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Subpopulations of B lymphocytes in patients with breast cancer depending on the PD-L1 status

Tashireva L.A., Kalinchuk A.Yu., Gerashchenko T.S., Savelyeva O.E., Perelmuter V.M.

Cancer Research Institute, Tomsk National Research Medical Center (NRMC) of the Russian Academy of Sciences (RAS)
5, Kooperativny Str., Tomsk, 634009, Russian Federation

ABSTRACT

Aim. To study the association between the functional potency and degree of maturity of B lymphocytes and PD-L1 expression in breast cancer patients.

Materials and methods. The study included 37 patients with the morphologically verified diagnosis of invasive breast cancer of no special type (IBC NST). The PD-L1 status was determined immunohistochemically using the Ventana SP142 assay (Roche, USA). Using the multiplex flow cytometry-based assay and high-throughput sequencing of the tumor microenvironment, subpopulations of B lymphocytes and their CD27 and PD1 expression profiles were determined, taking into account the PD-L1 status.

Results. In the tumor microenvironment, regardless of the PD-L1 status, expression signatures of five lymphocyte subpopulations were determined. However, in PD-L1-positive patients, the levels of B lymphocytes and immunoglobulin class-switched B lymphocytes were higher compared with PD-L1-negative patients. Evaluation of the number of different B lymphocyte subpopulations by flow cytometry showed that PD-1-positive B lymphocytes predominated in the tumor microenvironment in PD-L1-positive patients, regardless of the degree of lymphocyte maturity.

Conclusion. The results of the study showed predominance of mature committed B lymphocytes and memory B lymphocytes capable of synthesizing immunoglobulins of different classes and Th2 cytokines involved in type 2 immune response in PD-L-positive tumor microenvironment. It suggests that immunotherapy with PD-L1 inhibitors is highly likely to activate cells with protumor potential and can ultimately contribute to breast cancer progression.

Keywords: breast cancer, tumor microenvironment, B lymphocytes, PD-L1

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee at Cancer Research Institute, Tomsk NRMC (Protocol No.7 of 25.08.2020).

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✉ *Tashireva Lyubov A.,* tashireva@oncology.tomsk.ru

Субпопуляции В-лимфоцитов у больных раком молочной железы в зависимости от статуса PD-L1

Таширева Л.А., Калинчук А.Ю., Геращенко Т.С., Савельева О.Е., Перельмутер В.М.

Научно-исследовательский институт (НИИ) онкологии, Томский национальный исследовательский медицинский центр (НИМЦ) Российской академии наук
Россия, 634009, г. Томск, пер. Кооперативный, 5

РЕЗЮМЕ

Цель. Изучить, насколько функциональные потенции и степень зрелости В-лимфоцитов сопряжены со статусом экспрессии PD-L1 опухоли у больных раком молочной железы.

Материалы и методы. В исследование вошли 37 пациентов с морфологически верифицированным диагнозом инвазивной карциномы молочной железы неспецифического типа (ИКНТ). Статус PD-L1 определялся иммуногистохимически тестом Ventana SP142 (Roche, США). С использованием методов мультиплексной проточной цитофлуориметрии и высокопроизводительного секвенирования микроокружения были определены субпопуляции В-лимфоцитов, их профиль экспрессии CD27 и PD1 с учетом статуса PD-L1 опухоли.

Результаты. В микроокружении опухоли у пациентов независимо от статуса PD-L1 определяются экспрессионные сигнатуры пяти субпопуляций лимфоцитов. Однако у больных с позитивным статусом в микроокружении первичной опухоли уровни В-лимфоцитов и В-лимфоцитов с переключаемым классом Ig выше по сравнению с пациентами, имеющими негативный статус PD-L1. Оценка количества различных субпопуляций В-лимфоцитов методом проточной цитофлуориметрии показала, что у больных с PD-L1-позитивным статусом опухоли в микроокружении преобладают PD-1-позитивные В-лимфоциты независимо от степени их зрелости.

