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ONCOLOGY

Effects of *Tussilago farfara* L. Polysaccharides on the Expression of PD-1 (CD279) and PD-L1 (CD274) in Peripheral Blood and Tumor Tissue Lymphocytes in Mice with Lewis Lung Carcinoma

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One of prospective methods for immunotherapy of tumors is modulation via immunological checkpoints, specifically, via the PD-1(CD279)/PD-L1(CD274) system. Interactions between tumor cell receptor (CD279) and the ligand on lymphocytes (CD274) leads to lymphocyte inactivation, which allows tumor escape from the immune control. Experiments on C57BL/6 mice with Lewis lung carcinoma demonstrate the possibility of reducing the expression of CD279 and CD274 on the peripheral blood and tumor tissue lymphocytes under the effects of *Tussilago farfara* L. polysaccharides. This phenomenon can underlie the antitumor and antimetastatic effects of these substances.

Key Words: *polysaccharides; PD-1; PD-L1; immunotherapy; flow cytofluorometry*

Immunotherapy based on modulation via immunological checkpoints has changed and improved the treatment strategy used in patients with cancers [6]. Tumor cells suppress the immune response directed against them by modulating the PD-1/PD-L1 system. The programmed cell death-1 protein (PD-1) is expressed on the surface of activated T cells [2]. Interactions of PD-1 with its ligand PD-L1, located on the tumor and tumor microenvironment cells, lead to inactivation of T cells and hence, to immunosuppression and immune response inhibition [7]. Modulation of PD-1 protein and its ligand PD-L1 is a new promising approach to cancer control.

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By the present time PD-1/PD-L1 inhibitors, based on monoclonal antibodies, are approved for clinical use. Clinical trials and creation of new drugs, modulating the immunological checkpoints for malignant cell growth control, are in progress. Despite high efficiency of the available drugs for immunotherapy of tumors, their use is associated with the development of numerous side effects, such as dermal manifestations, gastrointestinal tract involvement (colitis, diarrhea, endocrinopathy and hepatotoxicity of varying severity), the gravest of these adverse reactions are severe allergic reactions and autoimmune diseases [8]. In addition, the price of innovation drugs is extremely high. The WHO experts think that no country in the world can fully compensate for the cost of treatment with immunological drugs [4]. That is why creation of new effective and available drugs producing no untoward side effects remains a pressing problem. Drugs

of plant origin, for example, polysaccharides, capable of modulating the immune system, improving the efficiency of antitumor therapy, and attenuating the toxic effects of cytostatic drugs on intact organs and tissues, seem to be a promising trend of research [5].

We studied the effects of *Tussilago farfara* L. polysaccharides on the expression of PD-1 (CD279) and PD-L1 (CD274) in peripheral blood and tumor tissue lymphocytes in mice with Lewis lung carcinoma (LLC).

MATERIALS AND METHODS

Experiments were carried out on first category C57BL/6 females (19-20 g) bred at the Laboratory of Experimental Biomodels, E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine (quality certificate No. 188-05). The animals were kept and handled in accordance with Directive 2010/63/EU of the European Parliament and EC Council on Protection of Animals used for Scientific Purposes. The study was carried out in accordance with the Order No. 199n (Principles of Good Laboratory Practice, April 1, 2016) of the Ministry of Health of Russia, and Regulations for Experimental (Preclinical) Studies of New Pharmacological Substances [3].

Methods for isolation and studies of the chemical structure of *Tussilago farfara* L. water-soluble polysaccharides (WSPS) (monosaccharide composition, measurements of uronic acids) have been developed at the Center for Introduction of New Technologies and at the Laboratory of Innovation Pharmaceutical Technologies, Siberian State Medical University. Studies of the component composition have shown that the polysaccharide complex consisted of two main components: rhamnogalacturonane I (33%) and neutral polysaccharides (67%), presented by the sum of arabinogalactane, rhamnane, and galactomannane [1]. *Tussilago farfara* L. polysaccharides were injected to animals (n=3) intraperitoneally in a dose of 20 mg/kg from day 2 after tumor transplantation till the end of experiment. Controls with LLC receiving no WSPS (n=3) were injected with equivalent volumes of saline. Intact group consisted of 3 animals without tumors. Animals of experimental and control groups received transplantations of LLC homogenate in sterile saline. The homogenates were prepared as follows. Fragments of the tumors without necrosis were resected from donor animals, processed in a crusher press, diluted in saline in a glass flask to a concentration of 10-20%, fragmented with a syringe with a needle, and filtered through sterile Capron filter. Tumor cells were counted in a Goryaev's chamber under a microscope, after which saline was added to the needed concentration. The resultant homogenate was injected intramuscularly in a dose of 5×10^6 cells/0.1 ml.

