

PD-1 BLOCKAGE FACILITATES CYTOTOXIC T AND NK CELLS TUMORICIDAL PHENOTYPE IN A MURINE BREAST CARCINOMA

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ABSTRACT

In breast cancer therapy, as the leading cause of death in women, besides chemo-radiotherapy, immunotherapy has been increasingly used. PD-1/PD-L1 axis blockade primarily acts on T lymphocytes, the main effectors of acquired immune response. NK cells, which are part of the innate immune response, also play a role in the anti-tumor response through the blockade of this signaling pathway. The study was conducted to examine the effects of anti-PD-1 therapy on NK and T cells in mouse breast cancer. Female BALB/c mice were used, divided into two groups, one with induced breast cancer and one treated with anti-PD-1 antibody. Breast cancer cell line was used to induce the cancer, and the anti-PD-1 antibody was applied intraperitoneally. Cell populations in spleen and tumor microenvironment were examined using flow cytometry. Data were statistically analyzed using SPSS. The percentage of NK cells expressing FasL, NKG2D, and IFN- γ is significantly higher in spleen and tumor-infiltrating NK cells upon anti-PD-1 therapy, while the expression of inhibitory markers Foxp3 and IL-10 in regulatory NK cells is significantly lower. The percentage of T lymphocytes expressing CD107a and IL-17 is significantly higher in the spleen, while a higher number of T lymphocytes expressing CD69 is present in the tumor microenvironment. The study suggests that anti-PD-1 therapy can activate NK and T cells, and improve anti-tumor immune response in breast cancer. Further research is needed to understand the interplay between these cells during PD-1 blockade.

Keywords: PD-1, NK cells, cytotoxic T cells, mammary carcinoma.

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INTRODUCTION

Breast cancer is the most common malignant tumor globally and the first cause of death in the female population. Breast cancer is the leading cause of global cancer incidence in 2020, with an estimated 2.3 million new cases, and representing 11.7% of all cancer cases. The number of patients is constantly increasing throughout the world, especially in developed countries (1). GLOBOCAN 2020 data for Serbia show that in 2020, there were 6.724 newly discovered cases of breast cancer in our country, while the number of deaths was 2.342 (1, 2).

Chemoradiotherapy as the main form of treatment for advanced breast cancer has its limitations due to the manifestation of side effects and inadequate response to the drug (3-5). In recent years, treatments of breast cancer have been expanded with immunotherapy (6). Immunotherapy with so-called immune check point inhibitors, is used to alleviate the response of patients and promote the antitumor effect of T cells, and has become an important strategy in the treatment of many malignant diseases (6, 7).

The programmed cell death 1 molecule (programmed cell death 1, PD-1, CD279) was first discovered in 1992 in the LsD9 mouse hematopoietic progenitor cell lines and 2B4-11 mouse T-cell hybridoma in the stages of apoptosis. At that time, the PD-1 molecule was considered to be involved in apoptosis, hence its name (8). PD-1, primarily expressed on activated T cells' surface, but also by NK (natural killer) and NKT (natural killer T) cells (9, 10), can combine with its ligand PD-L1 (programmed cell death 1 ligand), which presents expression on tumor cells and immune cells, to deprive T cells of killing ability (7). However, PD-L1-to-PD-1 binding can suppress the function of lymphocytes and reduce the release of cytokines. It can also promote the apoptosis of lymphocytes. Therefore, the function of CD8⁺ T cells is inhibited, which may lead to the immune escape of tumors. PD-1/PD-L1 inhibitors are capable of blocking PD-L1-to-PD-1 binding and reducing the immune escape phenomenon of tumors (7).

Ligands for the PD-1 molecule are PD-1L, expressed on macrophages, activated T and B lymphocytes, dendritic cells and some epithelial cells, especially in inflammation, and PD-2L expressed on macrophages, peritoneal B lymphocytes and activated dendritic cells (11-13). In addition to the cells of the immune system, PD-1L is also expressed on tumor cells, which is associated with a worse prognosis (14).