Заключение. Результаты исследования показывают преобладание зрелых коммитированных В-лимфоцитов и В-лимфоцитов памяти, способных к синтезу иммуноглобулинов разных классов и цитокинов, относящихся к спектру иммуновоспалительных реакций Th2 типа в микроокружении PD-L1-позитивных опухолей. Это может являться неблагоприятным признаком при планировании иммунотерапии анти-PD-L1 ингибиторами, поскольку с высокой вероятностью ее применение может активировать клетки микроокружения с проопухолевыми потенциями, что в конечном итоге будет способствовать прогрессии карцином.

Ключевые слова: рак молочной железы, микроокружение, В-лимфоциты, PD-L1

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INTRODUCTION

In the last decade, doctors began to actively use a new class of immunotherapy drugs, immune checkpoint inhibitors (ICIs), which block the PD-1 / PD-L1-dependent mechanism of immune response suppression in the tumor. New options for medical oncologists were developed providing patients with many years to live. However, the analysis of the ratio of indications for prescription to the benefit from these drugs in a cohort of American patients clearly demonstrated an increase in the number of indications but no proper increase in effectiveness [1]. Moreover, the evaluation of the effectiveness of ICI

therapy indicates that a prolonged response is indeed observed only in some patients. The other group of patients have at best stabilization of the disease and at worst – progression and hyperprogression of pathology [2].

It is known that the type of the immune and inflammatory response in the breast cancer microenvironment can determine the response to therapy and the course and prognosis of the disease. However, to date, despite numerous studies, there are no acceptable predictors of the response to ICIs other than the assessment of PD-L1 expression and MMR deficiency [3].

The role of B lymphocytes in the tumor is quite controversial. It is well known that B lymphocytes contribute to tumor progression. In particular, this was shown in human ovarian cancer [4]. Being some of the cellular elements of type 2 immune and inflammatory responses, B lymphocytes are the source of corresponding cytokines, which reduce the antitumor response [5]. By synthesizing transforming growth factor (TGF-β) and interleukin 10 (IL-10), B lymphocytes can promote differentiation and recruitment of regulatory T lymphocytes, resulting in an increased immunosuppressive microenvironment in the tumor and stimulating breast cancer metastasis [6]. Antitumor antibodies synthesized by plasma cells, which are descendants of B lymphocytes, can activate macrophages and promote invasive tumor growth and angiogenesis [7].

However, current data suggest that B lymphocytes in the tumor are a favorable prognostic marker in breast cancer [8]. In addition, there are observations indicating antitumor effects of B lymphocyte subpopulation with the CD27 memory cell phenotype. The presence of these cells in the microenvironment along with CD8+ T lymphocytes is associated with a favorable prognosis in ovarian cancer [9]. It is known that the presence of B lymphocytes in tumor tertiary lymphoid structures is associated with a more favorable disease prognosis [10]. Regarding the role of PD1 expression on B lymphocytes, it is known to be more a sign of maturity and weaker ability to proliferate and switch antibody isotypes [11]. Thus, in the context of immunotherapy, the protumor or antitumor functional characteristics of B lymphocytes are of great importance. It is important to understand to what extent the functional potency and degree of maturity of B lymphocytes are correlated with the expression status of PD-L1 and the presence of the PD1 receptor.

MATERIALS AND METHODS

Patients. The study included 37 patients with a morphologically verified diagnosis of invasive breast carcinoma of no special type (IC NST). The average age of patients who were treated at Cancer Research Institute, Tomsk NRMC, with estrogen receptor positive (luminal A and B-1, -2) and triple-negative cancer subtypes ($T_{1–3}$ $N_{0–3}$ M_0) was 52.7 ± 9.3 years. The patients did not receive neoadjuvant chemotherapy prior to surgical treatment. Fresh primary tumor samples were obtained during surgery and were then stored at –80 °C. The study was carried out in accordance with ethical standards (the Declaration of Helsinki of the World Medical Association "Ethical principles for medical research involving human subjects" as amended in 2000 and "Rules of Good Clinical Practice in the Russian Federation", approved by the Order of the Ministry of Healthcare of the Russian Federation No. 266 of 19.06.2003). All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee at Cancer Research Institute, Tomsk NRMC (Protocol No. 7 of 25.08.2020).

