Autologous dendritic cells and activated cytotoxic T-cells as combination therapy for breast cancer

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Abstract. Breast cancer is the most common oncological pathology in women worldwide. Techniques for improving the clinical parameters of patients undergoing combination therapy for breast cancer are currently under development. A type of treatment employing dendritic cells (DCs) and cytotoxic DC-induced antigen-specific T lymphocytes efficiently eliminates residual cancer cells that are the key cause of tumor recurrence and metastasis. In the present study, DCs and activated lymphocytes (treated with IL-12 and IL-18) were isolated from the peripheral blood of patients with breast cancer, using a lysate of tumor tissue as antigen. The patients received the cells as part of adjuvant or neoadjuvant regimens (stage IV disease or progression). Evaluation of immunity was performed at 3 and 6 months after terminating immunotherapy. Evaluation of the disease-free period was performed for 3 years after surgery. The use of antigen-loaded autologous DCs combined with mononuclear cells with increased cytotoxic activity following Th1 polarization reduced the populations of immunosuppressive cells. The results of the present study demonstrated that the investigated cellular immunotherapy for breast cancer is safe, reduces the risk of relapse and metastasis, and improves immunity by reducing the number of regulatory T cells. Therefore, this therapeutic strategy may represent a novel approach to combating distant metastases of breast cancer.

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Abbreviations: DCs, dendritic cells; BC, breast cancer; TAAs, tumor-associated antigens; PBMCs, peripheral blood mononuclear cells; FBS, fetal bovine serum

Key words: breast cancer, dendritic cells, cytotoxicity, cytotoxic T lymphocytes, T-helper cell polarization, cancer immunotherapy

Introduction

According to the GLOBOCAN 2018 data interpretation, breast cancer (BC) is the most commonly diagnosed type of cancer and the leading cause of cancer-related mortality among women, followed by colorectal and lung cancer (in terms of incidence), and vice versa (in terms of mortality) (1). Several breast cancer patients exhibit immunosuppression, which is enhanced after surgery, radiotherapy and chemotherapy. Impaired immunity leads to T cell dysfunction, which allows tumor cells to escape immune surveillance. The insufficient inhibition of breast tumor growth may be explained by the heterogeneous expression of tumor antigens within the primary tumor or its metastases, the modification of the tumor's antigenic profile during disease progression, and the low levels of tumor-associated antigens (TAAs), MHC proteins and other costimulatory proteins required to generate an efficient immune response (2). However, as recently exemplified by metastatic non-small-cell lung cancer, tumor types not traditionally considered as responsive to immunotherapy may become immunogenic following appropriate immune activation (3). Thus, immunotherapy is currently widely recognized as a key element in the treatment of cancer, including BC (4,5).

Dendritic cells (DCs), which are considered to be the strongest stimulators of T cell responses, play a crucial role in the initiation of the primary immune response (5,6). DCs modulate the activities of immunocompetent cells, and may correct the disrupted presentation of TAAs and stimulate the production of antigen-specific cytotoxic T lymphocytes (5-10). It has also been demonstrated that DC vaccines may display powerful Th1-polarizing ability that stimulates antitumor activity against autologous tumor cells *in vitro* (11). Therapy employing DCs and cytotoxic DC-induced antigen-specific T lymphocytes efficiently eliminates residual cancer cells that are the key cause of tumor recurrence and metastasis. Therefore, the potential effectiveness of DC-based vaccines has been employed in the treatment of BC (11-13).

The selection of the DC antigen-loading strategy may affect treatment efficiency. The basic principles of obtaining antigen-loaded DCs are actively updated with new methods of *ex vivo* and *in vivo* cell modifying. The simplest methods for antigen loading of DCs are using tumor-associated antigenic peptides (14) or mRNA from tumor antigens (15). In

addition, DCs may be transfected *ex vivo* with DNA constructs encoding tumor antigens (16,17) or loaded *in vivo* using DNA vaccines (18). Although highly efficient in antigen loading and combating cancer, the DC transfection technologies and DNA vaccine approaches are labor-intensive and more costly.

The main advantage of the widely used *ex vivo* DC antigen priming approach is that it allows circumventing the dysfunction of endogenously activated DCs (19) that occurs in numerous patients with BC, and the transfer of highly active induced cells may improve the effector mechanisms involved in tumor cell lysis (20). Furthermore, the use of autologous tumor cell lysates for immune system priming allows the induction of cellular immune responses against specific tumor tissues and promotes specific antitumor response, as tumor tissues contain the most actual set of TAAs that can be unique to each patient (21). Thus, the approach involving *ex vivo* DC activation and T cell priming and the use of autologous tumor cell lysate for antigen loading is currently considered as a promising strategy for cancer treatment.

The present study modeled the naturally occurring activation induced by stimulating type 1 T-helper cell and cytotoxic T cell cytotoxicity with IL-12 and IL-18 in co-cultures of DCs and mononuclear cells. IL-18 is a pleiotropic cytokine that contributes to the regulation of innate and adaptive immunity. In the presence of IL-12 or IL-15, IL-18 powerfully induces the secretion of interferon-γ by natural killer cells and type-1 CD4 helper T cells, and modulates the activity of CD8 cytotoxic cells and neutrophils, depending on their microenvironment (22-24).

Thus, the aim of the present study was to determine whether the combination of *ex vivo* priming of DCs with antigens present in tumor lysates and the *in vitro* formation of a pool of antigen-specific cytotoxic cells is promising for efficient antitumor immune response activation in BC patients.

