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Original Article

Enhanced *in vivo* anti-tumor efficacy of whole tumor lysate in combination with whole tumor cell-specific polyclonal antibody

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Abstract

Background and purpose: Despite the widespread utilization of cancer vaccines with specified antigens, the use of whole tumor cell lysates in tumor immunotherapy would be a very promising approach that can overcome several significant obstacles in vaccine production. Whole tumor cells provide a broad source of tumor-associated antigens and can activate cytotoxic T lymphocytes and CD4+ T helper cells concurrently. On the other hand, as an effective immunotherapy strategy, recent investigations have shown that the multi-targeting of tumor cells with polyclonal antibodies, which are also more effective than monoclonal antibodies at mediating effector functions for target elimination, might minimize the escape variants.
Experimental approach: We prepared polyclonal antibodies by immunizing rabbits with the highly invasive 4T1 breast cancer cell line.
Findings/Results: *In vitro* investigation indicated that the immunized rabbit serum inhibited cell proliferation and induced apoptosis in target tumor cells. Moreover, *in vivo* analysis showed enhanced anti-tumor efficacy of whole tumor cell lysate in combination with tumor cell-immunized serum. This combination therapy proved beneficial in significant inhibition of the tumor growth and the established tumor was entirely eradicated in treated mice.
Conclusion and implications: Serial intravenous injections of tumor cell immunized rabbit serum significantly inhibited tumor cell proliferation and induced apoptosis *in vitro* and *in vivo* in combination with whole tumor lysate. This platform could be a promising method for developing clinical-grade vaccines and open up the possibility of addressing the effectiveness and safety of cancer vaccines.

Keywords: Breast cancer; Cancer vaccine; Immunotherapy; Polyclonal antibodies; Tumor cell lysate.

INTRODUCTION

Despite recent developments in understanding breast cancer's molecular and cellular mechanisms and advances in tumor treatment, breast cancer-related death had increased (1). Since it is an extremely heterogeneous cancer, it is associated with various biological problems and thus responds to treatment differently (2). Prevention methods, including mammography population screening, are still the most effective strategy to overcome breast cancer and keep its mortality mostly stable over the last decade (3). Nonetheless, the global incidence of breast cancer has continued to rise over the last three decades.

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The low therapeutic efficiency and the worst prognosis for breast cancer patients are currently related to drug resistance and relapse due to metastasis and the presence of breast cancer stem cells (4).

To overcome these shortcomings, high-throughput technology and omics platforms have improved our knowledge of the genomic complexity within the tumor cell and intra-tumoral heterogeneity. Recent advances in our understanding of the underlying molecular mechanisms have led to the development of molecular-targeted therapies and combination therapies, including immune-based therapy (5,6). Recent research has shown that using whole tumor cell lysates rather than a single antigen, which may include the whole repertoire of cancer cell target antigens, increases the cytotoxic CD8+ T cell immune response (7,8). When compared to full-length recombinant tumor proteins/antigens or tumor-derived peptides, whole tumor antigens offer a promising alternative source of antigens for dendritic cell (DC)-based immunotherapy. Whole tumor lysate therapy, unlike specified tumor-derived peptides and proteins, regardless of the patient's human leukocyte antigen type, is applicable to all patients. Whole tumor cell lysates have been used in several clinical trials as an ideal and broad source of tumor-specific antigens for DC maturation. As a result, using entire tumor cell lysates as a method of increasing the efficacy of immunotherapy for cancer treatment is a feasible alternative (9).

Indeed, antibodies have gained immense attention as an effective immune therapy for various tumors. Numerous tumor-antigens have currently been discovered in breast cancer, including the basic fibroblast growth factor, vascular endothelial growth factor, and epidermal growth factor receptor family (Erbb family) (10,11). Targeted monoclonal antibodies are currently being applied in clinical trials because these antigens play an important role in tumor proliferation, survival, angiogenesis, and metastasis (12,13). Despite the advantages of specificity, monoclonal antibodies are unsuccessful in therapeutic applications caused by tumor heterogeneity and redundancy of molecular pathways leading to cancer cell survival, and there are still concerns with high recurrence rates and medication resistance (14). Bispecific antibodies are molecules that have been engineered to bind to two distinct antigens simultaneously. Furthermore, bispecific antibodies have drawbacks such as restricted tissue penetration and a short half-life (15,16). Hence, targeting multiple antigens simultaneously by efficient polyclonal antibodies is an efficient and faithful alternative approach (17). Polyclonal antibodies have unique properties, including multi-epitope binding capacity, and high-avidity binding which could provide multi-targeted cancer treatments that are effective at mediating effector activities while minimizing the risk of tumor cell escape (18). Recently, polyclonal anti-ovarian cancer antibodies were prepared *via* immunizing rabbits with the human ovarian tumor cell line SKOV3. The results from this study indicated that polyclonal antibodies bound to the targeted tumor cell line and inhibited the proliferation of tumor cells by blocking the caspase signaling pathway (19).