By blocking the PD-1/PD-1L axis, the antitumor response of the immune system is enhanced (15). Through the increased expression of PD-1L on tumor cells, the activation of primarily T lymphocytes is inhibited. In this way, the tumor avoids the antitumor immune response (16). The main effector cells in antitumor immunity are cytotoxic T lymphocytes and helper T lymphocytes (17, 18). They achieve their antitumor role through the production of cytotoxic substances containing granzyme and perforin and through the induction

of apoptosis through FasL. Cytotoxic and helper lymphocytes also secrete large amounts of cytokines into the tumor microenvironment, especially IFN- γ , which is responsible for the expression of ligands for PD-1 (19, 20). Cells of innate immunity, NK cells, also play an important role in the antitumor immune response. The degree of tumor tissue infiltration by these cells is a good prognostic indicator (21). They achieve an antitumor effect by different mechanisms, stimulate the migration of T lymphocytes into tumor tissue and directly eliminate tumor cells (22). They also play a role in preventing metastases by eliminating circulating tumor cells (23).

The role of the PD-1/PD-1L axis in tumor biology is well known. However, the data are very modest when it comes to the effect of PD1 blockade on T lymphocytes and NK. Our data suggest that these cells may also be important for more effective anti-PD1 therapy in malignancy, and may contribute to an overall effective immune response to breast cancer, as anti-PD1 therapy induces phenotypic changes in T lymphocytes and NKT cells. In addition, research in animal models has shown that blockade of the PD-1/PD-1L axis significantly delays palpable tumor onset and slows primary tumor growth in experimental breast cancer models.

MATERIAL AND METHODS

The study was conducted, in the period from 2019 to 2021, at the Center for Molecular Medicine and Stem Cell Research of the Faculty of Medical Sciences, University of Kragujevac, as well as at the Institute for Medical Research of the Faculty of Medicine of the Military Medical Academy in Belgrade.

Experimental animals

For the research study, we used syngeneic female BALB/c (wild type, WT) mice, aged 6 to 8 weeks. During the experiments, the mice were kept in the vivarium, and in accordance with standard laboratory conditions (22 ± 2 °C, relative humidity $51 \pm 5\%$ and a 12-hour light-dark cycle). The animals had access to a source of food and water throughout the duration of the experiment.

Experimental animals are divided into experimental groups:

- 1) female BALB/c mice with breast cancer induced;
- 2) female BALB/c mice induced with breast cancer and treated with anti PD-1 antibody;

Tumor induction

Weakly immunogenic breast cancer cell lines (4T1) compatible with the BALB/c genetic background were purchased from ATCC (American Type Culture Collection, ATCC, USA). An earlier protocol (24) was used. A dose of 5×10^3 4T1 cells was used, which was determined based on the results of a series of pre-experiments that preceded this research study.

Monitoring of palpable tumor appearance and tumor growth was followed by previously described methods (25).

Administration of anti PD-1 antibodies

Mouse anti PD-1 antibody was purchased from BioXcell (clone number: RMP1-14). The antibody was applied intraperitoneally to BALB/c mice on the third, sixth, ninth and eleventh days, starting from the day of tumor induction, in a dose of 150 µg dissolved in 150 µl of PBS (Phosphate-buffered saline). Mice that did not receive anti PD-1 antibody received intraperitoneally 150 µl of PBS on the same days.

Examination of cell populations in the spleen and tumor microenvironment - flow cytometry

Single-cell suspensions of spleen cells were obtained by mechanical dispersion, while single-cell suspensions of primary tumors were obtained by an enzymatic digestion process, as previously described (25). Fluorochrome-labeled anti-mouse antibodies specific for CD3, CD49, CD69, NKG2D (natural killer group 2D) and isotype controls (BD Pharmingen, NJ/Invitrogen, Carlsbad, CA) were used in the research. For intracellular staining, which was performed according to an established protocol (25), anti-mouse antibodies specific for CD107a, Foxp3 (forkhead box P3), IFN-γ, IL-10 (Interleukin - IL), IL-17 were used (BD Pharmingen/ BioLegend/eBiosciences). From 20,000 to 100,000 cells per sample were analyzed by flow cytometry. Flow cytometry was performed on a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA) and data were analyzed using FlowJo software (Tree Star).

Ethical statement

The research was approved by the competent Ethical Commission for the welfare of laboratory animals of the Faculty of Medical Sciences of the University of Kragujevac (approval number: 01-12188 dated 26.10.2018). All research procedures were conducted in compliance with the Helsinki Declaration and the Principle of Good Laboratory Practice.