Materials and methods

Study design and eligibility criteria. The present phase I/II prospective study was designed to evaluate the toxicity, antitumor activity and immune responses to vaccination. The eligibility criteria were as follows: Age 28-65 years, leukocytes >3,000/mm^3, neutrophils >1,500/mm^3, platelets >100,000/mm^3, and negative tests for human immunodeficiency virus, and hepatitis B and C viruses. The exclusion criteria were as follows: Cerebral metastases, positive pregnancy test, autoimmune diseases or other medical conditions, such as decompensated heart failure, severe anemia and pancytopenia, that constitute contraindications. Prior chemotherapy and treatment with cytokines was permitted; however, concomitant immunotherapy was not. The trial was conducted in accordance with the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from each patient prior to inclusion. Patients were enrolled between December 2013 and October 2015 at the Third Oncological Department of the Novosibirsk City Clinical Hospital No. 1. The patients received DC therapy in the Clinic of Immunopathology of the Institute of Clinical and Fundamental Immunology.

Historical controls were recruited from the database of three oncology departments of the Novosibirsk City Hospital. The medical records of patients treated between 2012 and 2014 were reviewed. For each patient from our group, a patient who did not receive immunotherapy was selected. The patients and control subjects were matched for age, stage, molecular type and treatment regimen. Selected cases were analyzed to determine the clinical outcome at 3 years after surgery.

The study protocol was approved by the local Ethics Committee of the Institute of Clinical and Fundamental Immunology (protocol no. 78, September 12, 2013). The study is registered at www.clinicaltrials.gov (NCT 03113019). The patent of the Russian Federation was obtained for the approach described (no. 2645464).

Evaluation of patients and treatment schedule. Clinical evaluation included a complete medical history, chest X-ray, tumor staging, histological and immunohistochemical analysis of tumors, blood chemistry, hematology and urine analysis. Patients received three vaccinations at 1-week intervals. Autologous cells were injected into patients with stage IIa-IIIc BC after completion of chemotherapy. Patients with underlying progressive disease or those initially diagnosed with stage IV disease were injected with autologous immune cells during the interval between hormone therapy cycles.

Preparation of tumor cell lysates. Tumor cell lysates were prepared from tissue samples after radical surgery or incisional biopsy. A 1-3-cm^3 tumor tissue sample was placed into a sterile tube, the adjacent (macroscopically unaltered) tissues were removed, and the sample was subjected to mechanical homogenization and four freeze-thaw cycles (-80˚C and room temperature, respectively). Larger particles were removed by centrifugation at 266 x g and 24˚C (room temperature) for 2 min. The lysate was filter-sterilized (filter pore diameter=0.45 μm; TPP Techno Plastic Products AG), and the protein concentration of the lysate was measured using a NanoDrop 2000 (Thermo Fisher Scientific, Inc.). The tumor cell lysate was divided into aliquots, frozen, and stored at -80˚C.

DC preparation and characterization. Mononuclear cells were isolated from the peripheral blood of the patients using Ficoll gradient centrifugation. The isolated peripheral blood mononuclear cells (PBMCs) were washed in RPMI-1640 medium (Biolot) and centrifuged twice at at 266 x g and 24˚C (room temperature) for 10 min. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS (HyClone; GE Healthcare Life Sciences), 2 mM L-glutamine (Biolot), 10 mM HEPES (Biolot), 80 μg/ml gentamycin (KRKA), and 100 μg/ml ampicillin (Sintez). Mononuclear cells (1-1.5x10^6 cells/ml) in RPMI-1640 complete medium supplemented with 10% FBS were placed into 150-cm^2 (690 ml) culture flasks (TPP Techno Plastic Products AG) with vented caps. The cells were allowed to adhere to the flasks in a CO_2 incubator at 37˚C and 100% humidity for 30 min. Viable non-adherent mononuclear cells (non-adherent PBMCs) were cultured in complete RPMI-1640 medium with partial media changes on days 3 and 5. The adherent cells were removed using a cell scraper and washed with RPMI-1640 medium. The adherent cell fraction was used to generate mature antigen-activated DCs. For this purpose, 100 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; BioVision, Inc.) and 50 ng/ml IL-4 (BioVision,

Inc.) were added to the cells, which were cultured in a 75-cm^2 (270 ml) (TPP Techno Plastic Products AG) culture flask in complete RPMI-1640 medium supplemented with 10% FBS for 72 h to generate immature DCs. The tumor tissue lysate (100 μg/ml) was added to immature DCs for 24 h. To obtain mature DCs, TNF-α (25 ng/ml) (BioVision, Inc.) was added to the fresh medium within 48 h. The DC preparations were subjected to quality control tests (viability, cell count, purity) and then to flow cytometry using a FACSVerse flow cytometer (Becton-Dickinson and Company). Monoclonal antibodies (mAbs) were as follows: CD11c (cat. no. 371508, cloneS-HCL-3), CD83 (cat. no. 305310, clone HB15e), HLA-DR (cat. no. 307604, clone L 243), CD86 (cat. no. 305406, clone IT2.2), CD123 (cat. no. 306012, clone 6H6) (BioLegend, Inc.), Lineage Cocktail (cat. no. 348801, clone UCHT1, HCD14, 3G8, HIB19, 2H7 and HCD56) (BioLegend, Inc.); CD205 (cat. no. 558069, clone MG 38) and CD209 (cat. no. 558263, clone DCN 46) (Becton-Dickinson and Company). All antibodies from BioLegend were added as 5 μl per 1 million cells in 100 μl staining volume (antibody:cell suspension volume ratio, 1:20). The Lineage Cocktail, CD205 and CD209 (Becton, Dickinson and Company) were added as 20 μl per 1 million cells in 100 μl staining volume (ratio, 1:5). All dilutions mentioned were as recommended by the manufacturers.

