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MONOCLONAL ANTIBODIES AGAINST CTLA-4 AND PD-L1 RECEPTORS OF THE CATTLE IMMUNE SYSTEM

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Abstract

With the progression of bovine leukemia virus (BLV), the concentration of T-cells, as well as CTLA-4 and PD-1 receptors on their cytoplasmic membrane increases. Elevated concentrations of regulatory T-cells lead to increased production of transforming growth factor-β (TGF-β), suppression of interferon-γ (IFN-γ) expression, tumor necrosis factor-α (TNF-α), and inhibition of natural killer (NK) cells. Effector and cytotoxic T-lymphocytes, as well as the production of cytokines IFN-γ and TNF-α, play a crucial role in immune response against viral infections. However, at late subclinical stages, T-lymphocyte activity decreases due to the activity of regulatory T-lymphocytes, contributing to infection growth and progression to clinical disease. Blockade of CTLA-4 and PD-1/PD-L1 receptors with specific antibodies restores the immune response against BLV.

Monoclonal antibodies (mAbs) against recombinant bovine CTLA-4 and PD-L1 were obtained using hybridoma technology methods. The obtained mAbs were analyzed using enzyme-linked immunosorbent assay (ELISA) and western blotting methods.

As a result of the study, hybridoma cell lines producing mAbs to recombinant bovine CTLA-4 and PD-L1 receptors were obtained. The hybridomas produced IgG1 class mAbs that specifically reacted with standard proteins and had a binding constant: 3b3 - 2.9×108 M-1, 4h10 - 1.9×108 M-1. The obtained mAbs effectively blocked the reaction of commercial bovine CTLA-4 and PD-L1 proteins with specific polyclonal antibodies in an indirect fluorescent antibody assay (IFA).

Key words: bovine leukemia virus; CTLA-4 and PD-1 receptors; ELISA; immune system; monoclonal antibodies.

Introduction

Immunotherapy of chronic infections in cattle represents one of the alternative approaches to prevent disease spread within this population. The efficacy of checkpoint blockade-based immunotherapy has been demonstrated in studies related to bovine leukemia virus (BLV) [1]. Interest in this disease stems from the fact that BLV is a delta retrovirus belonging to the Retroviridae family. It is closely related to human T-cell leukemia viruses' types 1 and 2 (HTLV-1 and HTLV-2) and simian T-cell leukemia viruses (STLV) [2, 3]. BLV is widespread among dairy and beef cattle and manifests as a non-neoplastic disease [4, 5]. In 70% of cases, BLV infection is asymptomatic, significantly complicating control over infection spread in the animal population due to the virus's high replication rate. Approximately 30% of infected cattle develop persistent lymphocytosis, and 1-5% of the population develops malignant B-cell lymphosarcoma [6].

Disruption of the immune system function in BLV-infected cattle occurs at all stages of infection, leading to decreased productivity. BLV infection can affect both innate and adaptive immune system cells and alter the proper functioning of uninfected cells. It has been found that the PD-1/PD-L1 signaling pathway is activated during BLV infection, exerting immunoinhibitory effects [7,8]. Another immune system inhibitor is the CTLA-4 receptor, a co-stimulatory receptor of the B7 receptor family. By competing with the CD28 receptor, the CTLA-4 receptor blocks the CD28:B7 signaling pathway, thereby reducing immune activation. Additionally, the CTLA-4 receptor can disrupt the TCR and MHC signaling pathways and, by blocking the B7.1 receptor on dendritic cells, prevent the development of anti-tumor immunity [9,10,11]. On the other hand, blocking the binding of CTLA-4 and CD80 or CD86 with antibodies restores the immune response against these diseases. In several studies using recombinant bovine CTLA-4-Ig, the inhibitory immune function of bovine CTLA-4 has been demonstrated. Immunization of mice with recombinant bovine CTLA4-Ig induced the formation of anti-CTLA-4 antibodies. Administration of anti-CTLA-4 antibodies significantly increased IFN-γ production by immune system cells in healthy and leukemia-infected cows. According to the authors, antibodies against CTLA-4 may be useful for developing new therapies against BLV infection [12,1]. A similar effect was observed with PD-1/PD-L1 pathway blockade, which enhanced T-cell function and led to BLV proliferation inhibition. It has been demonstrated that reducing the concentration of PD-1+ T-cells by binding them with PD-L1 on B-cells contributes to viral infection progression. According to the authors, PD-1/PD-L1 pathway blockade has potential clinical application for enhancing host antiviral immunity in the treatment of chronic infections [13].

