035).

The constant affinity of mAbs ranged from $2\times10^{7}M^{-1}$ to $2\times10^{8}M^{-1}$, which indicates the sufficient power binding of mAbs to this protein. The results of assessing the subclass of mAbs show that the mAbs to the rPD-1 protein synthesized by hybridoma belongs to the class G1 antibody. In the ELISA all mAbs specifically reacted with commercial recombinant human PD-1 protein (Abcam).

Specificity and sensitivity of monoclonal anti-PD-1 antibodies

The main biological characteristics of mAbs are specificity, sensitivity and affinity. The specificity demonstrates the ability of mAbs to react only with the certain protein to obtain antibody selectively. mAbs specificity was determined by Western blotting and ELISA with heterogeneous proteins. The analysis

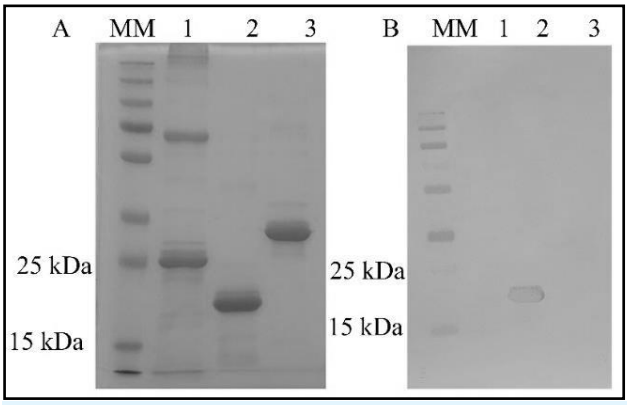

Fig. 3. Electrophoresis (A) and Western blot (B) determine the specificity of mAbs (H8) to rPD-1 protein. Line 1 - rPlyG; line 2 - rPD-1; line 3 - rHexon bAdv3; MM - molecular markers (# 26619, Thermo Scientific)

showed a specific reaction of mAbs only with rPD-1 protein, the molecular weight of which is 20 kDa (**Fig. 3**).

Sensitivity characterizes the ability of mAbs to bind to the lowest concentration of a specific protein. The rPD-1 protein was used at a concentration of 12 µg to 0.15 µg to determine the sensitivity of mAbs. As a result, mAbs in the Western blot, in the dilution at a 1:1000, reacted with 0.15 µg/mL rPD-1 protein (**Fig. 4**).

DISCUSSION

PD-1 is a receptor for various cells of the immune system, the blocking of which enhances the activity of

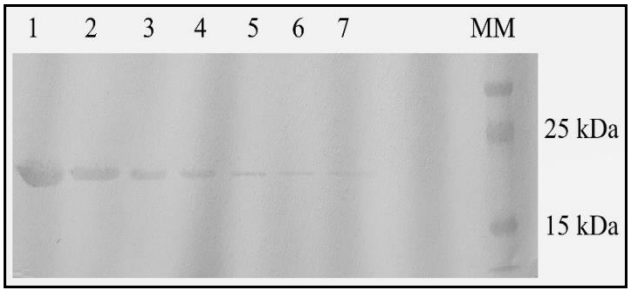

Fig. 4. Western blot for determining the sensitivity of monoclonal antibodies to rPD-1 protein. Protein concentrations: 1 - 12 μg; 2 - 6 μg; 3 - 3 μg; 4 - 1.5 μg; 5 - 0.7 μg; 6 - 0.3 μg; 7 - 0.15 μg; MM - molecular marker (# 26619, Thermo Scientific)

CD4 + and CD8 + T cells against tumor and virus-infected cells. Various human tumor cells highly expressed PD-L1 and PD-L2 receptors, which are the main PD-1 receptor ligands. The interaction of PD-1 with ligands allows tumor cells to evade the surveillance of the immune system. Therefore, PD-1 could be potential candidate in cancer treatment (Freeman et al. 2000, Latchman et al. 2001, Nomi et al. 2007, Pardoll 2012; Alemayehu, et al, 2016). mAbs against PD-1 and its ligands have been successfully used as a treatment for various tumors and occupy a stable position in the pharmaceutical market of the world. The most widelyused are three humanized anti-PD-1 mAbs: ipilimumab, pembrolizumab and nivolumab (Li et al. 2017).

mAbs can be used for treatment different types of malignant tumors. Japanese scientists have seen a positive effect in the treatment of diseases such as NSCLC, renal cell carcinoma and melanoma in 18, 27 and 28% of patients, respectively. In these patients, also was observed the formation of long-term antitumor immunity. mAbs restrained the proliferation of some malignant tumors for 20-30 months (Mandai et al. 2016).

Wang et al. (2019), conducted studies on the effect of PD-1 receptor glycosylation on binding to mAbs. For these purposes, the authors obtained murine mAbs for recombinant human PD-1 (amino acid residues 21-167) obtained from *Escherichia coli* (Wang et al. 2019). Mouse mAbs specifically bind to human PD-1 protein and inhibit the interaction of PD-1 and ligands. The mAbs obtained by the author had a binding constant of $3.55\times10^{7}M^{-1}$. Then, the authors received humanized antibodies following the humanization of frame sequences of antibodies without changing affinity and specificity. Structural analysis showed that the specificity of mAbs is associated both with interaction with loops and with the glycan, parts of PD-1. The results showed that the N-glycosylation of PD-1 does not affect the binding strength of antibodies (Wang et al. 2019).

In this work, five of hybridoma cells producing mAbs to PD-1 receptor were obtained. To get the lines of hybridoma cells, we used the rPD-1 receptor obtained from *E. coli*. Analysis of the resulting murine mAbs showed a specific reaction with the extracellular fragment of the PD-1 receptor. mAbs had a sufficient affinity ranged from $2\times10^{7}M^{-1}$ to $2\times10^{8}M^{-1}$, specificity and sensitivity to rPD-1. In our study, the resulting mAbs provides their unique specificity for PD-1 due to the interaction, which is likely to help in the development of therapeutic antibodies.

CONCLUSION

As a result of the work were obtained lines of hybridoma cells producing mAbs to a fragment of the extracellular domain of PD-1. The lines of hybridoma cells have high productivity in vitro and in vivo. mAbs react with rPD-1 protein, belong to the class of IgG1, and have a high binding constant. Obtained mAbs efficiently bind to the commercial rPD-1 protein. Characterization of mAbs rPD-1 allows you to use them to obtain recombinant humanized mAbs to human PD-1 receptor.

ACKNOWLEDGEMENTS

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