Here, we immunized rabbits with a highly tumorigenic and invasive breast cancer cell line, 4T1, to produce rabbit anti-breast cancer polyclonal antibodies. Immunized rabbit serum in combination with tumor cell lysate could decrease and inhibit proliferation and induce apoptosis of breast cancer *in vitro* efficiently. Moreover, this combination therapy reduced tumor growth in subcutaneous breast cancer mouse models. Accordingly, this study proposes that the therapeutic effects are attributed to multiple targets on breast tumor cells. Thereby, such approaches could provide potential therapeutic alternatives based on the total characteristics of tumors, facilitating the achievement of the personalized medicine goal.

MATERIALS AND METHODS

Animals and cell lines

New Zealand White Rabbits and 6-8-week-old BALB/c mice were provided by the Royan institute (Isfahan, Iran). All animal experiments were carried out based on Iran's Guidelines for Care and Use of Laboratory Animals during the investigation, which was performed in the experimental animal section of Isfahan University of Medical Science (20). Ethical

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approval for this study was obtained from the institutional review boards at the Isfahan University of Medical Science (Ethic No. IR.MUI.RESEARCH.REC.1398.668). Prior to the experiment, the mice were housed for at least a week. In addition, tubes, boxes, and climbing structures were provided in order to enrich, which is renewed once a week.

The 4T1 mouse mammary tumor cell line and murine monocyte/macrophage cell line J774 were purchased from Pasture Institute (Iran) and were cultured in RPMI-1640 (Bio-idea, Iran), supplemented with 10% fetal bovine serum (FBS, BioIdea, Iran), 100 U/mL penicillin, and 100 mg/mL streptomycin (Bio-idea, Iran) in a humidified 5% CO_2 incubator at 37 °C.

Rabbit immunization for polyclonal antibodies preparation

Blood was taken from the ears before inoculation of tumor cells and normal rabbit serum was isolated before immunization. By immunizing New Zealand White Rabbits, anti-tumor cell polyclonal antibodies were generated with 6×10^6 4T1 cells (in 1 mL normal saline) mixed with 500 µL of Freund's complete adjuvant (Sigma Aldrich, USA) subcutaneously into the right flank. Booster injections were given with incomplete adjuvant on D7, D14 and, D21. Before each injection, rabbits were weighed, and their body temperature was controlled.

One week after the last immunization through ear vein bleeding, the serum was collected. Blood samples were kept at 4 °C for coagulation, and the serum was separated from the coagulated blood after centrifugation at 5000 rpm for 5 min. The serum was stored at -20 ℃.

Investigation of specific binding of polyclonal antibodies to tumor cells using flow cytometry

For this purpose, 1×10^6 cells of each cell line (4T1 tumor cells and J774 cells as control) in the logarithmic phase, were incubated with serum-containing polyclonal antibody (1:1000) and control rabbit serum for 30 min. The cells were then resuspended and washed with 500 µL of phosphate-buffered saline (PBS, 1×). Flow cytometry was then performed using fluorescein isothiocyanate (FITC)-conjugated IgG-goat anti-rabbit antibody (Abcam, USA) by FACS Calibur flow cytometer (BD Bioscience, USA) by accumulating up to 200,000 cells per tube. Data analysis was performed using FlowJo™ 10 (BD bioscience, California, USA). To indicate the specific binding of immunized serum to target cells, flow cytometry analysis was performed under the same condition for J774 cells as the control.