Statistical data processing

All obtained data were statistically processed using the software program (SPSS v.23), using Student's t test, Mann-Whitney U test, ANOVA or Kruskal-Wallis tests as needed. The difference in the appearance of the primary tumor was analyzed using the Kaplan-Meier curve and log-rank analysis.

RESULTS

Effect of PDL/PD-1 blockade on NK cells

The percentage of FasL-expressing CD3⁺CD4b⁺ NK cells was significantly higher in spleens of BALB/c mice on anti PD1 therapy compared to BALB/c mice without therapy ($p < 0.05$; Figure 1a). The percentage of CD3⁺CD4b⁺ NK cells that were expressing NKG2D was significantly higher in the spleen of anti PD-1 treated BALB/c mice ($p < 0.05$; Figure

1b). Anti PD-1 therapy significantly increased IFN-γ (interferon-gamma) expression in splenic CD3⁺CD4b⁺ NK cells BALB/c mice compared to WT mice without therapy ($p < 0.05$; Figure 1c). The percentage of CD3⁺CD4b⁺ NK cells producing IL-10 was significantly lower in WT mice on PD-1 therapy ($p < 0.05$; Figure 1d). In the tumor microenvironment, anti PD-1 therapy significantly increased accumulation of CD3⁺CD4b⁺ NK cells that were expressing NKG2D ($p < 0.05$; Figure 2a). Also, the percentage of FoxP3-expressing CD3⁺CD4b⁺ NK cells was significantly lower in tumors from WT mice on anti-PD-1 therapy compared to WT mice without therapy ($p < 0.05$; Figure 2b).

Effect of PDL/PD-1 blockade on T lymphocytes

Application of anti-PD-1 therapy has significantly increased expression of CD107a molecule in splenic CD3⁺CD49b⁺ T lymphocytes ($p < 0.05$; Figure 3a). Also, anti PD-1 therapy has significantly increased production of IL-17 in splenic CD3⁺CD49b⁺ T lymphocytes ($p < 0.05$; Figure 3b). In the tumor microenvironment, the application of anti-PD-1 therapy has significantly increased percentage representation of CD69⁺CD3⁺CD49b⁺ T lymphocytes ($p < 0.05$; Figure 4a). Anti-PD-1 therapy has significantly decreased percentage representation of IL10⁺CD3⁺CD49b⁺ T lymphocytes in compared to the experimental group without therapy ($p < 0.05$; Figure 4b).

DISCUSSION

Immunotherapy of breast carcinoma is currently evolving field, with usage of immune check-point inhibitors being in the limelight of scientific research (26). Anti-PD-1 therapy is still emerging as one of the leading therapeutics in breast cancer, with it being approved in triple-negative breast cancer (27). Effects of PD-1 blockage are mainly conveyed through facilitation of effector cells, such as T and NK cells (28). The roles of T and NK cells in immune response to breast carcinoma are intertwined. T cells eradicate tumor cells via presentation of tumor antigens, and NK cells additionally eradicate every transformed cell, regardless of tumor antigen presentation (29). In addition, activated NK cells can further on stimulate effector T cells. Anti-PD-1 therapy stimulates both types of cells, making overall immune response more efficient. To illustrate the exact effect behind T and NK cells activation during anti- PD-1 therapy, we conducted this study to analyze the phenotype changes of T and NK cells during immune response to breast carcinoma.

We firstly analyzed NK cells in both, spleen and tumor microenvironment. As mentioned above, NK cells have important role in eradicating all tumors cells, but chronic inflammation that takes place constantly in the tumor microenvironment, could induce inefficient regulatory phenotype of NK cells (30). On the other hand, some recent studies show that increased percentage of NK cells within tumor microenvironment are related to increased survival (31) Our results revealed that administration of anti-PD-1 antibodies increases the expression of activating markers such as FasL,

