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Analysis of Antibody Induction by Macrophages Treated *Ex Vivo* with Human Proteins in Mice

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Abstract

Background: Macrophages are essential cellular components in various body tissues and tumor microenvironments. The high infiltration of macrophages into the tumor microenvironment determines the importance of *ex vivo* treatment of personalized macrophages with recombinant cytotoxic T-lymphocyte-associated protein 4 (rCTLA-4), programmed death-ligand 1 (rPD-L1), and programmed cell death protein 1 (rPD-1) proteins to block immune checkpoints.

Methods: We investigated the development of humoral immunity against CTLA-4, PD-L1, and PD-1 receptors by introducing macrophages treated *ex vivo* with the corresponding proteins into mice. Peritoneal macrophages from BALB/c mice were cultured in medium containing recombinant human CTLA-4, PD-L1, and PD-1 proteins. Macrophages processing recombinant proteins were analyzed via immunofluorescence staining using antibodies against CTLA-4, PD-L1, and PD-1. The treated macrophages were administered intraperitoneally to mice to induce anti-CTLA-4, anti-PD-L1, and anti-PD-1 antibodies. The antibody titer in vaccinated mice was determined via enzyme-linked immunosorbent assays, followed by statistical analysis of the results.

The specificity of the antibodies was determined via immunofluorescence staining in MCF7 cells.

Results: The *ex vivo* treatment of macrophages with rCTLA-4, rPD-L1, and rPD-1 induced the formation of specific antibodies in vaccinated mice. The various rPD-L1 and rPD-1 concentrations used to treat macrophages had no significant effect on the specific antibody titers, while the anti-rCTLA-4 titer was dependent on the protein concentration in the culture medium. Immunofluorescence analysis revealed that anti-CTLA-4 and PD-L1 antibodies reacted with MCF7 cells.

Conclusions: The *ex vivo* treatment of macrophages with rCTLA-4, rPD-L1, and rPD-1 can help induce humoral immunity and develop new approaches for cancer immunotherapy.

Keywords: Cytotoxic T Lymphocyte-Associated Protein 4, Immunotherapy, Macrophages, Programmed Death-Ligand 1, Programmed Cell Death Protein 1.

Introduction

Since the discovery of cancer cell susceptibility to immunotherapy, research has focused on the application of autologous T-lymphocytes and antitumor vaccines. Several decades ago, the use of immunotherapy in applications with autologous T-lymphocytes and antitumor vaccines had limited success. The low efficacy of this application of immunotherapy is associated with the limited availability of antigens and the lack of methods to stimulate immune responses in cancer patients. However, the discovery of dendritic cell (DC) function in generating and modulating immunity has provided a basis for the induction of antitumor immunity by activating T cells to cancer cell antigens (1).

Given the central role of antigen-presenting

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cells (APCs) in T-cell activation, DCs have become a rational target for research in many clinical scenarios, including transplantation, allergies, autoimmune diseases, resistance to infections, and tumors. Unfortunately, many tumor components do not elicit a specific T-cell response in patients. However, specific immunity arises upon *ex vivo* loading of DCs with tumor antigens and repeated administration. In animals, this strategy provides protection against tumors and even reduces the size of established tumors (2). Research is currently underway to develop conventional APCs for cancer patients in whom APC and effector cell functions are impaired. Protocols are being developed to create APCs in finished products from induced pluripotent stem cells, construct major histocompatibility complex (MHC) class I-deficient cells, and develop artificial APCs based on paramagnetic nanoparticles (3,4).

APCs also include macrophages that can present exogenous antigens to CD4+ T helper cells via MHC class II, CD86, and CD83 molecules. Macrophages derived from the same progenitor cells as DCs express MHC class II and co-stimulatory molecules after interferon gamma (IFN-γ) activation. They also circulate in the blood, enter areas of infection or tissue damage, and participate in antigen cross-presentation. Several recent studies have shown that an increase in number of M1 macrophages is a prognostic indicator capable of inducing anticancer immunity and phagocytosis of cancer cells (5,6). An increase in the number of CD169+F4/80+CD11b+CD45+ macrophages in the tumor microenvironment and spleen has been found, confirming the role of CD169+ macrophages in the activation of CD8+ T lymphocytes in tumor destruction. In addition, macrophages show low co-stimulatory and cross-presentation activities and are generally absent in alternatively activated anti-inflammatory macrophages. The lack of cross-presentation in certain macrophage types is desirable in autoimmune diseases (7). Considering the influence of the components of the tumor cell microenvironment on the therapeutic effect of monoclonal antibodies, the hypothesis of creating personalized immunity against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death-ligand 1 (PD-L1), and programmed cell death protein 1 (PD-1) receptors is of interest.