Assessment of tumor-infiltrating lymphocyte (TIL) density in a primary tumor. The density of TILs in the primary tumor was assessed according to the recommendations of the International TILs Working Group for the evaluation of tumor-infiltrating lymphocytes [12]. To do this, a representative tumor sample was stained with hematoxylin and eosin according to the standard operating procedure. Next, the proportion of stroma occupied by mononuclear leukocytes was determined (Axio Scope A1 (Zeiss, Germany)). Areas with artifacts, areas of necrosis, and pronounced hyalinosis were excluded from the assessment. The value was expressed as a percentage of the stromal area.

Determination of the PD-L1 status. PD-L1 expression was assessed using the PD-L1 test (SP142, Ventana) in the BenchMark ULTRA system (Roche, USA). Staining of immune cells (ICs) which are present in the intratumoral and adjacent peritumoral stroma was assessed. These cells included lymphocytes, macrophages, dendritic cells, and polymorphonuclear leukocytes. ICs were estimated as the fraction of the tumor area occupied by them with PD-L1 immunostaining of any intensity. Human tonsil tissue was used as a control. The test was considered positive at IC ≥ 1%.

Flow cytometry. Fresh frozen samples of the primary tumor were used as the study material.

To obtain a suspension of primary tumor cells, its fragment was placed in the Medicon (50 μm, BD Biosciences) and homogenized in 1 ml of phosphate-buffered saline (BD Biosciences) for 1 min twice. The suspension was then filtered through a cell separation mesh (70 μm, Falcon, Japan). The cells were then washed twice and resuspended in 100 μl cell staining buffer (Sony Biotechnology) and stained with a cocktail of monoclonal antibodies: BV570-anti-CD45 (clone HI30, mouse IgG1, Sony Biotechnology, Japan), PerCP/Cy5. 5-anti-CD3 (clone UCHT1, Mouse IgG1, Sony Biotechnology, Japan), APC/Cy7-anti-CD20 (clone 2H7, Mouse IgG2b, Sony Biotechnology, Japan), BV785-anti-CD27 (clone O323, Mouse IgG1, Sony Biotechnology, Japan), BV510-anti-CD28 (clone CD28. 2, Mouse IgG1, Sony Biotechnology, Japan), BV605-anti-CD279 (PD-1) (clone EH12.2H7, Mouse IgG1, Sony Biotechnology, Japan), BV421-anti-CD274 (PD-L1) (clone MIH3, Mouse IgG1, Sony Biotechnology, Japan), and AF647-anti-CD326 (EpCAM) (clone 9C4, Mouse IgG2b, Sony Biotechnology, Japan). Unstained and isotype controls were used. Isotype antibodies were added to the corresponding isotype control in a similar concentration. The analysis was performed on the NovoCyte 3000 flow cytometer (ACEA Biosciences, Agilent, USA). Cell populations were gated based on determination of forward scatter (FSC) and side scatter (SSC) parameters. Then the cells were analyzed for fluorescence in Density Plot and Dot Plot modes. The number of cells was presented as a fraction of all lymphocytes.

Sequencing of primary tumor cell microenvironment. Frozen tumor samples were taken from eight patients (PD-L1 negative – four patients, PD-L1 positive – four patients). Seven-micrometer-thick sections were prepared on PEN membrane frame slides (Carl Zeiss, Oberkochen, Germany) pre-treated with RNAZap (Thermo Fisher Scientific, Waltham, MA, USA) and stained with hematoxylin and eosin. PALM microbeam laser capture microdissection (Carl Zeiss, Oberkochen, Germany) was used to isolate cells from the tumor microenvironment. Four samples of stromal fragments adjacent to tumor cells were isolated from each tumor. A total of 32 samples were collected. RNA was isolated using the Single Cell RNA Purification Kit (Norgen, Canada). cDNA libraries were prepared using the SMARTer Stranded Total RNA-Seq kit v2 (Takara, USA). The size of cDNA libraries was estimated using the HS D1000 ScreenTape kit and 2200 Tape Station (Agilent, USA) and varied from 200 to 700 bp, with an average peak size of 340 bp.