Thus, PD-1, PD-L1, and CTLA-4 receptors are potential targets for restoring the function of exhausted T-cells in chronic viral infections. Studies on checkpoint blockade in cattle with specific antibodies have shown increased IFN-γ production in BLV-infected cattle [1,12,13]. Watari K. et al., (2019) used recombinant bovine CTLA4-Ig to produce antibodies that blocked only the region of the CTLA-4 receptor that reacts with the B7 receptor of T lymphocytes. The aim of this study was to obtain mAbs against recombinant bovine PD-L1 and CTLA-4 receptors of the immune system and study their biochemical properties. To obtain mAbs, recombinant extracellular fragments of PD-L1 and CTLA-4 receptors carrying several epitopes, including the region reacting with the B7 receptor, were used.

Materials and methods

Ethical approval

The article was prepared within the framework of project AP14870156. To implement the project, a positive decision was received from the local ethical commission at the National Center for Biotechnology LLP (IRB 00013497).

Balb/c mice (n=10) 6-8 weeks old (Institute of Cytology and Genetics, Novosibirsk, Russian Federation) were immunized with recombinant bovine CTLA-4 and PD-L1 proteins using a two-week schedule. Recombinant proteins were administered intraperitoneally at a concentration of 25 μg/mL for CTLA-4 (NCB, Astana, Kazakhstan) and 125 μg/mL for PD-L1 (NCB, Astana, Kazakhstan) in 0.1 mL of incomplete Freund's adjuvant. Three days after the final immunization, the serum of immunized mice was examined for the presence of antibodies to recombinant CTLA-4 and PD-L1 proteins. Splenocytes from mice with high antibody titers were used for hybridization. Hybridization of myeloma cells X63-Ag8.653 and immune splenocytes was carried out according to the method described by Oi and Herzenberg [14]. Hybridoma clones producing antibodies were transferred into the wells of a 24-well plate (Corning Incorporated, Kennebunk, USA) as they grew. Cells were cultured until an average monolayer density or concentration of 5x105 cells/ml was achieved. Cloning of hybrid cultured cells was carried out using the limiting dilution method [15].

To obtain preparative quantities of mAbs, hybridoma cells were cultured in 25-50 mL tissue culture flasks containing complete growth medium RPMI-1640 (Sartorius, Beit HaEmek, Israel) for 3-4 days in a CO2 incubator at 37°C. The cells were harvested from the flasks surface by pipetting and centrifuged at 1000 rpm for 7 minutes. The cell pellet was resuspended in incomplete growth medium and 2×106 cells were injected intraperitoneally into BALB/c mice previously injected with pristane (Sigma, St. Louis, USA) at a dose of 0.5 mL per head 7-10 days before hybridoma injection.

Purification of mAbs from as cites fluid was carried out by salting out with ammonium sulfate to 50% saturation with stirring for 12 hours at 4 °C. The resulting precipitate was centrifuged at 5000 rpm for 30 minutes at 4 °C. The antibody pellet was resuspended in PBS (pH 7.2) and dialyzed for 24 hours. The concentration of mAbs was determined using Bradford method [16]. For additional purification, the MAbTrap™ Kit (GE Healthcare, Uppsala, Sweden) was used. Ascitic fluid was diluted with prepared binding buffer at a 1:1 ratio. Subsequently, the sample was applied to a prepared column using a syringe.