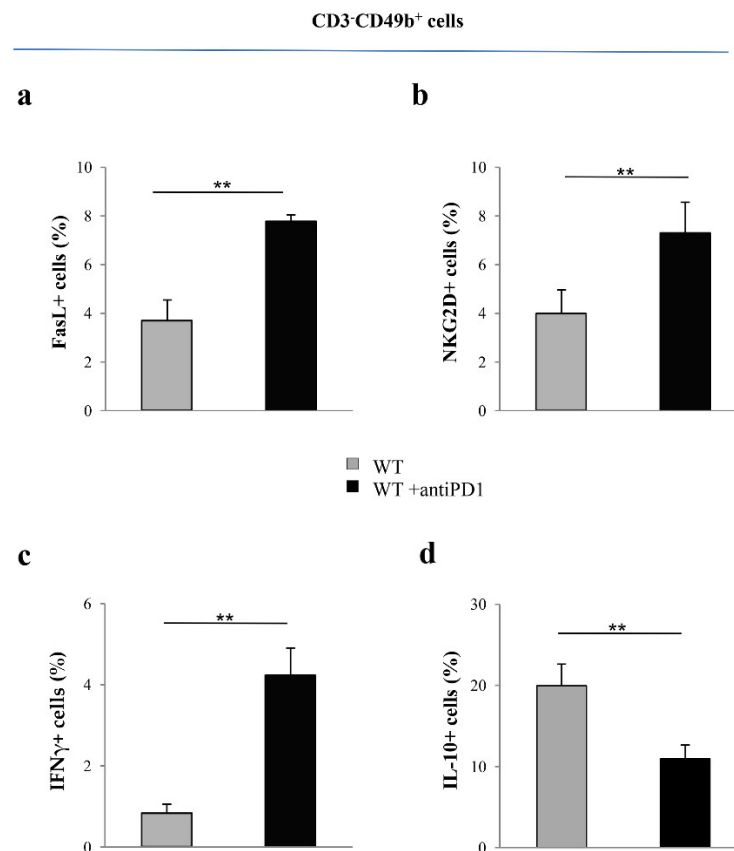
NKG2D and IFN- γ in both, splenic and tumor-infiltrating NK cells (Fig 1-2). These results are in line with previously published data (28). On the other hand, anti- PD-1 therapy significantly decreased the expression of inhibitory markers such as Foxp3 and IL-10 in NK cells (Fig 1-2). Foxp3 and IL-10 are thought to be markers of a regulatory NK cells phenotype (32). The application of anti- PD-1 therapy therefore efficiently reduces phenotype switch of NK cells towards regulatory one within spleen, and more importantly, within tumor environment.

The important role of T cells in antitumor immunity is well established. When it comes to T cells, anti PD-1 therapy increases the percentage of splenic CD107a⁺ and IL-17⁺ T cells (Fig 3-4). Our results are in line with previously published studies (33, 34). CD107a/LAMP-1 (Eng. *Lysosome-Associated Membrane Protein 1*) is one of the main glycoproteins in the membrane of cytolytic granules and also a marker of degranulation of NK cells and CTLs (35). CD107a expression correlates with cytokine secretion and cytotoxic function of NK cells. IL-17 is a marker of increased inflammatory response (36).

Within tumor microenvironment, after application of anti-PD-1 antibody, there is an increased percentage of CD69⁺ T lymphocytes, and, on the other hand, there is decreased level of immunosuppressive IL-10 (Fig 3-4). The C-type lectin receptor CD69 represents an early activating marker and may play a direct role in mediating cytotoxicity against tumor target cells (37). These results are in line with previously published studies (38, 39). When it comes to IL-10, its increased expression could be indicative of exhaustion in T cells, skewing T cells towards immunosuppressive phenotype (40). As anti PD-1 blockage lowers IL-10 expression in tumor microenvironment, tumor-infiltrating T cells during anti PD-1 therapy are evidently more active conveyers of anti-tumor immunity. These phenotype changes of T cells in spleen and tumor microenvironment altogether illustrate a more active T cell, leading to overall more effective anti-tumor response.

In conclusion, our presented results indicate that anti- PD-1 therapy activates both, NK and T cells, producing a more improved anti-tumor immune response to breast carcinoma, which could potentially lead to better prognosis. More studies are required to further elucidate the interplay between NK and T cells during PD-1 blockage in breast carcinoma.

Figure 1. The percentage of FasL⁺ cells was significantly higher in spleens of BALB/c mice on anti PD1 therapy compared to BALB/c mice without therapy (1a).



The percentage of cells that were expressing NKG2D was significantly higher in the spleen of anti PD-1 treated BALB/c mice (1b). Increased IFN- γ expression in NK cells BALB/c mice compared to WT mice without therapy (1c). The percentage of NK cells producing IL-10 was significantly lower in WT mice on PD-1 therapy (1d). Statistical significance is defined using Student's t test.