This study evaluated the development of humoral immunity in mice against human CTLA-4, PD-L1, and PD-1 receptors. Humoral immunity was induced using mouse macrophages treated *ex vivo* with recombinant PD-L1 (rPD-L1), CTLA-4 (rCTLA-4), and PD-1 (rPD-1) human proteins.

Materials and Methods

Mice and reagents

Inbred female BALB/c mice (n= 96) aged 6–8 weeks were used to obtain macrophages and for immunization. The research protocols were approved by Animal Ethics Committee of the Republican State Enterprise **"**National Center for Biotechnology" (Nur-Sultan, Kazakhstan). The organization has all the necessary conditions for keeping mice by the requirements of their physiology. Fetal calf serum (FCS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and RPMI 1640 medium was purchased from VWR Life Science AMRESCO (Radnor, PA, USA). Anti-mouse IgG peroxidase antibody produced in rabbits was purchased from Sigma–Aldrich (St. Louis, MO, USA). Incomplete Freund**'**s adjuvant was purchased from Sigma–Aldrich.

Mice immunization

To obtain positive sera, 400 μg of the recombinant protein in 500 μL of phosphate-buffered saline (PBS) containing 100 μL of incomplete Freund's adjuvant (Sigma–Aldrich) was intraperitoneally injected into six BALB/c mice (6–8 weeks old). Two mice were injected with each recombinant protein. On days 7, 11, 12, and 13 of immunization, the animals were injected with 400 μg of recombinant protein in PBS. Three days after the last immunization, the immunized mice sera were analyzed for the presence of antibodies against the recombinant proteins using enzyme-linked immunosorbent assay (ELISA).

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Preparation and administration of macrophage cell preparations treated with rCTLA-4, rPD-L1, and rPD-1

To obtain peritoneal macrophages, the mice were euthanized using ether and immobilized via rapid displacement of the cervical vertebrae. The abdominal area of each mouse was treated with 70% alcohol and an incision was made along the midline using sterile scissors. Next, 10 mL of cold RPMI 1640 was injected into the abdominal area. The medium was carefully removed from the abdominal cavity and transferred into a 50-milliliter conical polypropylene tube. A suspension of peritoneal macrophages isolated from BALB/c mice was precipitated via centrifugation at 200 × g for 10 min. The cell sediment was resuspended in RPMI 1640 medium containing 5% FCS at 5×10^5 cells/mL, and 25 mL were seeded into TPP culture mattresses (TPP Techno Plastic Products AG, Trasadingen, Switzerland). Cells were cultured in a CO_2 incubator at 37 °C in an atmosphere of 5% CO_2 for 24 h. After the formation of a cell monolayer, rCTLA-4, rPD-L1, and rPD-1 were sterilized via filtration through 0.22-micron filters and added to the culture medium at 250, 500, and 1,000 μg/mL, respectively. Peritoneal macrophages expressing recombinant proteins were cultured at 37 °C in an atmosphere of 5% CO_2 for 72 h. The cells were washed by centrifugation and resuspended at a density of 5×10^6 cells/mL in PBS, pH 7.2.

Three groups of mice were intraperitoneally injected with the various macrophage preparations at a concentration of 5×10^6 cells/mouse diluted in 500 μL of PBS. Mouse macrophages were treated with rCTLA-4, rPD-L1, and rPD-1 and inoculated into the mice of the first, second, and third groups, respectively. Ten days after the primary vaccination, the mice were given secondary inoculations with the same cellular preparations. Seven days after the last vaccination, serum samples were collected to determine the specific antibody titers using ELISA.

ELISA

The Nunc-Immuno™ MicroWell ™ **"**Maxisorp" 96-well microplates (Sigma-Aldrich) were treated with 10 μg/mL of rCTLA-4, rPD-L1, and rPD-1 in bicarbonate buffer (0.1M $NaHCO_3$, pH= 9) and incubated for 60 min at 37 °C. The microplates were blocked with 1% (w/v) bovine serum albumin (BSA) in PBS for 60 min at 37 °C. The immunized mice sera were double-diluted in PBS and added to the recombinant proteins for 60 min at 37 °C. The microplates were washed with PBS containing 0.1% Tween 20 (PBS+Tw20), and the working dilution of anti-mouse IgG–peroxidase antibody produced in rabbit (Sigma–Aldrich) was added for 60 min at 37 °C. The microplates were washed with PBS+Tw and PBS, and bound peroxidase activity was measured by adding 100 μL/well of TMB substrate solution (Thermo Fisher Scientific) for 15 min at room temperature (22-25 °C). H_2SO_4 (2M) was added to stop the reaction in the well, and color development was measured at 492 nm using a microplate reader 680 (BioRad, Japan).