Activation, characterization and injection of mononuclear cells. Mature DCs and non-adherent PBMCs were mixed at a ratio of 1:10 and cultured in the presence of 8 ng/ml IL-12 and 80 ng/ml IL-18 (both from BioVision, Inc.) to stimulate Th1 polarization of the activated T cells over 96 h. The resulting cell suspensions (autologous tumor lysate-loaded mature DCs and activated PBMCs) were washed and frozen in Freezing Medium (Biolot) supplemented with 10% FBS and 10% DMSO. The cells were stored at -150˚C and defrosted on the day of injection. The cryotube containing the cells was placed in a water bath and the cells were then transferred to a sterile 15-ml test tube containing 4 ml RPMI-1640 medium and 1 ml FBS. The cells were then thoroughly resuspended and centrifuged at 266 x g and 24˚C (room temperature) for 10 min. An aliquot of cells was used for analysis. The thawed cells were cultured for 3 h in RPMI-1640 medium supplemented with 10% FBS, and the non-adherent fraction was prepared for intravenous administration. The cells were washed with 0.9% sodium chloride (saline) solution three times, and cell count and viability (≥95%) were determined. The cells for intravenous administration (20-30x10^6) were resuspended in 100 ml 0.9% saline solution supplemented with 2 ml 10% albumin.

The adherent fraction was removed from the culture flask surface using a cell scraper, the cells were washed three times with saline, and cell count and viability (≥95%) were determined. The cells for subcutaneous administration (2-3x10^6) were resuspended in 800-1,000 μl 0.9% saline solution and injected subcutaneously into three sites within the intrascapular region. Intravenous administration of the cells was performed along with 8 mg dexamethasone. All the cell preparations were subjected to quality control (viability, cell count and purity) and then to flow cytometry analysis using a FACSVerse flow cytometer (Becton-Dickinson and Company). The mAbs (20 μl per 100 μl staining volume) used were as follows: 6-color TBNK (cat. no. 644611; Becton-Dickinson and Company) containing CD45, CD3, CD4, CD8, CD19 and CD16/56.

Evaluation of immunity. To evaluate the relative content of $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, $CD16/56^+$ and $CD4^+CD25^+$ $FoxP3^+$ cells, circulating DCs and the expression of HLA-DR on monocytes, PBMCs were subjected to flow cytometry before surgery, before immunotherapy, and at 3 and 6 months after completion of the immunotherapy.

Cytotoxicity assay. Cytotoxicity was assessed by determining the lactate dehydrogenase (LDH) activity in the conditioned medium of DC and non-adherent PBMC co-cultures and the BC-derived cell line MCF-7 (Russian Collection of Cell Cultures, Institute of Cytology), using a CytoTox 96 Non-Radioactive Cytotoxicity Assay (G1780; Promega Corporation). PBMCs were isolated using Ficoll gradient centrifugation (PanEco) from peripheral blood before the immunotherapy and at 3 and 6 months after completion of the immunotherapy. Cells were frozen in Freezing Medium (Biolot) supplemented with 10% FBS and stored at -150˚C. Samples acquired from each patient before and after therapy were tested in the same experiment. PBMCs (1-10^5 cells per well) were incubated in triplicate in 96-well round-bottom tissue culture plates (10:1, PMBCs:tumor cells) for 16 h. LDH activity in the culture supernatants was measured using a 30-min coupled enzymatic assay that measures the conversion of the tetrazolium salt INT into red formazan. The absorbance of visible light was determined using a standard 96-well plate reader. The amount of color was proportional to the number of lysed cells.

Evaluation criteria and statistical analysis. Adverse events were classified according to the CTCAE ver. 4.03 (2010) (25). The World Health Organization criteria (WHO Handbook for Reporting the Results of Cancer Treatment, 1979, Geneva) (26) were used for patients with stage IV disease or progression of the underlying disease. A complete response) was defined as the complete disappearance of all clinically detectable disease. A partial response was defined as a ≥50% decrease in all measurable lesions, without an increase in the size of any target lesion or the appearance of new lesions. Stable disease was defined as the absence of a significant change for 4 weeks or an increase of <25% or a decrease of <50% in tumor size, and no new lesions. Progressive disease was defined as a ≥25% increase in the sum of the products of the measurable lesions or appearance of new lesions. For patients with stages 2a-3c, blood was collected after 3 and 6 months to evaluate immunological and clinical parameters.

GraphPad Prism 6 software (GraphPad Software, Inc.) was used to analyze the data, which are presented as the median and the interquartile ranges (upper and lower quartile, UQ and LQ, respectively). Significant differences between the means were determined using ANOVA for repeated measurements and Tukey's multiple comparisons test. The Kaplan-Meier method was used to generate survival curves. The medical records of patients of the same age and disease stage were analyzed to generate the Kaplan-Meier curves. $P<0.05$ was considered to indicate statistically significant differences.