Cell-based enzyme-linked immunosorbent assay

For the enzyme-linked immunosorbent assay (ELISA) experiment, the J774 and 4T1 tumor cells (5×10^3) were cultured overnight on a 96-well plate. The cells were washed three times with PBS (1×) after the medium was removed. The cells were washed and fixed in 10% ethanol for 10 min, and then blocked for 1 h at room temperature with 0.5 bovine serum albumin. Before the addition of the immunized rabbit serum, the cells were washed repeatedly with PBS. The immunized and non-immunized rabbit serums were diluted at 1:1,000, 1: 10,000, and 1:100,000, and incubated with 4T1 tumor cells and J774 cells overnight. Cells were then washed three times with PBST (PBS containing 0.05 % tween-20), and treated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000, Cytomatingen, Iran), then incubated for 30 min. Finally, 100 µL of 3,3', 5,5;-tetramethylbenzidine solution (TMB; Cytomatingen, Iran) was added and absorbance at 450 nm was read. Each experiment was performed in triplicate.

Tumor cell viability assay

Cell viability analysis was performed by 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) assay. In brief, 4T1 and J774 cells were cultured (5×10^4 cells/well in 48-well cell culture plate) and incubated for 24 h at 37 °C. The cells were treated with rabbit serums (1:1000-1:100000) for 24 and 48 h. The cells were then cultured in a CO_2 incubator for 2 h before the MTT experiment was completed by adding 50 µL of MTT solutions from the stock (5 mg/mL). After removing the media, formazan crystals that the cells had generated

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were dissolved in 500 μL of dimethyl sulfoxide (DMSO) and then transferred to a 96-well plate. On a Multiwell plate reader, the absorbance was measured at 570 nm using 630 nm as the reference wavelength (Biotech Instruments, USA).

Apoptosis detection by annexin V-FITC-propidium iodide staining

The 4T1 breast cancer cell line was utilized to evaluate the anti-tumor activity of immunized rabbit serum using annexinV-FITC and propidium iodide (PI) staining. In a brief, the 4T1 cells (2×10^5) were cultured and treated with non-immunized control serum or polyclonal antibodies. After 24 h incubation, the cells were harvested, washed, and then resuspended at 1×10^6/mL in recommended binding buffer. The apoptotic cells were evaluated by flow cytometry using an apoptosis detection kit (Life Technologies, USA) according to the manufacturer's recommendations.

Preparation of tumor cell lysate

To prepare a tumor cell lysate, 4T1 cells were divided among 1.5-mL tubes (10^6 per 1 mL PBS), and the tubes were subjected to 5 freeze-thaw cycles using liquid nitrogen and a 37 °C water bath. The lysates were stored at -80 °C until use.

Primary tumor implantation

The 4T1 cells were cultured in RPMI-1640 media with 2 mM glutamine, 1% penicillin/streptomycin, and 10% FBS. Cells were allowed to grow at 37 °C with 95% humidity for two days before use in animals. To prepare cells for injection implantation, 4T1 cells were trypsinized and verified to be in single-cell suspension, and the viability of the cell was then accessed by the trypan blue staining. The cells were then washed with PBS (1×), pelleted and suspended in 1 mL of fresh PBS. A 1 cc syringe without a needle was used to draw cell suspension. A 25-gauge needle was then placed into the syringe and 200 μL of cell suspension was injected subcutaneously into the right flanks of BALB/c mice. All efforts were made to minimize animal distress, which was assessed by weekly weight measurements and the presence of physiological signs of distress. The mice were randomly divided into five groups (5-7 per group) once the tumor nodules were palpable, and treated with PBS, non-immunized rabbit serum, immunized serum, tumor cell lysate, and tumor cell lysate in the combination with immunized serum *via* the tail vein. For three weeks, all injections were given every three days and the tumor's volume was calculated using a caliper and the following equation:

$$\text{Volume} = \text{length} \times \text{width}^2 \times 0.52$$

Blood was collected from the tail vein one day before treatment and at 24 days after treatment to assess interferon gamma (IFN-γ) cytokine level in serum using ELISA. The tails were cleaned and 100 μL of whole blood was collected in ethylenediaminetetraacetic acid (EDTA) containing tubes. Serums were separated as described aforementioned and INFγ levels were measured using the ELISA kit (Abcam, UK), according to the manufacturer’s instructions (sensitivity: 4 pg/mL). The animals were sacrificed after 24 days after tumor injection and the size of the tumor was analyzed.

Statistical analysis

Data, expressed as means ± SD, were analyzed by a two-tailed Student’s t-test or Mann-Whitney test. A *P*-value of less than 0.05 was regarded as statistically significant.