The concentration of cDNA libraries was estimated using the Qubit 4.0 fluorometer (Thermo Fisher Scientific, USA) and varied from 2.5 to 14 ng / μl depending on the number of cells in the microdissected samples. Samples of cDNA libraries were pooled, denatured, and sequenced on NextSeq 500 (Illumina, USA) in a single-end sequencing read mode for 75 cycles. The number of clusters was 220 K / mm^2 (93% of clusters after filtration), and the number of reads was ~12 million per sample. The bioinformatic analysis included mapping of reads using the STAR software (the GRCh38 reference genome assembly and GENCODE.R27 annotation). The number of reads in the coding and non-coding regions of the genome in each sample was estimated using the featureCounts tool. The xCell algorithm was used to identify cell types in the microenvironment [13]. The sequencing data were published in the GEO database (No. GSE184196).

Statistical analysis. Categorical parameters were compared using the Fisher's exact test. Differences in independent quantitative variables were assessed using the Mann–Whitney test. All criteria were two-sided and considered significant at $p < 0.05$. The analysis was performed using GraphPad Prizm 9 software.

RESULTS

Patient characteristics. A total of 54% (20/37) of all patients included in the study had PD-L1 positive status and 46% (17/37) had PD-L1 negative status. The clinical and pathological characteristics of the two groups of patients were comparable. Menopausal patients over 50 years of age, with a tumor size between 20 and 50 mm, stage IIA grade 2 luminal B subtype prevailed.

Expression signatures of B lymphocytes in the breast cancer microenvironment. xCell analysis detects signatures of five B lymphocyte subsets. Signatures of all five lymphocyte subpopulations were determined in the tumor microenvironment in patients regardless of the PD-L1 status (Fig. 1).

The numbers of B lymphocyte subpopulations were not equal within the study groups (Table). Thus, PD-L1 negative patients had lower levels of naive B lymphocytes compared to the levels of B lymphocytes and memory B lymphocytes. The levels of B lymphocytes differed more markedly in PD-L1 positive patients. Thus, B lymphocytes and Ig class-switched B lymphocytes were predominant.

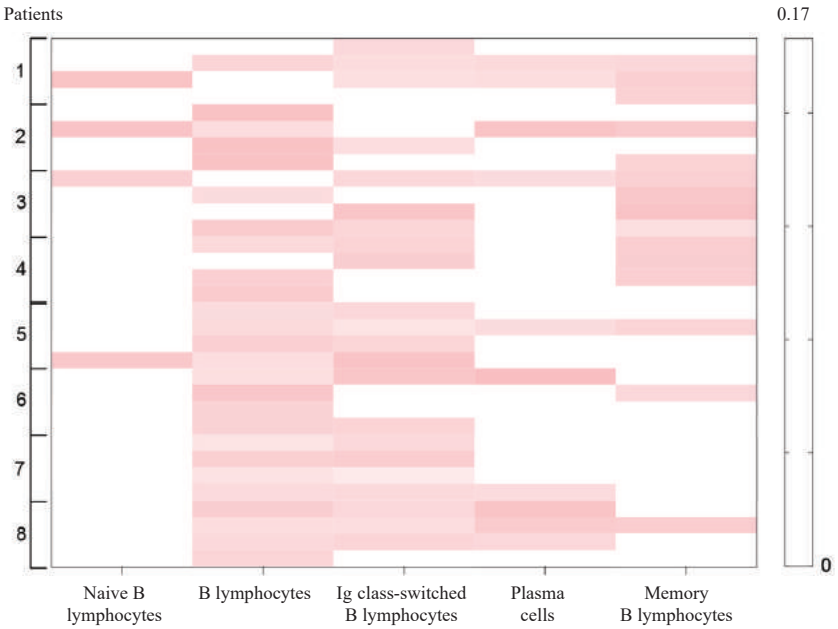

Fig. 1. Heat map of B lymphocyte signatures in the primary tumor microenvironment in breast cancer patients, *Me* (Q_1–Q_3)

Table

The number of cells in the primary tumor microenvironment of breast cancer patients depending on the PD-L1 status according to the xCell analysis, units, *Me* (Q_1–Q_3)