The immunochemical properties were determined as follows: the binding constant of mAbs was determined using the method described by J. Beatty [17]. The class and subclass of immunoglobulins were determined using the Rapid ELISA Mouse mAb Isotyping Kit Pierce™ (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. The specificity of mAbswas determined using an indirect ELISA. For this, wells of a 96-well plate (Corning Incorporated, Kennebunk, USA) were sensitized with commercial bovine PD-L1 and CTLA-4 proteins (Kingfisher Biotech, Inc., Saint Paul, USA) at a concentration of 5 µg/mL in 0.05 M carbonate buffer with a pH of 9.6 and incubated for 12 hours at 4 °C. After washing the plate and blocking with a 1 % BSA solution (PAA Laboratories GmbH, Pasching, Austria) the wells were filled with serial dilutions of mAbs, starting from a concentration of 10 µg/mL, and incubated at 37 °C for 1 hour. After washing, an anti-species conjugate was added at a dilution of 1:1000 and incubated at 37 °C for 1 hour. The reaction was developed using a substrate, and the results were recorded on a spectrophotometer iMark (BioRad, Tokyo, Japan) at a wavelength of 492 nm.

Results

One of the key conditions for successful hybridoma generation producing mAbs is the increase in the concentration of positive B-lymphocytes. Therefore, in the optimization of mouse immunization parameters, two different protein administration regimens were employed. In the first case, antigens were injected twice into the hind paw pads with an interval of two weeks. The principle of the second variant of immunization is described in the materials and methods section. It was found that the highest antibody titers were obtained using 5-fold intraperitoneal injections of protein (Table 1).

Table 1 – Optical density of serum from mice immunized with recombinant PD-L1 and CTLA-4 proteins in ELISA

Scheme Number	Route of protein administration	Protein injection dose,µg/mL	OD at 492 nm, M/m				
			day1	day7	day11	day12	day13
Recombinant bovine PD-L1							
Scheme1	Hind foot pad	100	0,155 0,042	-	-	-	0,500 0,047
		200	0,262 0,020	-	-	-	1,107 0,062
Scheme2	Intraperitoneally	100	0,103 0,006	0,252 0,024	0,361 0,031	0,456 0,028	0,541 0,040
		125	0,163 0,023	0,271 0,031	0,431 0,034	0,833 0,046	1,646 0,039
		300	0,322 0,031	0,725 0,041	lethal outcome	-	-
Recombinant bovine CTLA-4							
Scheme1	Hind foot pad	50	0,348 0,028	-	-	-	1,341 0,040
		100	0,665 0,040	-	-	-	lethal outcome

Continuation of Table 1

Scheme2	Intraperitoneally	25	0,213 0,047	0,576 0,029	0,937 0,036	1,345 0,029	1,652 0,057
		100	0,322 0,018	0,742 0,023	lethal outcome	-	-
		200	0,537 0,027	lethal outcome	-	-	-

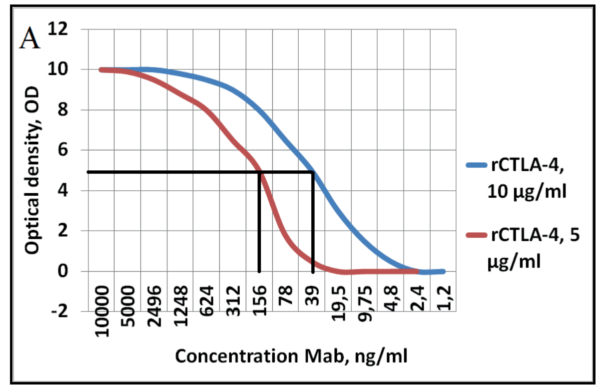
From Table 1 it can be seen that during immunization according to scheme 2, the optical density in the ELISA were higher and amounted to 1.646 for PD-L1 and 1.652 for CTLA-4. The optimal immunization dose for recombinant PD-L1 and CTLA-4 proteins was 125 and 25 µg/mL, respectively. The obtained results demonstrate that scheme 2 is preferable, since when using lower concentrations of proteins, it made it possible to obtain more intense immunity than scheme 1.