Specimens of peripheral blood and tumor tissue were tested for the presence of $CD279^+$ and $CD274^+$ cells in the leukocyte population, which was identified by the presence of CD45 marker. The parameters were evaluated at the early stage of tumor process (on day 7 after LLC transplantation) and later (day 14 after LLC transplantation). Separation of peripheral blood and tumor tissue cells into populations was carried out by front (FSC) and side (SSC) light scatter gating. The total count of lymphocytes was taken for 100% and the percentage of $CD274^+$ and $CD279^+$ cells was evaluated. Analysis was carried out according to the instruction with the use of labeled antibodies PerCP-Cy5.5.Rat Anti-Mouse CD45, BV421 Hamster Anti-Mouse CD279, and PE Rat Anti-Mouse CD274 (BD Biosciences) on a CytoFLEX flow cytofluorimeter (Beckman Coulter). The results were processed using CytoExpert 2.3 software for device setting up and data collection, processing, and visualization.

The data were statistically processed using Statistica 8.0 software (StatSoft, Inc.) using Mann—Whitney test. The differences were assumed to be significant at $p<0.05$. The data were presented as the means for group with the standard errors in the means.

RESULTS

The peripheral blood level of $CD274^+$ lymphocytes at the early stage of tumor development (day 7 after transplantation) was several-fold lower in control mice

TABLE 1. Effects of *Tussilago farfara* L. Polysaccharides on Expression of CD274 and CD279 in Peripheral Blood and Tumor Tissue Lymphocytes of C57BL/6 Females on Day 7 after Transplantation of LLC (%; n=3; $M\pm SD$)

Group	Peripheral blood		Tumor tissue	
	$CD274^+$	$CD279^+$	$CD274^+$	$CD279^+$
Intact	5.65±2.09	15.34±4.10	—	—
Control (LLC)	0.07±0.01*	38.68±0.97*	0.10±0.04	72.96±2.10
LLC+WSPS, 20 mg/lg (6 injections)	0.06±0.02	27.60±5.48$^+$	0.04±0.02$^+$	42.45±9.52

Note. Here and in the Table 2: $p<0.05$ in comparison with *intact group, $^+$control.

(with tumors) than in intact ones. In animals receiving polysaccharides, the level of these cells remained the same as in controls with LLC. The expression of CD279 on lymphocytes of untreated mice with tumors was 2.5-fold higher ($p<0.05$) than in animals without LLC. Polysaccharide treatment promoted a significant (by 1.4 times) decrease in the count of $CD279^+$ cells in comparison with the control (Table 1; Fig. 1).