Cell type		PD-L1 status		*p* (intergroup)
		negative	positive	
Naive B lymphocytes	a	0.0000 (0.0000–0.0001) *p* (a–b) = 0.0075 *p* (a–e) = 0.0323	0.0000 (0.0000–0.0001) *p* (a–b) < 0.0001 *p* (a–c) < 0.0001	0.7876
B lymphocytes	b	0.0092 (0.0000–0.0362)	0.0530 (0.0299–0.0713) *p* (b–d) < 0.0001 *p* (b–e) < 0.0001	0.0001
Ig class-switched lymphocytes	c	0.0168 (0.0000–0.0482)	0.0388 (0.0107–0.0506) *p* (c–d) = 0.0005 *p* (c–e) < 0.0001	0.0490
Plasma cells	d	0.0001 (0.0000–0.0087)	0.0008 (0.0000–0.0159)	0.9170
Memory B lymphocytes	e	0.0213 (0.0046–0.0310)	0.0000 (0.0000–0.0029)	0.0847

A comparison of B lymphocyte levels between patient groups depending on their PD-L1 status revealed that patients with PD-L1 positive status had higher levels of B lymphocytes and Ig class-switched B lymphocytes in the primary tumor microenvironment compared to PD-L1 negative patients (Fig. 2).

Assessment of B lymphocyte maturation and PD1 expression. As in the transcriptome analysis, we detected B lymphocytes in the primary tumor microenvironment in all patients. The variability of the determined population composition is shown in Fig. 3.

The assessment of CD27 (lymphocyte maturation marker) and PD1 (functional receptor molecule for PD-L1) expression showed that CD27+PD1- B lymphocytes were not found in the primary tumor tissue in all patients, regardless of the PD-L1 status in the primary tumor. CD27-PD1- B lymphocytes were the most rare (in 12.5% (2 / 16) of patients in both groups). CD27+PD1+ and CD27-PD1+ B lymphocytes were found with comparable frequency in the primary tumor tissue (81.2% (13 / 16) and 81.2% (13 / 16), respectively, $p > 0.9999$).

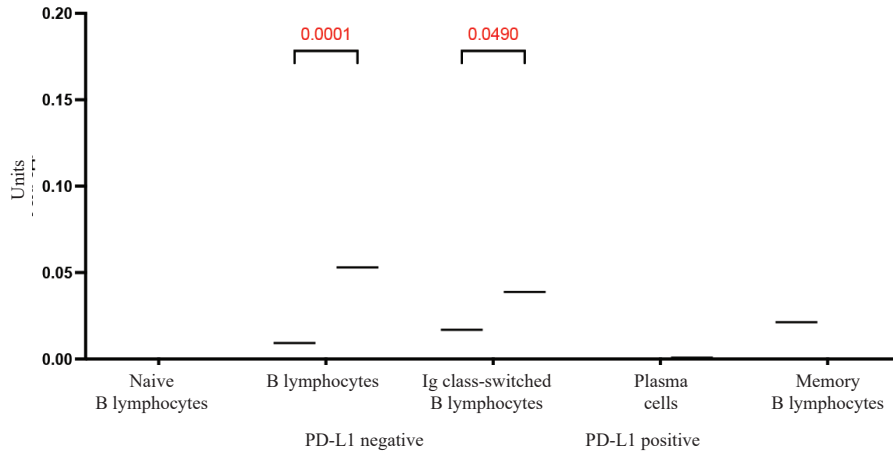

Fig. 2. B lymphocyte subpopulations in PD-L1 negative and PD-L1 positive breast cancer patients