To obtain cell lines producing mAbs against recombinant bovine PD-L1 and CTLA-4 proteins, B-lymphocytes from immunized mice were subjected to hybridization with X-63 myeloma cell line. Several experiments were conducted, and the results of the hybridizations are presented in Table 2.

Table 2 – Indicators of hybridization of B-Lymphocytes from immunized mice and X-63 cell line

Number of hybridizations	Number of myeloma cells	Number of lymphocytes	Number of wells	Number of formed clones/%	Number of clones producing mAbs/%
Recombinant bovine PD-L1					
3	5x10^6	40x10^6	576	126/22	12/10
Recombinant bovine CTLA-4					
3	5x10^6	40x10^6	576	100/14	8/8

To obtain stable hybrid cell lines producing mAbs, hybridomas were cloned using the limiting dilution method in 3 replicates. The binding affinity constant (Kaff) of the mAbs was determined using OD-50 sigmoidal curves in ELISA. The method is based on the law of mass action and utilizes the total concentration of antibodies added to the antibody well, rather than the ratio of bound to free antibodies. The ELISA results of the antibodies, 3b3 against rCTLA-4 and 4h10 against rPD-L1, are presented in Figure 1.



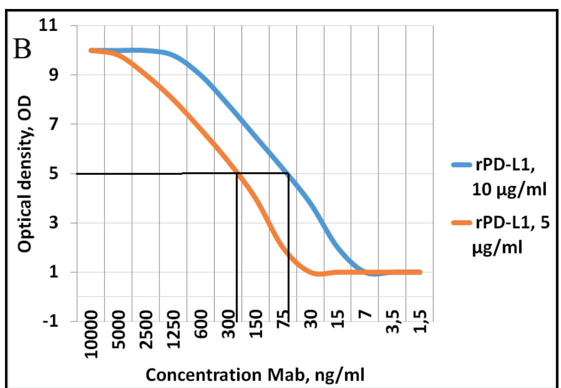
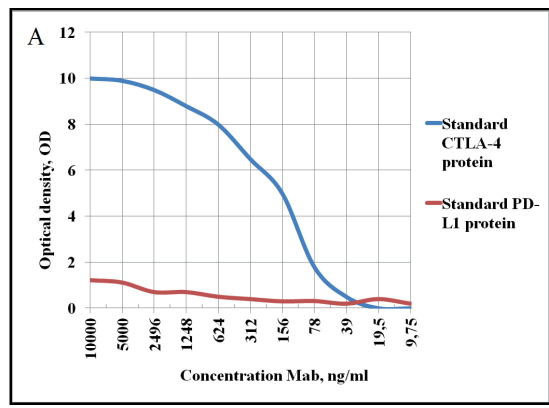


Figure 1 – Experimental ELISA curve for mAbs against two concentrations of bovine rCTLA-4 and rPD-L1 proteins. (A) 3b3 mAb; (B) 4h10 mAb

From the figure 1, it can be observed that the OD-50 sigmoidal curves of the 3b3 mAb strain at two concentrations of rCTLA-4 protein were 156 and 39 ng/ml, while for 4h10 it was 225 and 58 ng/ml. After calculations using the formula, the binding affinity constant (Kaff (M-1)) was determined to be 2.9×108M-1 for 3b3 and 1.9×108M-1 for 4h10.

The subclass of mAbs was determined using the Rapid ELISA Mouse mAb Isotyping Kit Pierce™. The results showed that the mAbs to bovine rCTLA-4 and rPD-L1 proteins belong to the immunoglobulin class IgG1.

To determine the specificity of the obtained mAbs, commercialbovineCTLA-4 and PD-L1 proteins from Bovine CTLA-4 ELISA Kit and Bovine PD-L1 ELISA Kit (Kingfisher Biotech, Inc., USA), were used. The results of specificity testing are presented in Figure 2.