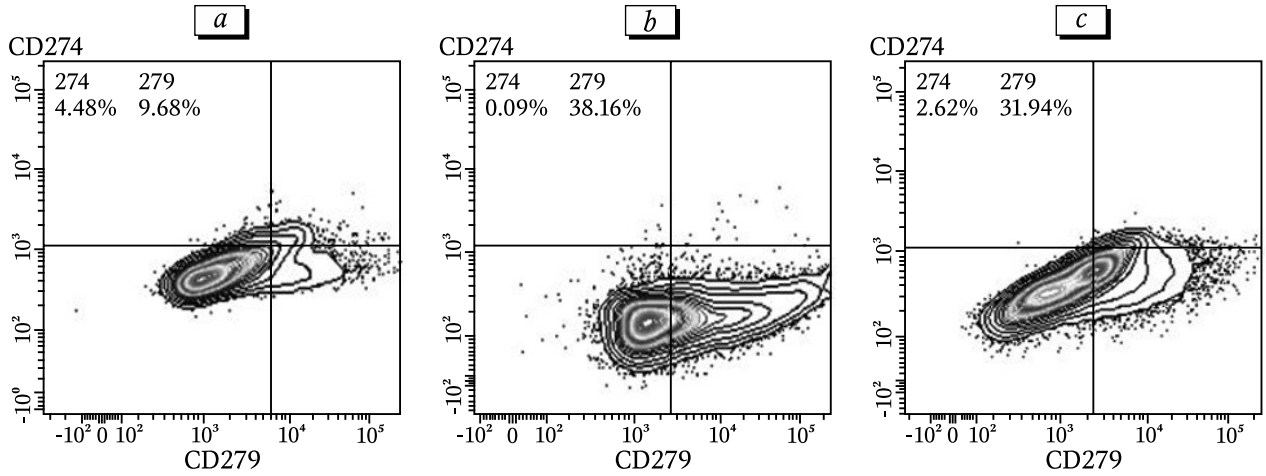

Fig. 1. Analysis of mouse peripheral blood lymphocytes labeled with CD274 and CD279 antibodies, on day 7 of tumor development. *a*) Intact animals (no tumors); *b*) control (LLC); *c*) LLC+WSPS, 20 mg/kg.

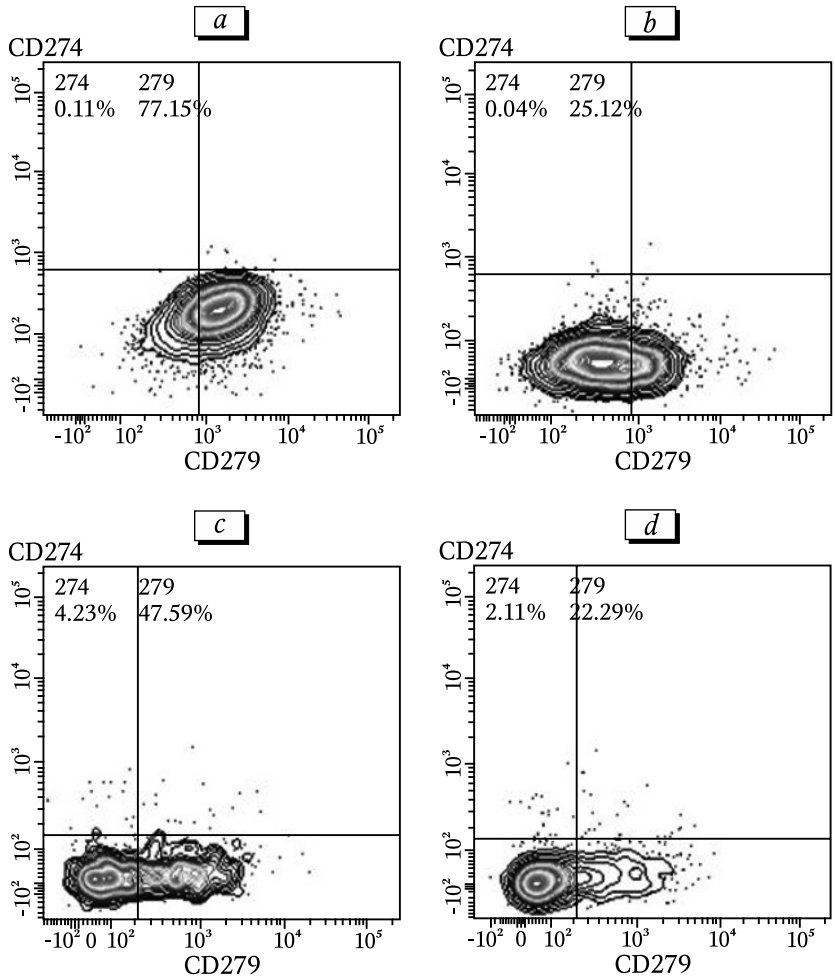

Fig. 2. Analysis of tumor tissue lymphocytes, labeled with CD274 and CD279 antibodies, on day 7 (*a*, *b*) and day 14 (*c*, *d*) of tumor development. *a*, *c*) Control (LLC); *b*, *d*) LLC+WSPS, 20 mg/kg.