Immunofluorescence

Plates with macrophages, and MCF7 cells (Ekaterinburg Research Institute of Viral Infections, Yekaterinburg, Russia) were washed in PBS and fixed with 4% paraformaldehyde (Sigma–Aldrich, St. Louis, MO, USA) for 20 min at 22 °C. Paraformaldehyde was pre-sterilized via filtration through a filter with a pore size of 0.22 μm (Millipore, Burlington, MA, USA). After cell fixation, the wells were washed three times for 5 min in PBS+Tw20. Triton X-100 (0.1%) was added to the wells to permeabilize the cell membranes. Plates were incubated with Triton X-100 for 3 min at 22 °C and washed with PBS+Tw20. The wells were blocked with 1% (w/v) BSA in PBS for 60 min at 22 °C and washed with PBS+Tw20. Serum containing antibodies against rCTLA-4, rPD-L1, and rPD-1 proteins at a 1:10 dilution was added to the wells and incubated for 12 h at 22 °C. The plates were then washed

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with PBS+Tw20. Secondary antibodies against mouse IgG labelled with fluorescein isothiocyanate at a dilution of 1:250 were added to the wells and incubated for 60 min at 22 °C. The wells were washed three times for 5 min in PBS+Tw20. 4′,6-Diamidino-2-phenylindole (blue) at a dilution of 1:2,000 was added to the wells and incubated for 5 min at 22 °C. Images were taken using AxioObserver A1 (Carl Zeiss AG, Oberkochen, Germany) at 10× magnification. The scale line was 10 μm.

Statistical analysis

Plots were created using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). Statistics were analyzed using GraphPad Prism version 9.3.1. The statistical tests used, and p values are indicated in the figure captions. A p value of 0.05 was considered significant.

Results

Ex vivo treatment of macrophages with rCTLA-4, rPD-L1, and rPD-1

Macrophages are known to activate T and B cells by presenting antigens on the membrane surface, thereby inducing specific immunity. However, the influence of macrophages treated *ex vivo* with rCTLA-4, rPD-L1, and rPD-1 on the induction of specific antibodies capable of blocking homogeneous receptors is unclear. Therefore, we cultured peritoneal macrophages in media containing human rCTLA-4, rPD-L1, and PD-1, and injected them intraperitoneally into mice. Each experiment used 15 mice, and an additional three mice served as negative controls. Immunofluorescence staining was performed to examine the macrophages presenting human rCTLA-4, rPD-L1, and PD-1 on the outer membrane (Fig. 1). Peritoneal macrophages cultured in media containing rCTLA-4 (row A), rPD-L1 (row B), and rPD-1 (row C) carried antigenic epitopes of proteins on their membranes.

Anti-rCTLA-4, anti-rPD-L1, and anti-PD-1 antibody titers were measured via ELISA 14 d after the administration of recombinant protein-treated macrophages (Fig. 2). Notably, all groups of mice had high specific antibody titers after vaccination with treated macrophages. The average antibody titers against the three proteins in mice ranged from 1:800–1:3,200.

Effect of rCTLA-4, rPD-L1, and rPD-1 concentrations upon macrophage treatment on antibody titers in vaccinated mice

Significant differences were found in mouse antibody titers depending on protein concentrations during macrophage treatment with rCTLA-4. When macrophages were treated with rPD-L1 or rPD-1, no significant differences in antibody titers were observed after administration of the cell preparations. However, the mice sera showed high antibody titers and optical densities in the ELISA reactions (Fig. 3). Thus, B cells may target the recombinant protein epitopes processed on macrophages.

Reaction of anti-rCTLA-4 and anti-rPD-L1 antibodies with MCF7 cells

Despite the induction of anti-rCTLA-4, anti-rPD-L1, and anti-PD-1 antibodies in vaccinated mice, the ability of the antibodies to bind to homologous receptors was not determined. Therefore, we investigated the ability of macrophage preparation-stimulated antibodies to bind to the CTLA-4 and PD-L1 receptors on oncogenic cells. The binding of anti-rCTLA-4 and anti-rPD-L1 antibodies to homologous receptors was analyzed via immunofluorescence staining

To analyze the reaction of the antibodies with homogeneous receptors, we used the MCF7 breast adenocarcinoma cell line. This cell line was selected based on the difference in surface expression of CTLA-4 and PD-L1 receptors. Despite the low intensity of the surface expression of the CTLA-4 and PD-L1 receptors, antibodies bound to them on the MCF7 cell membranes (Fig. 4). MCF7 cells were incubated with anti-CTLA-4 (row A) and anti-PD-L1 (row B) mouse sera.