Figure 2. Increased accumulation of CD3⁺CD4b⁺ NK cells that were expressing NKG2D (2a) and significantly lower percentage of FoxP3-expressing CD3⁺CD4b⁺ NK cells in tumors from WT mice with anti-PD-1 therapy compared to WT mice without therapy ($p < 0.05$; Figure 2b). Statistical significance is defined using Student's t test.

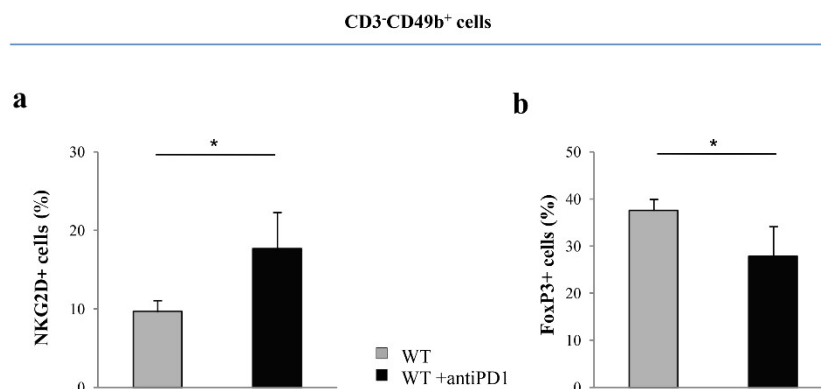


Figure 3. Significantly increased expression of CD107a molecule in splenic CD3⁺CD49b⁻ T lymphocytes (3a) and significantly increased production of IL-17 in splenic CD3⁺CD49b⁻ T lymphocytes (3b) in WT mice with anti-PD-1 therapy compared to WT mice without therapy. Statistical significance is defined using Student's t test.

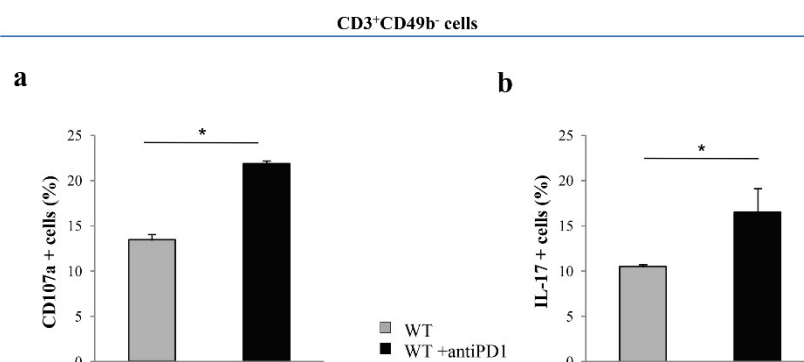
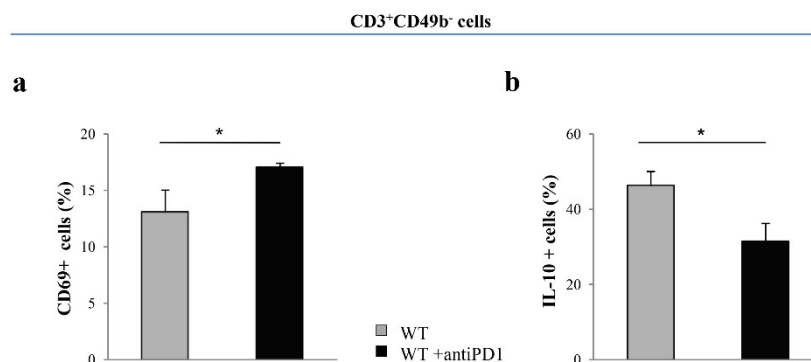


Figure 4. Significantly increased percentage representation of CD69⁺ CD3⁺CD49b⁻ T lymphocytes (4a) and significantly decreased percentage representation of IL10⁺ CD3⁺CD49b⁻ T lymphocytes (4b) in the tumor microenvironment of WT mice with anti-PD-1 therapy compared to the experimental group without therapy. Statistical significance is defined using Student's t test.



DECLARATION OF INTEREST

The authors declare that they have no competing interests.

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