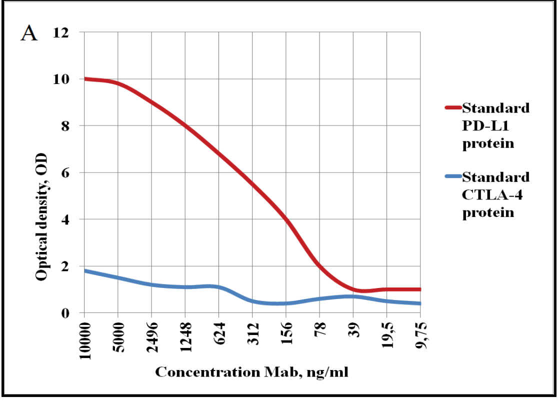


Figure 2 – Experimental ELISA curve for mAbs against two commercial bovine CTLA-4 and PD-L1 proteins. (A) 3b3 monoclonal antibody; (B) 4h10 monoclonal antibody

From Figure 2, it is evident that the mAbs 3b3 and 4h10 specifically bind to commercial bovine CTLA-4 and PD-L1 proteins at concentrations ranging from 5 μg/ml to 78 μg/ml. No reactions of the mAbs with heterogeneous proteins were observed.

Discussion

Despite the veterinary measures being implemented, the significant prevalence of BLV has intensified the interest in studying immune checkpoints for treating chronic infections in cattle. It has been determined that the progression of bovine leukemia virus-related disease (BLV-RS) increases the concentration of regulatory T lymphocytes, leading to elevated production of TGF-β, which, in turn, suppresses the expression of IFN-γ and TNF-α, as well as NK cell suppression [18]. A correlation with the PD-1/PD-L1 signaling pathway and lymphocyte activation gene 3 (LAG-3) has been noted in BLV-RS infection [8]. Additionally, it has been reported that increased expression of CTLA-4+ T cells leads to the progression of BLV infection [19].

Information is available on obtaining mAbs by immunizing mice with CTLA-4-Ig protein. Purified mAbs were found to bind to bovine cells expressing CTLA-4 and did not bind to CD28-expressing cattle Cos-7 cells. The application of anti-CTLA-4 antibodies significantly increased IFN-γ production by the immune cells of healthy and BLV-infected cattle. According to the authors, anti-CTLA-4 antibodies could be useful for developing new therapies against BLV infection [1]. A similar effect was observed with PD-L1 blockade antibodies, which enhanced T-cell function and inhibited BLV proliferation [20].

As a result of the research, an effective mouse immunization regimen and protein injection dose were selected, which allowed for a high level of antibody concentration in immunized mice. Thus, when mice were immunized with rPD-L1 protein, the OD value in the ELISA assay was 1.651, while immunization with rCTLA-4 protein yielded an OD of 1.731. Hybridization of immune mouse B-lymphocytes with the myeloma cell line X-63 resulted in 12 hybrid clones producing antibodies against bovine rPD-L1 and 8 against bovine rCTLA-4 proteins. For further work, clones 3b3 (anti-CTLA-4) and 4H10 (anti-PD-L1), demonstrating the highest antibody activity in ELISA, were selected. The obtained clones produced IgG1 class antibodies, specifically bound to commercial bovine CTLA-4 and PD-L1 proteins, and had a binding constant: 3b3 - 2.9×10^{8} M^{-1}, 4H10 - 1.9×10^{8} M^{-1}.

Conclusion

Blocking CTLA-4 and PD-L1 receptors of cattle with antibodies contributes to increasing the concentration of T lymphocytes and the expression of IFN-γ, thereby effectively inhibiting BLV. As a result of the study, hybridoma cell lines producing mAbs to bovine CTLA-4 and PD-L1 receptors

were obtained. The obtained mAbs belong to the IgG1 class, specifically reacted with commercial bovine proteins, and had a binding constant: 3b3 - 2.9×10^{8} M^{-1}, 4H10 - 1.9×10^{8} M^{-1}. The obtained mAbs efficiently blocked the reaction of commercial bovine CTLA-4 and rPD-L1 proteins with specific polyclonal antibodies in ELISA.

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