RESULTS

Evaluation of specific binding of immunized-rabbit serum to target cells

Flow cytometric analyses were carried out to evaluate the specific binding capacity of the immunized rabbit serum-containing polyclonal antibodies to 4T1 tumor cells. In addition, we assess the binding capacity of immunized rabbit serum-containing polyclonal antibodies against non-target J774 cells and there was no significant change in fluorescent signal from immunized rabbit serum-treated J774 cells compared to the control group (non-immunized rabbit serum-treated cells) (Fig. 1, A1 and A2). In comparison to non-immunized rabbit serum (Fig. 1, A3), flow cytometry data using FITC-conjugated IgG-goat anti-rabbit antibody demonstrated that polyclonal antibodies could target tumor cells with 1000 times dilution (Fig. 1. A4).

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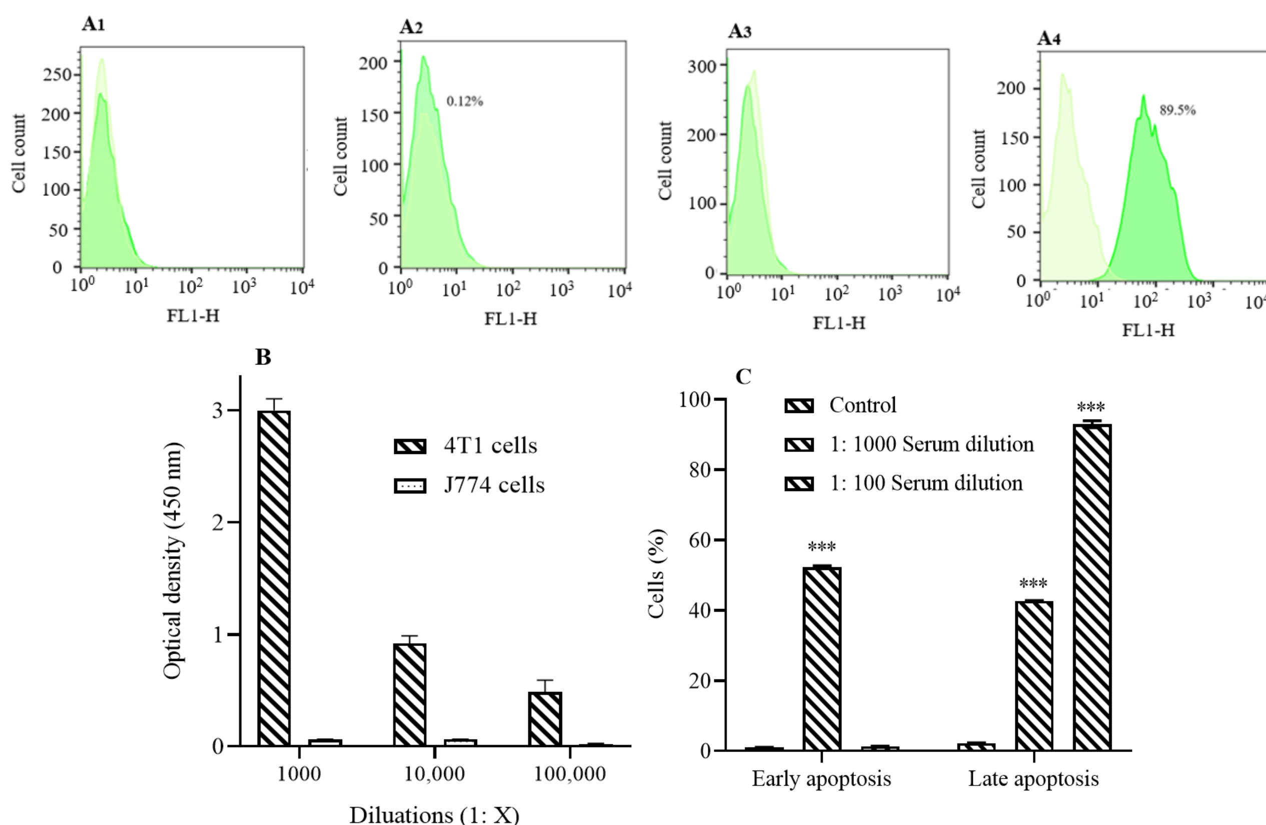

Fig. 1. The *in vitro* assays indicated specific binding capacity and anti-tumor efficacy of immunized serum against 4T1 cells. (A) Flow cytometry analysis was performed to evaluate the specific binding of polyclonal antibodies-containing immunized serum against 4T1 target cells in comparison with non-target J774 cells: (A1) non-immunized serum-treated J774 cells, (A2) immunized serum-treated J774 cells, (A3) non-immunized serum-treated 4T1 cells, and (A4) immunized serum-treated 4T1 cells. (B) Specific binding of polyclonal antibodies to the 4T1 cell line also was confirmed by cell-based ELISA in compression with J774 cells as the control group. (C) Annexin V-FITC and PI staining was performed to quantitatively assess the target cell apoptosis by flow cytometric analysis. The results are presented as the means ± SD of three independent replicates. ***$P < 0.001$ indicates significant differences compared to the control group.