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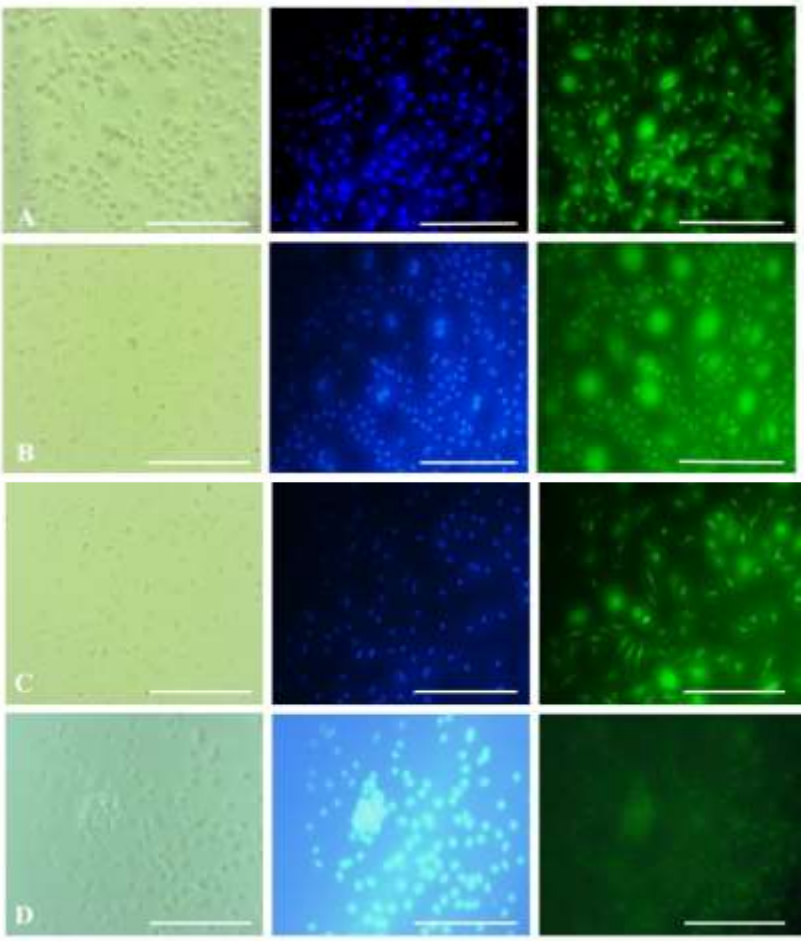

Fig. 1. Immunofluorescence of macrophages containing epitopes of human (A) rCTLA-4, (B) rPD-L1, and (C) rPD-1 proteins on their outer membranes and the (D) control group. A macrophage monolayer (left column) was stained with 4′,6-diamidino-2-phenylindole (blue, center column) and secondary anti-mouse IgG antibodies labeled with fluorescein isothiocyanate (green, right column). Scale bars: 100 µm. rCTLA-4, cytotoxic T-lymphocyte-associated protein 4; rPD-L1, recombinant programmed death-ligand 1; and rPD-1, recombinant programmed cell death protein 1.