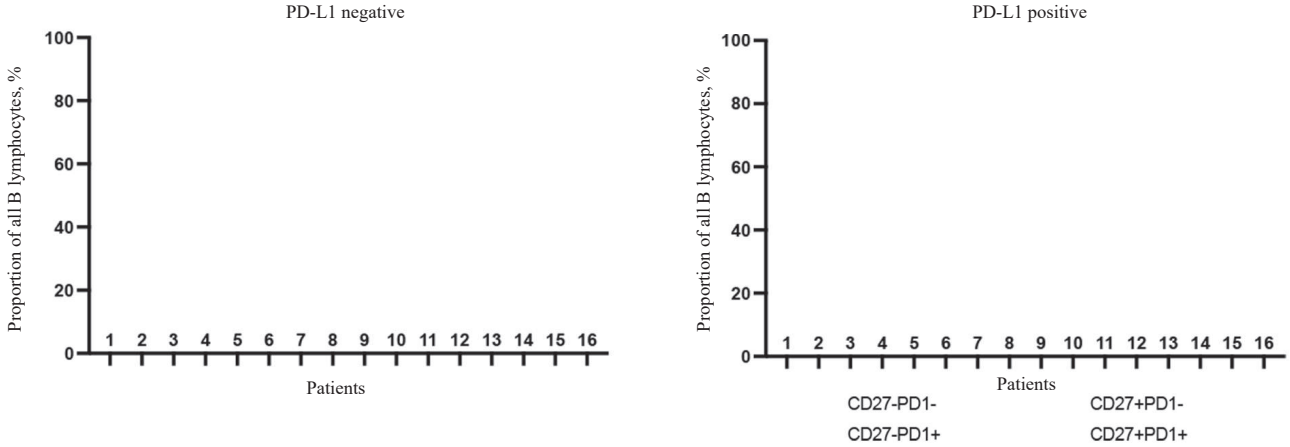

Fig. 3. Intrapersonal heterogeneity of B lymphocyte composition in PD-L1 negative and PD-L1 positive breast cancer patients

The assessment of the number of different B lymphocyte subpopulations in patients depending on the PD-L1 status showed that PD-1 positive B lymphocytes prevailed in the tumor microenvironment in PD-L1 positive patients, regardless of their maturity (Fig. 4).

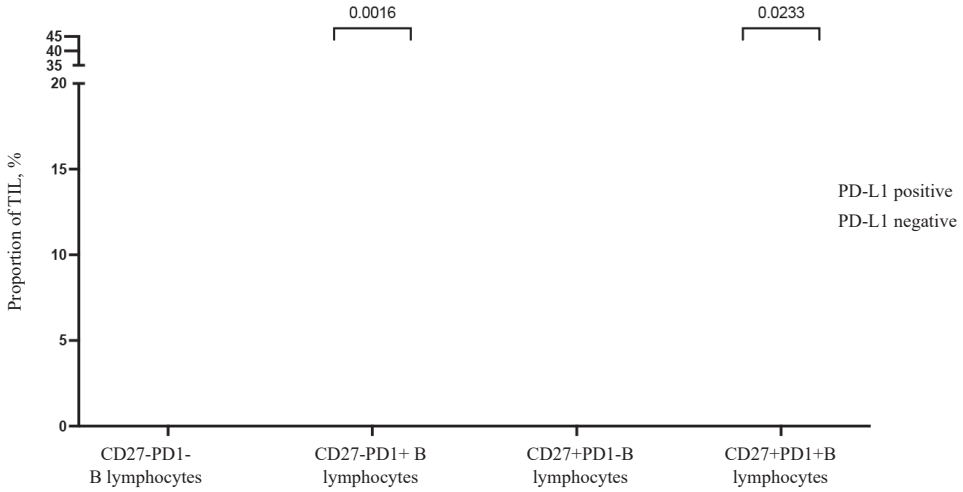

Fig. 4. The number of B lymphocytes with different combination of CD27 and PD1 expression in patients, depending on the PD-L1 status

The number of CD27+PD1+ B lymphocytes in PD-L1 negative patients was 0.09 (0.00–0.89) versus 1.67 (0.61–3.22) % in PD-L1 positive patients (p = 0.0233). The number of CD27-PD1+ B lymphocytes was 0.05 (0.00–1.53) % in PD-L1 negative patients and 1.41 (0.25–7.23) % in PD-L1 positive patients (p = 0.0016).