Cell-based ELISA assay indicates specific binding of immunized serum-containing polyclonal antibodies to tumor cells

Immunized serum-containing polyclonal antibodies bind to 4T1 cells at 100,000 times dilution, according to cell-based ELISA analysis. In addition, non-specific binding was omitted using control groups. To test whether immunized serum could bind to non-target cells, the J774 cell line was treated with different dilutions of immunized serum (Fig. 1B). Also, 4T1 and J774 cell lines were treated with non-immunized serum. Moreover, no significant signals were observed in each of the control groups (data not shown).

Polyclonal antibodies induced tumor cell apoptosis in vitro

Quantitative assessment of the cytotoxic effect of polyclonal antibodies containing immunized serum was further done by flow cytometry to detect the number of early and late apoptotic cells. The 1:1000 concentration of immunized serum treatment induced 93.7% late apoptosis on 4T1 cells. The specific targeting of tumor cells was evaluated by treatment of target cells with non-immunized serum and 2.3% of 4T1 cells showed late apoptosis (Fig. 1C).

Polyclonal antibodies inhibited tumor cell proliferation in vitro

Cell viability was assessed using MTT analysis to evaluate the possible anti-proliferative effect of polyclonal antibodies on the tumor cells. After 24 and 48 h of incubation, the immunized rabbit serum substantially reduced the proliferation of tumor cells in a concentration-dependent manner as compared to the non-immunized rabbit serum. We also found that polyclonal antibodies did not show a significant inhibitory effect on the J774 cell line (Fig. 2).

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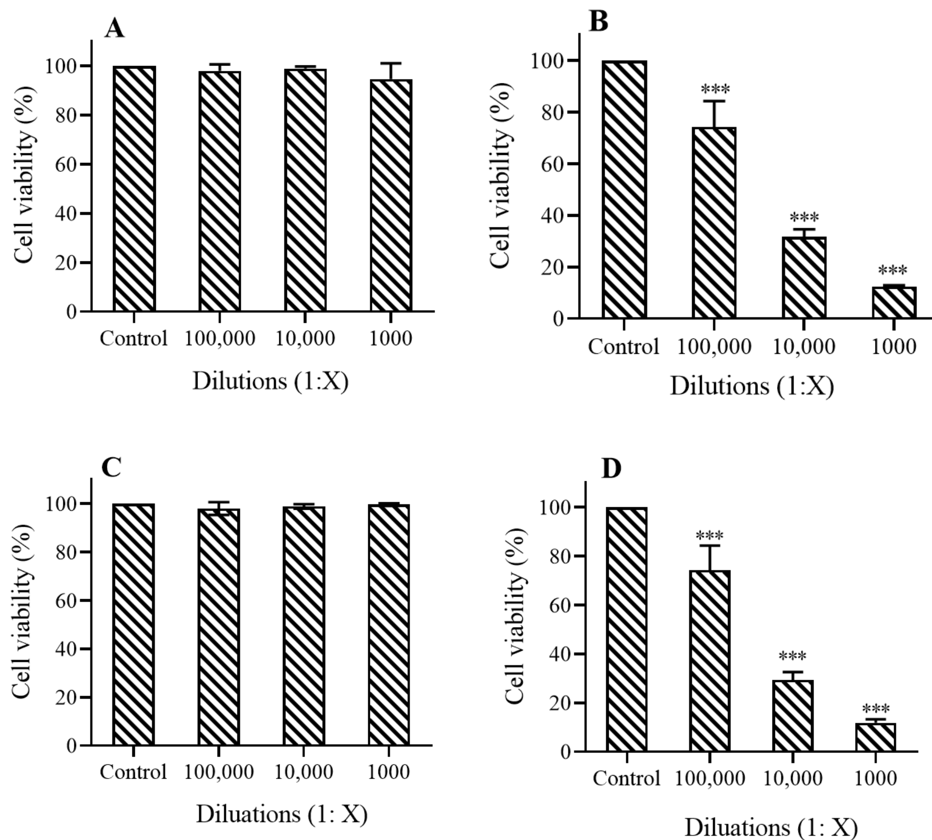