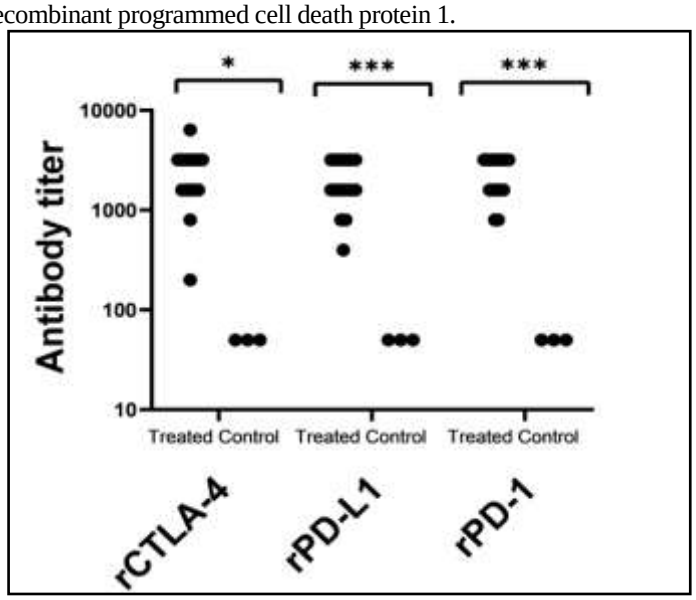

Fig. 2. Antibody titers of mice vaccinated with macrophages treated with rCTLA-4, rPD-L1, and rPD-1 *ex vivo*. Each character represents a single mouse. Statistics were analyzed on log-transformed data using Student's t-test: *p, 0.05, ***p, 0.005. rCTLA-4, cytotoxic T-lymphocyte-associated protein 4; rPD-L1, recombinant programmed death-ligand 1; and rPD-1, recombinant programmed cell death protein 1.

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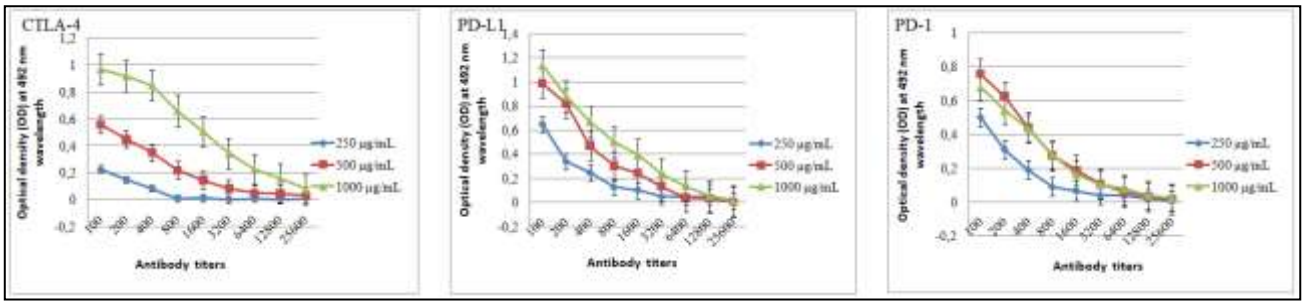

Fig. 3. Mouse antibody titers versus rCTLA-4, rPD-L1, and PD-1 concentrations in *ex vivo* macrophage treatment. rCTLA-4, cytotoxic T-lymphocyte-associated protein 4; rPD-L1, recombinant programmed death-ligand 1; and rPD-1, recombinant programmed cell death protein 1.