Results

Patient characteristics. The clinical characteristics of the patients (n=30) are listed in Table I. A total of 25 patients underwent surgery, 3 patients had stage IV disease, 1 patient experienced disease progression after surgery, and 1 patient progressed during neoadjuvant chemotherapy. In the remaining 5 patients, tumor samples were obtained using incisional biopsy of the metastases (skin). The sites of metastasis included the lungs, skin, lymph nodes and bones. A tumor sample was obtained during radical surgery (radical mastectomy or radical sector resection together with axillary lymphadenectomy) in 25 patients. All patients received chemotherapy (anthracyclines and/or taxanes) and radiotherapy after surgery.

Characterization of DCs and activated mononuclear cells used for injection. DCs were prepared from autologous monocytes cultured in the presence of GM-CSF and IL-4. The DC preparations were subjected to quality control tests (viability, cell count and purity) and then to flow cytometry using a FACSVerse flow cytometer. Cell viability was 90-95% as assessed by Trypan blue staining. The purity of the mature DC population was determined by flow cytometry. To determine the phenotype of mature DCs, lineage-negative ($CD3^-$, $CD14^-$, $CD16^-$, $CD19^-$, $CD20^-$ and $CD56^-$) $HLA\text{-}DR^+$ cells were isolated from the population of $CD45^+$ cells, and the levels of CD123-plasmacytoid and CD11c-myeloid DCs were determined. Their populations were 2.35% (UQ; LQ: 4.45; 1.25) and 51.75% (UQ; LQ: 64.35; 23.925), respectively. To assess the expression of specific molecules expressed by DCs in the population of large granular lymphocytes, the $CD11c^+HLA\text{-}DR^+$ cell population was isolated, and the abundance of $CD83^+CD86^+$ and $CD205^+CD209^+$ double-positive DCs was 97.65% (UQ; LQ: 94.925; 99.3) and 97.5% (UQ; LQ: 99.1; 92.9), respectively (Table II).

The main subpopulations were measured using the gating scheme recommended by the manufacturer (6-color TBNK, Becton, Dickinson and Company). Typical dot plots displaying the gating scheme used are provided as supplementary material (Fig. S1). The cellular compositions of the fraction of injected mononuclear cells were characterized accordingly (Table II). $CD3^+$ and $CD3^-$ T cells were isolated from the $CD45^+$ T cell population. $CD4^+$ and $CD8^+$ T cell counts were determined in the $CD3^+$ T cell population, while $CD16^+/56^+$and $CD19^+$cell counts were determined in the $CD3^-$ T cell population. DCs and activated non-adherent PBMCs were generated in all patients. The cultured adherent cells developed elongated, stellate cell processes, which are characteristic of DCs. Following co-culture with non-adherent PBMCs, these cells formed characteristic clusters.

Clinical activity for patients with stage IV or progressive disease. Among patients with stage IV disease, 2 remained stable for 6 months. Patient no. 25 developed local progression, distant foci were undetectable, and chemotherapy was not administered. Patient no. 23 succumbed to disease progression (two injections were administered). Three lines of chemotherapy did not achieve positive response. After cell infusion, the patient noted a decrease in the pain associated with the affected breast; however, it was impossible to perform confirmatory tests. The general condition of patient no. 24 deteriorated (multiple bone metastases, pathological fracture of the femoral neck). For personal reasons, the patient refused treatment with the recommended antitumor drugs (except zoledronic acid). After the introduction of the cells, the patient noted a decrease in pain in the lumbar spine.

Immune responses. No significant differences were observed in the numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD16/56^+$ cells before surgery and after immunotherapy. Statistically significant differences were detected only for $CD19^+$ B cells (Fig. 1). There was a consistent reduction in this cell population when the immunotherapy commenced compared with that prior

Table I. Clinical characteristics of the patients.

Characteristics	Number of patients (%)
Disease stage	
T2N0M0 (IIA)	10 (34.48)
T1N1M0 (IIA)	3 (10.34)
T2N1M0 (IIB)	6 (20.68)
T2N2M0 (IIIA)	2 (6.9)
T2N3M0 (IIIC)	2 (6.9)
T1N3M0 (IIIC)	1 (3.45)
TxN3M0 (IIIC)	1 (3.45)
T2N2M0 (IIIA), progression after neoadjuvant therapy	1 (3.45)
T2N2M0 (IIIA), progression after therapy	1 (3.45)
T2NxM1 (IV)	1 (3.45)
T4N3M1 (IV)	1 (3.45)
TxNxM1 (IV)	1 (3.45)
Histological characteristics of the tumor after radical surgery	
Moderately differentiated invasive ductal carcinoma (GII)	25 (100.0)
Solid-glandular growth type	22 (88.0)
Solid-glandular with areas of scirrhous growth pattern	1 (4.0)
Solid-trabecular	1 (4.0)
Solid-cribriform	1 (4.0)
Molecular subtypes of the tumor after radical surgery	
Isolated expression or overexpression of HER-2	7 (28.0)
Triple-negative	5 (20.0)
Luminal A	10 (40.0)
Luminal B (overexpression of HER-2/neu)	1 (4.0)
Luminal B (Ki-67 >20%).	2 (8.0)

HER-2, human epidermal growth factor receptor 2.

Table II. Cellular composition of the vaccine used to immunize patients who underwent the immunotherapy with autologous antigen-activated DCs. The data are presented as the median and interquartile range (n=25).