Fig. 2. The *in vitro* assays indicated the anti-tumor efficacy of immunized serum against 4T1 cells. MTT assay was used to assess the antiproliferative effect of polyclonal antibodies on the target and non-target cell lines' viability and data indicated that polyclonal antibodies inhibited tumor cell proliferation. MTT assay results after 24 h incubation of (A) J774 cells and (B) 4T1 cells with immunized serum, and after 48 h incubation of (C) J774 cells and (D) 4T1 cells with immunized serum. The results are presented as the means ± SD of three independent replicates. ***$P < 0.001$ indicates significant differences compared to the control group.

Combination therapy with polyclonal antibodies enhanced the anti-tumor efficacy of whole tumor cell lysate in vivo

We used the 4T1 tumor model to investigate the tumor growth inhibition efficacy of whole tumor cell lysate in combination with tumor cell-immunized rabbit serum. The 4T1 subcutaneous mice models were treated for 3 weeks *via* tail vein (Fig. 3A). The levels of IFN-γ in the serum were compared between the controls and the treated mice. The mean value for serum IFN-γ in the control mice was 56.67 ± 2.5 pg/mL. The mice in the whole tumor cell lysate in combination with tumor cell-immunized rabbit serum-treated group displayed significantly higher serum IFN-γ levels on day 24 than the untreated animals. The whole tumor cell lysate alone (318.33 ± 25.16 pg/mL) and the tumor cell-immunized rabbit serum-treated mice (289.66 ± 13.21 pg/mL) did show a significant increase in IFN-γ serum level in comparison with the control group (Fig. 3B). Compared with the control group, administration of whole tumor cell lysate in combination with tumor cell-immunized rabbit serum significantly inhibited the tumor growth and the established tumor was completely eradicated in treated mice. In contrast, tumorigenesis was observed in all of the mice receiving PBS, in addition, as compared with the control group, the antitumor potency observed in the groups treated with whole tumor cell lysate alone or immunized rabbit serum alone was statistically significant, whereas that in the groups treated with non-immunized serum was not significant (Fig. 3C). Tumors were excised and further proved that polyclonal antibodies significantly decreased mean tumor size in combination with whole tumor cell lysate (Fig. 3D).

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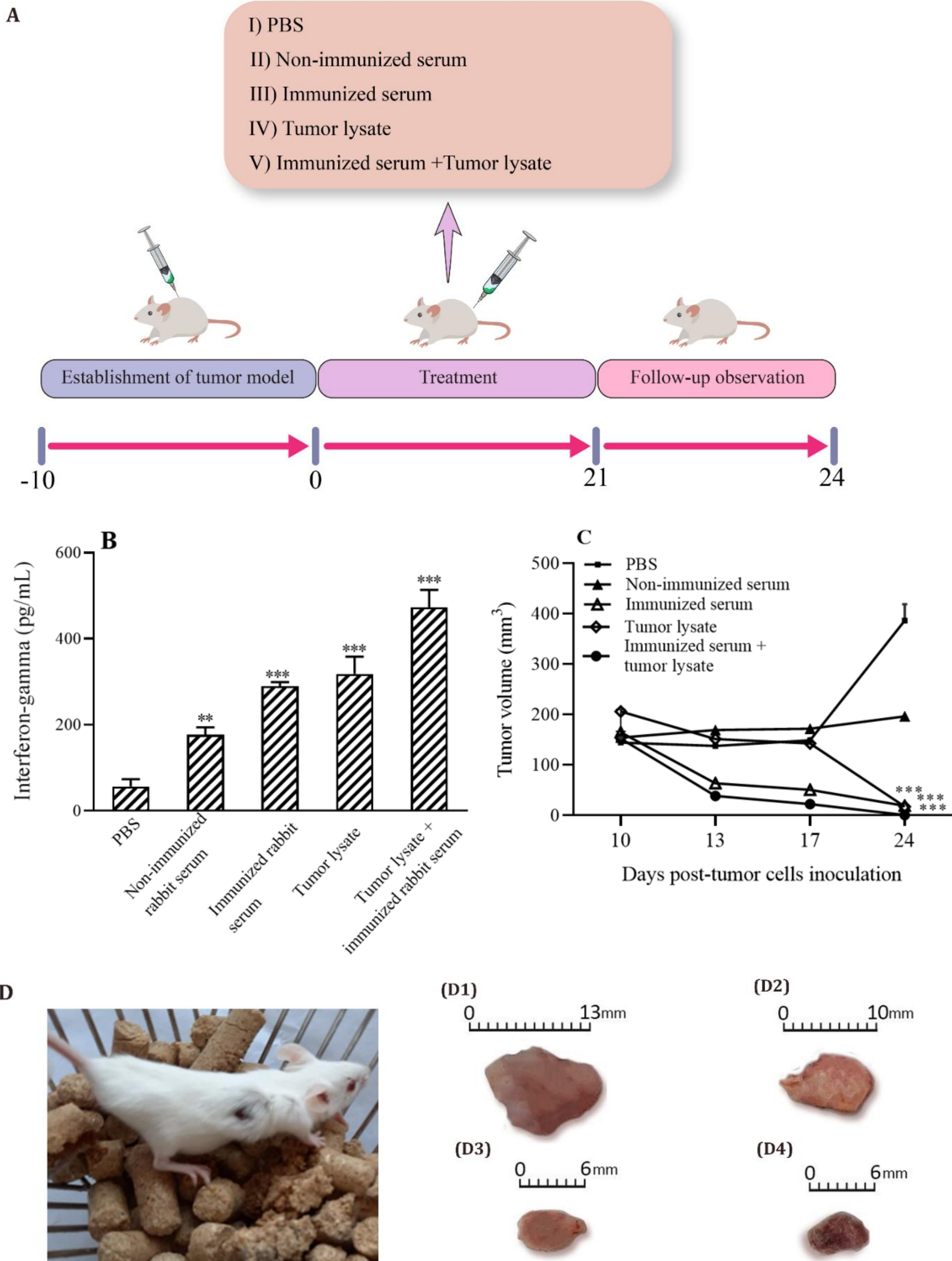