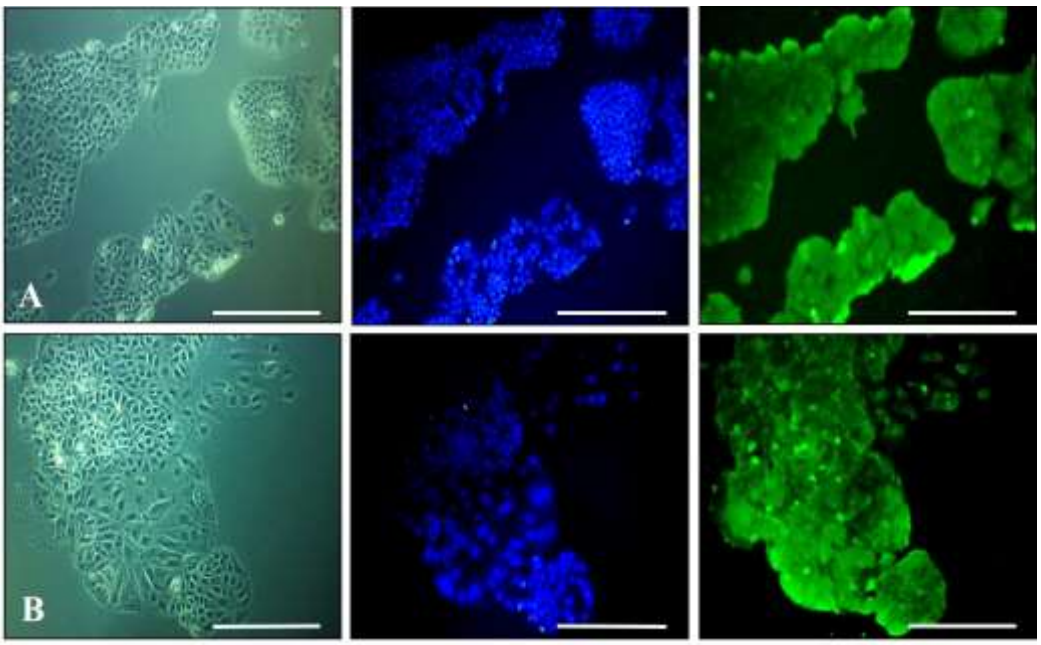

Fig. 4. Immunofluorescence of MCF7 cells with (A) anti-CTLA-4 and (B) anti-PD-L1 mouse sera. The cell monolayer (left column) was stained with 4′,6-diamidino-2-phenylindole (blue, middle column) and secondary antibodies against mouse IgG labeled with fluorescein isothiocyanate (green, right column). Scale bars: 100 µm. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PD-L1, recombinant programmed death-ligand 1.

Discussion

Currently, the therapy of oncological diseases, diagnosis of cancer using target antigens, and development of new platforms for cancer vaccines have shown progress. Combining the blockade of immunological checkpoints with therapeutic cancer vaccines has led to successful clinical results. APCs are the most suitable candidates for developing cellular cancer vaccines because of their ability to take up, process, and present tumor antigens (8). Only specialized APCs induce immunity via antigen presentation by MHC molecules, expression of co-stimulatory molecules, and secretion of cytokines/chemokines. Using all three mechanisms, specialized APCs activate T lymphocytes to recognize and destroy cells that contain oncogenic or viral antigens. Macrophages and DCs internalize pathogens and cellular debris via phagocytosis and present T cells with the necessary co-stimulatory molecules for activation (9).

DCs are the most effective APCs capable of presenting tumor antigens and mediating antitumor immunity (10). Moreover, the efficiency of immune activation by DCs has increased with the improvement of methods for isolation, cultivation, and antigen treatment under various conditions (11-13). In addition to DCs, macrophages can cross-present heterogeneous antigens to activate CD8+ T

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lymphocytes. Moreover, there are different macrophage types depending on their location in body tissues, and their use represents a promising strategy for anticancer therapy (14). Currently, studies are being conducted on the activation of T cell memory in tissues and the lymphatic system to induce a rapid humoral response. Tissue-resident macrophages can play a crucial role in the development and reactivation of resident CD8+ T lymphocytes via local cytokine secretion (15).

In this study, we induced antibodies against CTLA-4, PD-L1, and PD-1 in mice by administering macrophages treated *ex vivo* with rCTLA-4, rPD-L1, and rPD-1. The macrophages were found to process these proteins in forms suitable for recognition by T and B cells and activate the production of antibodies that react with homogeneous receptors on cancer cells. Analysis of the effect of protein concentration during macrophage treatment on antibody titers in mice showed a difference in results between rPD-1/rPD-L1 and rCTLA-4. The difference in anti-rPD-1/anti-rPD-L1 antibody titers in mice was insignificant despite significant differences in the protein concentrations in the culture medium. In contrast to rPD-1 and rPD-L1, the anti-rCTLA-4 antibody titer depended on the protein concentration upon *in vitro* macrophage treatment.

The efficiency of adaptive immunity development depends on the delivery conditions of APCs and effector T-cells to the tumor microenvironment. Significant progress has been made in improving existing cancer immunotherapy strategies using new DC-based anticancer drug platforms Our result

demonstrate the possibility of using macrophage-based drugs to block immune checkpoints. These drugs effectively activate the production of specific antibodies capable of blocking homogeneous receptors on cancer cells. However, safety and efficacy of the use of macrophage-based anti-CTLA-4, anti-PD-L1, and anti-PD-1 drugs remain unclear. Therefore, further research is required to achieve the ultimate goal of creating safe and effective personalized cancer treatment (16-18).

The ability of macrophages to activate cellular and humoral immunity has become the basis of their use in oncological and infectious disease therapy. The success of macrophage-based treatment is associated with their ability to activate immunity, independent of the conditions for loading cells with antigens. Macrophages loaded *ex vivo* with rCTLA-4, rPD-L1, and rPD-1 induced sufficiently high antibody titers in the vaccinated mice. Furthermore, immunofluorescence staining showed that antibodies against rCTLA-4 and rPD-L1 specifically reacted with MCF7 cells. Further studies on macrophages and DCs presenting with rCTLA-4, rPD-L1, and rPD-1 may lead to new approaches for immunizing against oncological and infectious diseases.

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Conflicts of Interest

The authors declares that there is no conflict of interest regarding the publication of this paper.

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