$CD45^+$ leukocytes (100%)				$CD45^+$ leukocytes (100%)	
$CD3^+$ 85.8% (UQ; LQ: 88.05; 80.75)		$CD3^-$ 14.2% (UQ; LQ: 19.25; 11.95)		Lineage-negative ($CD3^-$, $CD14^-$, $CD16^-$, $CD19^-$, $CD20^-$ ans $CD56^-$) HLA-DR	
$CD4^+$	$CD8^+$	$CD16/56^+$	$CD19^+$	CD123-plasmacytoid DCs 2.35% (UQ; LQ: 4.45; 1.25)	CD11c-myeloid DCs 51.75% (UQ; LQ: 64.35; 23.925)
59.3%	21.8%	36.7%	23%		$CD83^+CD86^+$ 97.65% (UQ; LQ: 94.925; 99.3)
(UQ; LQ: 63.83; 51.03)	(UQ; LQ: 31.15; 18.28)	(UQ; LQ: 43.55; 28.83)	(UQ; LQ: 27.25; 15.8)		$CD205^+$ $CD209^+$ 97.5% (UQ; LQ: 99.1; 92.9)

UQ, upper quartile; LQ, lower quartile; DCs, dendritic cells.

to surgery, and the population of these cells was found to be increased at 3 and 6 months after the immunotherapy.

To assess the levels of regulatory T cells among $CD45^+$ leukocytes, the $CD4^+CD25^+$ cell population was isolated, and the number of cells expressing the FoxP3 marker was determined (Fig. 2). Typical dot plots displaying the gating scheme used for the FoxP3 expression evaluation are provided as supplementary material (Fig. S2). There was a consistent decrease in the level of regulatory T cells 3 months after the immunotherapy, which was maintained for another 3 months.

To assess the percentages of activated HLA-DR-positive and -negative $CD14^+$ monocytes (monocytes and suppressor myeloid precursor cells, respectively) (27), $CD14^+$ monocytes were isolated from $CD45^+$ leukocytes, and HLA-DR expression was assessed (Fig. 3) The relevant dot plots are provided as supplementary material (Fig. S3). The levels of $CD14^+$HLA-DR^+ monocytes consistently increased 3 months after the immunotherapy, while the levels of myeloid suppressor cells consistently decreased and remained low 6 months after the immunotherapy.

The relative amounts of $CD45^+CD123^+$ cells (plasmacytoid DCs) and $CD45^+CD11c^+$ cells (myeloid DCs) were also assessed [(typical dot plots displaying the gating scheme used are provided as supplementary material (Fig. S4)]. Lineage-negative/HLA-DR-positive cells were isolated from the population of $CD45^+$ leukocytes, and the levels of $CD123^+$-plasmacytoid and $CD11c^+$-myeloid DCs were determined (Fig. 4). The data are presented as the percentage of CD123- and CD11c-positive cells among the $CD45^+$ cells. During immunotherapy, the numbers of myeloid DCs increased, while those of plasmacytoid DCs gradually decreased 3 and 6 months after the immunotherapy.

The cytotoxicity of mononuclear cells against the MCF-7 cell line gradually increased during months 3 and 6 after the immunotherapy compared with baseline (Fig. 5).

Among the patients who were administered adjuvant immunotherapy, hyperplasia of the subclavian lymph node, which was difficult to access, was detected in patient no. 1 who had stage IIIc BC in 2014. No further progression was observed. Progression of the underlying illnesses and fatal outcomes of 2 patients was characterized by metastasis to the brain (patient no. 15), and recurrence in the axillary lymph nodes and further spread to the liver (patient no. 17). Patient no. 22 experienced local recurrence.

Side effects. The treatment was generally well-tolerated. The patients were monitored by medical personnel for 24 h after injection to assess toxicity (CTCAE ver. 4.03, 2010). The most common adverse events were flu-like symptoms, such as fever and fatigue, that did not require additional treatment or prolonged hospitalization. All symptoms spontaneously resolved without treatment after 2-3 h.

Disease-free period. The 3-year relapse-free periods of the 25 patients who received adjuvant immunotherapy were compared with those of the control group who were not immunized (historical controls) (Fig. 6). The clinical characteristics of patients in the control group and patients after immunotherapy are compared in Table III. The day of surgical treatment was considered as the starting point.

Table III. Clinical characteristics of patients in the control group (n=28) and after receiving immunotherapy (n=25).

Characteristics	Immunotherapy	Control
Number	25	28
Age, years [mean (range)]	48.8 (28-65)	46.48 (28-62)
Disease stage, n (%)		
IIA	13 (52)	13 (46.43)
IIB	6 (24)	8 (28.57)
IIIA	2 (8)	4 (14.29)
IIIC	4 (24)	3 (10.71)
Molecular subtypes of the tumor after radical surgery, n (%)		
Isolated expression or overexpression of HER-2	7 (28)	5 (28)
Triple-negative	5 (20)	8 (20)
Luminal A	10 (40)	12 (40)
Luminal B (overexpression of HER-2/neu)	1 (4)	2 (4)
Luminal B (Ki-67 >20%).	2 (8)	1 (8)
Recurrence rate for different molecular subtypes, n (%)		
Isolated expression or overexpression of HER-2	2 (8)	3 (10.71)
Triple-negative	2 (8)	4 (14.29)
Luminal A	1 (4)	5 (17.86)
Luminal B (overexpression of HER-2/neu)	0	2 (7.12)
Luminal B (Ki-67 >20%).	0	0
General recurrence rate, %	25 (5/25)	50 (14/28)
Median time to progression, months	20.5	16.5

HER-2, human epidermal growth factor receptor 2.