Fig. 3. Polyclonal antibody combined with whole tumor cell lysate enhanced anti-tumor efficacy *in vivo*. (A) Schematic representation of the *in vivo* treatments in mouse 4T1 breast tumor model; (B) interferon-gamma secretion assay was performed in samples of control and different treatments; (C) tumor progression curves of mice treated with various treatments; (D) anti-tumor effects of combination therapy with polyclonal antibody and whole tumor cell lysate in (D1) PBS-treated, (D2) non-immunized serum-treated, (D3) immunized serum-treated, and (D4) whole tumor lysate-treated mice. There was a non-visible tumor mass in mice treated with a combination of immunized serum and whole tumor lysate. The results are presented as the means ± SD of three independent replicates. ***$P < 0.001$ indicates significant differences compared to the control group (PBS treated).

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DISCUSSION

Recently, accumulating data indicate the efficacy of immune-based therapeutic strategies in treatment, prevention, and long-term survival in patients with breast cancer. Monoclonal antibodies and immune checkpoint inhibitors have previously received approval for cancer immunotherapy. However, only a few patients benefit, and the therapeutic effectiveness of these antibodies is limited due to the existence of resistant variations. The effective approach to stimulate the anti-tumor immune response is to use polyclonal antibodies rather than monoclonal or bispecific antibodies. Polyclonal antibodies can target several epitopes of the same or different tumor-associated antigens. According to the findings, targeting human epidermal growth factor receptor (HER) 1 and HER2 by particular polyclonal antibodies reduced the viability of a panel of human tumor lines with different HER1 and HER2 expression levels, overcoming the tumor resistance mechanism (21). However, non-specific interaction with the non-tumor antigens or non-target antigens can limit polyclonal antibody applications. Recently, polyclonal antibodies were prepared by immunizing rabbits with basic fibroblast growth factor-activated fibroblasts. Results from this study revealed that the polyclonal antibodies triggered apoptosis *in vitro* and in mice with murine CT26 colon cancer, serial intravenous infusions of polyclonal antibodies reduced tumor development (22).