DISCUSSION

Inflammatory infiltration in the tumor microenvironment reflects a combination of innate immune responses and specific adaptive immune responses to various antigens: tumor, tissue and pathogenic antigens contaminating tumor tissue. The ratio of these components of immune and inflammatory responses in the microenvironment is individual in each case. B lymphocytes are a key link in the antibody genesis, a source of cytokines belonging to "proinflammatory" ("protumor") Th2 cytokines. Accordingly, the effect of the B cell link of the immune response on tumor elements is possible due to the synthesis of antibodies specific to tumor antigens. The effect depends on the type of immunoglobulins. Theoretically, both antibody-dependent cytotoxic reactions involving granulocytes and macrophages and "masking" of tumor antigens by antibodies preventing cytotoxic effects of CD8+ T lymphocytes are possible. Tumor cells are also affected by cytokines. Moreover, regardless of B lymphocyte specificity to the tumor or non-tumor antigen, activation of such cells by the corresponding antigen causes secretion of Th2 cytokines, which contributes to carcinoma progression: epithelial – mesenchymal transition, invasion, angiogenesis, and, finally, metastasis.

The results of our study allowed us to evaluate the presence and activity of B lymphocytes at different stages of functional differentiation from naive B lymphocytes and Ig class-switched B lymphocytes to memory B lymphocytes. The main results of the study are the following: 1) B lymphocytes express PD1 and PD-L1; 2) the number of differentiated forms of B lymphocytes depends on the PD-L1 status. The number of Ig class-switched B lymphocytes and memory B lymphocytes is greater when PD-L1 is expressed in the tumor infiltrate. Moreover, there are no cells without PD1 expression among CD27+ B lymphocytes. These results imply that B lymphocytes actively involved in antibody genesis (with the ability to switch from early IgM to IgG, IgA, or IgE immunoglobulins) as well as memory B lymphocytes (with the potential to develop secondary immune responses to repeatedly exposed antigens) are most likely blocked through the PD1 / PD-L1 pathway. This phenomenon corresponds to normal inflammation when there is an increase in PD-L1 expression on various cells in the foci of inflammation [5]. This prevents nonspecific damage to the tissues where inflammatory responses occur and ensures selective local activation of B lymphocytes during ligand blockade.

CONCLUSION

The results show the predominance of mature committed B lymphocytes and memory B lymphocytes capable of synthesizing immunoglobulins of different classes and cytokines belonging to type 2 immunity in the microenvironment of PD-L1 positive tumors. This may have an adverse effect, since the use of immunotherapy with anti-PD-L1 inhibitors is highly likely to activate cells with protumor potentials in the microenvironment, which ultimately will contribute to the progression of carcinomas.

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Authors contribution

Tashireva L.A. – conception and design, analysis and interpretation of the results, drafting of the manuscript. Kalinchuk A.Yu., Gerashchenko T.S., Savelyeva O.E. – conducting the research. Perelmuter V.M. – final approval of the manuscript for publication.

Authors information

Tashireva Lyubov A. – Cand. Sci. (Med.), Senior Researcher, Department of General and Molecular Pathology, Cancer Research Institute, Tomsk NRMC, Tomsk, tashireva@oncology.tomsk.ru, http://orcid.org/0000-0003-2061-8417

Kalinchuk Anna Yu. – Research Assistant, Department of General and Molecular Pathology, Tomsk NRMC, Tomsk, anya98.tomsk@gmail.com, http://orcid.org/0000-0003-2106-3513

Gerashchenko Tatiana S. – Cand. Sci. (Med.), Researcher, Laboratory of Tumor Progression, Cancer Research Institute, Tomsk NRMC, Tomsk, t_gerashenko@list.ru , http://orcid.org/0000-0002-7283-0092

Savelyeva Olga E. – Dr. Sci. (Med.), Leading Researcher, Department of General and Molecular Pathology, Cancer Research Institute, Tomsk NRMC, Tomsk, olga_chechina@mail.ru, http://orcid.org/0000-0002-0301-8455

Perelmuter Vladimir M. – Dr. Sci. (Med.), Professor, Distinguished Researcher of the Russian Federation, Principal Researcher, Department of General and Molecular Pathology, Cancer Research Institute, Tomsk NRMC, Tomsk, pvm@ngs.ru, http://orcid.org/0000-0002-7633-9620

(✉) **Tashireva Lyubov A.,** tashireva@oncology.tomsk.ru

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