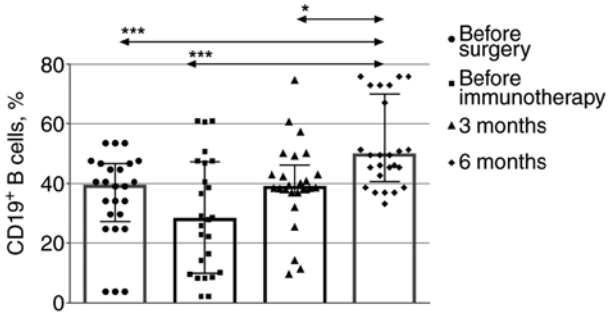

Figure 1. Levels of $CD19^+$ B cells in the peripheral blood of the patients (n=25). $^*P=0.05$, $^{***}P=0.001$.

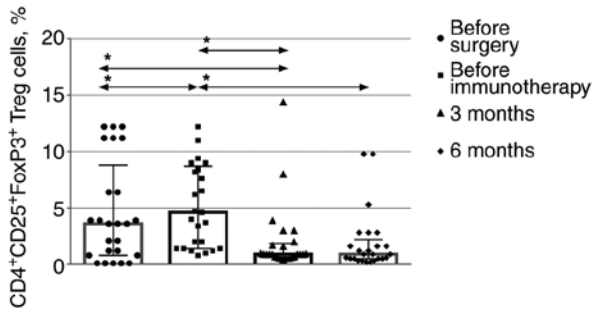

Figure 2. Levels of regulatory T (Treg) cells in the peripheral blood of the patients (n=25). $^*P=0.05$.

Discussion

The overall incidence of BC has increased over the past 30 years, reflecting the increase in absolute and relative incidence. The absolute increase, which is caused by socio-economic factors, reflects the increase in the number of newly diagnosed patients with BC.

Restoration of antitumor cell-mediated immunity, particularly that mediated by the T cell component, during combination cancer therapy is required to destroy cells in the primary tumor, as well as to eliminate metastatic cells. In our earlier preclinical studies using DCs to stimulate a cytotoxic response, the safety and effectiveness of using TAA-loaded autologous DCs were assessed (28,29). A number of studies have confirmed the ability of mature antigen-loaded DCs to successfully present tumor antigens to T lymphocytes *in vitro* and *in vivo* (30,31). Modification of T cells using both natural adjuvants and genetic engineering methods may help overcome the mechanisms of tumor immune escape (30). However, there remains the question of whether the endogenously activated T cell response is able to mediate tumor regression, since tumor progression is often observed, even in the presence of high levels of circulating blood cells or tumor-infiltrated T cells (33,34). It is known that cellular immunotherapy is not sufficient to completely destroy solid tumors, but the approach using T cells activated *ex vivo* by antigen-loaded DCs may be efficient for elimination of minimal residual disease represented by single tumor cells remaining in the body after

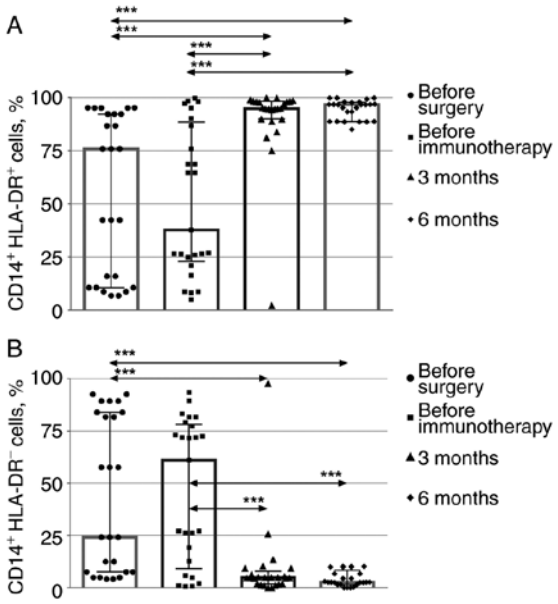

Figure 3. Levels of (A) HLA-DR-positive and (B) HLA-DR-negative CD14$^+$ cells in the peripheral blood of patients (n=25) who received immunotherapy using autologous antigen-activated dendritic cells. ***P=0.001.

eradication of the main tumor burden via surgery and/or chemotherapy and/or radiotherapy in order to prevent relapse or metastasis (35).

In the present study, antigen-loaded DCs were generated in all patients. DCs are adherent cells with an elongated stellate shape and exhibit the typical mature phenotype. A cell suspension produced by co-culturing DCs and non-adherent mononuclear cells in the presence of IL-12 and IL-18 was used for injection. The viability of the suspension after thawing was 95-98%. Examination of the subpopulations of the resulting mononuclear cells demonstrated that, compared with the standard peripheral blood parameters, the percentage of CD16$^+$/CD56$^+$ cells in the suspension injected into patients increased to 36.7% from the normal range of 4.2-25.2%, while the percentage of CD8$^+$, CD4$^+$ cells was within the normal range. Cell injection was tolerated well by all patients, and complications were not reported.