To maintain their proliferation and growth, tumor cells frequently recruited and regulated alternative signaling pathways to generate compensatory survival signals (23). As a result, combination therapy and targeting different epitopes at the same time could be a powerful method for avoiding tumor recurrence and resistance (24-27). One approach of immunotherapy currently employed in development and clinical studies is cancer vaccines. Cancer vaccinations, despite their efforts, can still be regarded as ineffective therapy. Such suboptimal responses could be associated, partially, with poor tumor-associated antigen delivery, tolerance induction by dominant tumor peptides, or the absence of immunological effectors during immunizations (28,29). The advantage of using whole tumor cells as a vaccine is to provide all potential tumor-associated antigens, which can lead to numerous tumor antigens targeting, eliciting immune responses to multiple tumor antigens, avoiding tumor antigen loss and immune escape of tumor cells (30,31). In a recent study, Gleisner *et al.* evaluated the anti-tumor efficacy of heat shock-treated tumor cell lysates combined with *Concholepas concholepas* hemocyanin as an adjuvant. This therapeutic vaccine effectively suppresses the weakly immunogenic and aggressive B16F10 melanoma tumor development and prolongs tumor-bearing mice's life (9). The tumor-associated antigens-loaded autologous DCs are an immune technique with great potential for cancer treatment (32). These promote the development of immunological memory and the anticancer response. In order to successfully deliver antigens to DCs, many antigen sources have been investigated, including autologous tumor lysates, tumor-associated proteins and peptides, tumor cells, and tumor-derived mRNA (33). A recent study employed DCs loaded with allogeneic tumor cell lysates to stimulate a strong immune response. Independent of major histocompatibility complex haplotypes or the availability of autologous tumor tissue, this approach offers a reproducible pool of almost all possible antigens appropriate for patient use. Allogeneic melanoma tumor cell lysate called Melacine® was combined with an immunological adjuvant called DETOX®. Although this therapy in stage IV melanoma patients in phases I and II trials showed moderate antitumor efficacy, the adjuvant setting offers the most promise for this vaccine. The human leukocyte antigen class I antigens A2 and/or human leukocyte antigen-C3-expressing subset of melanoma patients showed a survival advantage for Melacine® in a phase III trial. It is now necessary to prospectively confirm this finding (34).

In the present study, we investigated the synergic anti-tumor effect of whole tumor cell lysate and tumor cell-immunized rabbit serum in tumor growth inhibition *in vitro* and *in vivo*.

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In this regard, we prepared polyclonal antibodies by immunizing rabbits with the human breast cancer cell line 4T1 which is extremely tumorigenic and invasive. Various *in vitro* experiments showed the specific binding capacity of tumor cell-immunized rabbit serum to 4T1 target cells. Cell proliferation assay showed the polyclonal antibodies-containing immunized serum effectively caused *in vitro* cytotoxicity to the 4T1 cells at 1:100,000 dilution after 24 h of incubation. In our experimental settings, the non-specific binding capacity and *in vitro* cytotoxicity of polyclonal antibodies-containing immunized serum on non-target J774 cells were evaluated. In addition, annexinV-FITC/PI staining revealed that the percentage of apoptotic cells in immunized serum-treated tumor cells was higher compared with the control groups.

The *in vivo* experiments showed an enhanced anti-tumor effect caused by a combination treatment of whole tumor lysate with tumor cell-immunized rabbit serum, and all the mice survived throughout the 24 days of treatment. IFN-γ plays a key role in the activation of cellular immunity and subsequently, stimulation of antitumor immune response. Based on its cytostatic, pro-apoptotic, and anti-proliferative functions, IFN-γ is considered potentially useful for adjuvant immunotherapy for different types of cancer. Moreover, IFN-γ may inhibit angiogenesis in tumor tissue, induce regulatory T-cell apoptosis, and/or stimulate the activity of M1 proinflammatory macrophages to overcome tumor progression. Subsequently, we determined the tumor inhibition effect of combination treatment of whole tumor lysate with tumor cell-immunized rabbit serum *in vivo*.

CONCLUSION

Taken together, our results indicated that the whole tumor-specific polyclonal antibodies in combination with whole tumor cell lysate could decrease tumor cell growth and induce tumor cells apoptosis. Our research might pave the way for a clinically effective combinational cancer vaccine, as well as, according to our findings this platform could be an efficient strategy for the design of clinically graded vaccines to address cancer vaccine efficacy and safety.

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Conflict of interest statement

The authors declared that no conflict of interest in this study.

Authors' contribution

H. Khanahmad and I. Rahimmanesh conceived of the presented idea; I. Rahimmanesh and H. Hejazi developed the theoretical framework; I. Rahimmanesh and Y. Esmaili carried out the experimental methods; I. Rahimmanesh and E. Ghafouri analyzed the data and wrote the article. H. Khanahmad and H. Hejazi revised the final version of the article. The finalized article was approved by all authors.

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