TABLE 2. Effects of *Tussilago farfara* L. Polysaccharides on Expression of CD274 and CD279 in Peripheral Blood and Tumor Tissue Lymphocytes of C57BL/6 Females on Day 14 after Transplantation of LLC (%; *n*=3; *M*±*SD*)

Group	Peripheral blood		Tumor tissue	
	CD274+	CD279+	CD274+	CD279+
Intact	0.96±0.55	7.66±3.28	—	—
Control (LLC)	17.04±3.94*	44.98±7.27*	15.99±11.81	46.69±3.16
LLC+WSPS, 20 mg/lg (13 injections)	1.47±0.04+	4.92±0.28+	2.20±0.67	18.22±4.25+

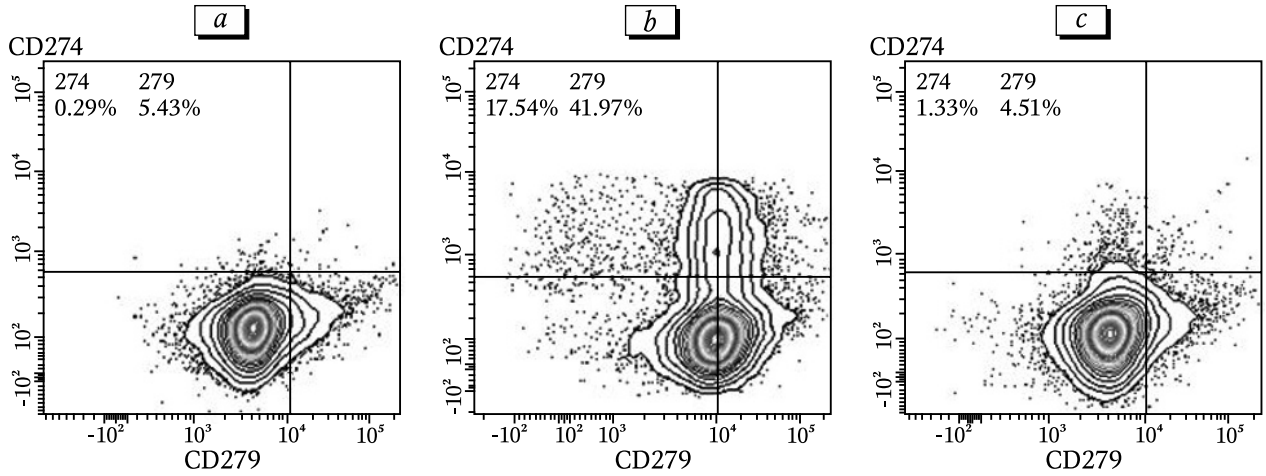

Fig. 3. Analysis of peripheral blood lymphocytes, labeled with CD274 and CD279 antibodies, on 14 of tumor development. *a*) Intact animals (no tumors); *b*) control (LLC); *c*) LLC+WSPS, 20 mg/kg.

The level of CD274+ cells in tumor tissue of animals treated with polysaccharides was significantly lower (by 2.5 times) and of CD279+ cells lower (by 1.7 times) than in controls (Table 1; Fig. 2, *a*, *b*).

On day 14 after LLC transplantation, the levels of CD274+ and CD279+ cells in the peripheral blood cells increased in comparison with those in intact mice (by 17.8 and 5.9 times, respectively; $p<0.05$). In animals treated with *Tussilago farfara* L. polysaccharides the expression of CD274 and CD279 was significantly lower on the peripheral blood lymphocytes (by 11.6 and 9.1 times) and on tumor tissue cells (by 7.3 and 2.6 times) in comparison with the control group (Table 2; Fig. 2, *c*, *d*).

The development of approaches to improvement of the efficiency of antitumor therapy is in progress at the Laboratory of Oncopharmacology of the E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine. The possibility of improving the antitumor and antimetastatic efficiencies of cytostatics with various mechanisms of action by using them in combination with *Tussilago farfara* L. polysaccharides is demonstrated on experimental tumors (LLC, B-16 melanoma, and lung cancer-67) [5]. Our study demonstrated a decrease in the level of CD279+ lymphocytes in the peripheral blood and tumor tissue and of CD274+ cells only in tumor tissue under the effect of *Tussilago farfara* L. polysaccharides during the early stage of LLC development. On day 14 after LLC transplantation, the decrease in the levels of CD274+ and CD279+ lymphocytes in response to polysaccharides is recorded for the peripheral blood and tumor tissue. These data indicate that blocking of the signal transmission from tumor cells to lymphocytes by means of lower expression of PD-1/PD-L1, as a result of which the tumor is no longer capable of escaping the immunological surveillance and is recognized and destroyed by effector T cells, is one of the mechanisms of antitumor and antimetastatic activity of *Tussilago farfara* L. polysaccharides.

Hence, *Tussilago farfara* L. polysaccharides seem to be a promising object for studies as a candidate drug for immunotherapy and for creation on this base of a new drug boosting the chemotherapy efficiency.

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