Measurements of the cytotoxic activity of activated mononuclear cells against the MCF-7 cell line displayed a consistent increase at 3 and 6 months after the immunotherapy compared with the baseline. Elevated numbers of CD19$^+$ B cells and myeloid DCs were detected, while the numbers of immunosuppressive myeloid precursors and regulatory T cells simultaneously decreased. Changes in the percentages of CD8$^+$, CD4$^+$ T cells were undetectable.

The growth of a tumor and its microenvironment leads to the appearance of immunosuppressive factors as well as the appearance of cells with suppressive properties, including regulatory T cells. The absence of tumor load and cell destruction during chemotherapy may lead to a decrease in the level of regulatory T cells. In this regard, the present results showing such a decrease are consistent with those of similar research on the treatment of kidney cancer (36).

Considering the changes in the numbers of regulatory T cells and increased cytotoxic activity, it is reasonable to conclude that a qualitative change in the T cell population occurred after the therapy. In addition to the changes in the numbers of regulatory T cells, those of myeloid suppressor cells and plasmacytoid DCs decreased, while the percentage of myeloid DCs increased. Thus, the protective response increased in patients within 6 months after the immunotherapy, while the size of the suppressor population decreased.

The predicted survival rates and durations of the relapse-free period of patients with BC are attributed to characterizing the tumor using the TNM system as well as determining the molecular subtypes of tumor cells, including expression levels of estrogen, progesterone and human epidermal growth factor receptor 2 (HER-2/neu) receptors along with the Ki-67 index. In the present study, patients with operable cancer had all four tumor molecular subtypes (Table I). Disease progression during the first year after immunotherapy was observed in 1 patient with triple-negative BC and relapse occurred in 2 patients with isolated expression of HER-2/neu (HER-2/neu 3$^+$) (i.e., patients with an initially less favorable outcome) (35,37). Progression in 2 patients occurred towards the end of the 3-year follow-up period.

The effectiveness of immunotherapy was assessed according to the regression of tumor foci in patients with tumor progression, or initially found to have stage IV disease. A total of 2 patients achieved a positive response, which lasted for 9 and 6 months, respectively. These patients continued treatment recommended by the case conference. A total of 2 patients received palliative cellular immunotherapy. Distant changes were undetectable in 1 patient with disease invading into the soft tissues of the breast, and only limited local progression was observed. The tumor in another patient changed its molecular subtype from luminal A to luminal B during progression (emergence of HER-2/neu expression), indicating that tumor aggressiveness increased. Moreover, all tumor samples (biopsies) in this group of patients were originally luminal A or B subtype.

It should also be taken into consideration that the therapy was safe and comfortable for the patients. A small sample of patients in our hospital exhibited fewer relapses after undergoing a course of immunotherapy and an increase in the median time to disease progression. Our patients experienced positive changes in immune responses, such as a decrease in the level of regulatory T cells. Similar changes, particularly those of regulatory T cells, in the patients' immune responses after treatment with DCs and cytokine-induced killer cells was demonstrated in a study using cellular therapy to treat renal cancer (36).

The principal features of our research may be summarized as follows: First, *ex vivo* DC activation was used, which enables avoiding impaired maturation and antigen loading of DCs that usually occur in the presence of a tumor and its microenvironment. Next, after *in vitro* antigen-loading of DCs and further *in vitro* activation of lymphocytes in non-adherent PBMC and DC co-culture, antigen-loaded DCs and DC-activated PBMCs were injected into the patients in order to eliminate tumor cells via the transferred DC-activated lymphocytes and to continue the activation of T cells by antigen-loaded DCs *in vivo*. There was an observed strong tendency for an increase in the protective immune response in patients within 6 months after the immunotherapy, while the size of the suppressor population decreased. Thus, it was inferred that the use of autologous DCs loaded with antigens in combination with mononuclear cells with increased cytotoxic activity during Th1 polarization

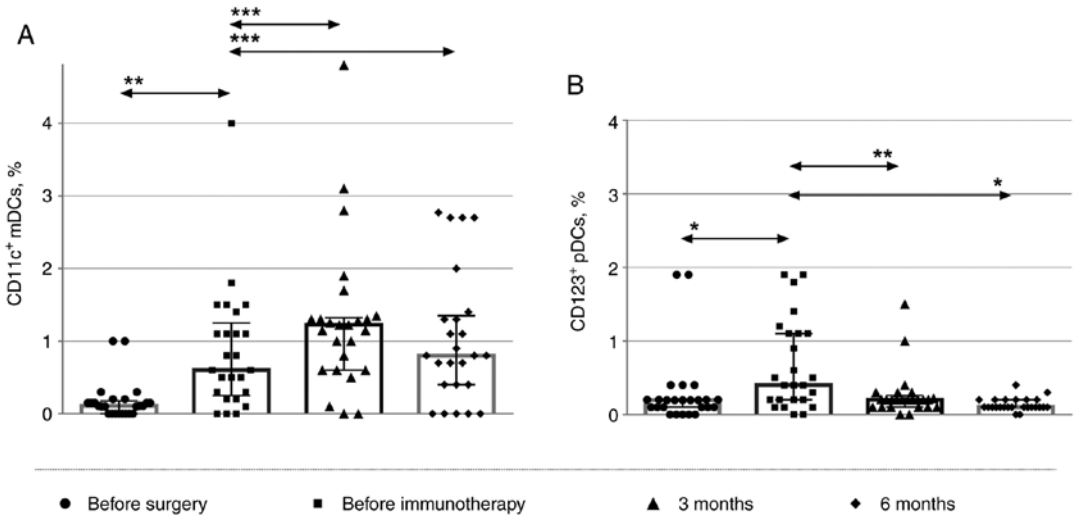

Figure 4. Levels of (A) myeloid and (B) plasmacytoid DCs among peripheral antigen-activated DCs. *P=0.05, **P=0.01, ***P=0.001. DCs, dendritic cells.

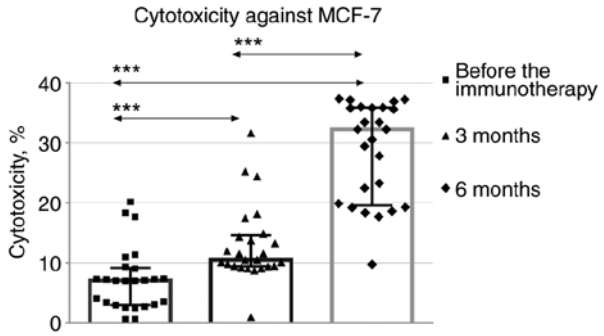

Figure 5. Cytotoxic activity of mononuclear cells against the MCF-7 cell line in patients (n=25) who received immunotherapy using autologous antigen-activated dendritic cells. ***P=0.001.

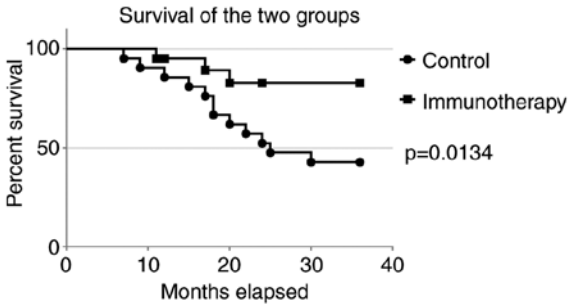

Figure 6. Analysis of the 3-year relapse-free period of the 25 patients who received adjuvant immunotherapy and of the control group (n=28) starting on the day of the surgery.

represents a promising immunotherapeutic approach to the prevention or treatment of metastatic foci and may be used in the treatment of patients with stage IV BC.

It should be noted as a limitation of the present study that no imaging data were provided. The case management of the patients was performed based on the data provided by the Third Oncological Department of the Novosibirsk City Clinical Hospital No. 1, where the patients underwent multispiral computed tomography examination at different treatment phases. The imaging data were only provided by the supervising clinics as official textual conclusions from certified specialists. We could not request imaging data from the Oncological Department that provided us with clinical data on the cases, as the results of imaging screenings are not collected there as images.

We herein demonstrated that the administration of cell suspensions containing autologous tumor lysate-loaded mature DCs and activated PBMCs is a feasible approach to inducing an antitumor response. However, the percentage of patients who achieved objective long-term tumor regression was very small. The most common outcome is an extended antigen-specific response in the absence of a pronounced clinical response (38,39). The aim of the present approach involved 'priming' naïve T cells using antigen-loaded DCs in the presence of IL-12 and IL-18 to elicit a T cell antitumor response. It should also be noted that the approach investigated in the present study helped us i) induce an antitumor response against breast cancer cells via generation of activated cytotoxic cells, which is demonstrated in our further studies (28,29), and ii) generate long-lived memory cells to prevent late relapses, which was also demonstrated in our recent research (40).

The clinical effectiveness of our cellular protocol was demonstrated in the present study and verified by the effects on the patients' antitumor immunity, which was characterized by enhanced cell-mediated cytotoxic immune responses and lower percentages of suppressor cells in the peripheral blood within 6 months after the initiation of immunotherapy. However, we believe that the maximum effect of cancer immunotherapy can only be achieved using a combined two-stage strategy. Therefore, it may be concluded that it is reasonable to conduct anti-suppressor therapy targeted against suppressor cell populations (41,42) or their mediators (e.g., targeted immunotherapy using monoclonal antibodies or immune cells) as the first stage and cellular immune-stimulating antitumor therapy (cellular immunotherapy) as the second stage.

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Availability of data and materials

The authors confirm that the data supporting the findings of the present study are available in this published manuscript. Additional data supporting the findings of this study and basic research materials are available from the corresponding author on reasonable request.

Authors' contributions

JAS contributed to the design, experimental work, and optimization of each experimental stage, analysis and interpretation of data, and drafting of the manuscript. AAK contributed to the conception, design, and data interpretation. VVK performed experimental work (flow cytometry) and contributed to data interpretation. MSK conducted experimental work (optimization of adherent PBMC isolation protocol) and revision of the manuscript. DDB performed all the injection procedures and tracking of clinical indicators and adverse events. NMS supervised the patients during the immunotherapy. SVSi performed patient recruiting, oncological treatment and obtained tumor material. SVSe contributed to the conception, design, revision, and final approval of the manuscript. All authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with the Declaration of Helsinki with written informed consent from all subjects. The protocol was approved by the local Ethics Committee of the Institute of Clinical and Fundamental Immunology (protocol no. 78, September 12, 2013).

Patient consent for publication

The publication of data provided in the study does not compromise the anonymity or confidentiality of the participants. Written informed consent was obtained from each patient prior to inclusion. The informed consent was approved by the local Ethics committee of the Institute of Clinical and Fundamental Immunology.

Competing interests

All the authors declare that they have no competing interests.

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