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Anti-PD-1 therapy activates tumoricidic properties of NKT cells and contributes to the overall deceleration of tumor progression in a model of murine mammary carcinoma

Anti-PD-1 terapija aktivira tumoricidna svojstva NKT ćelija i doprinosi ukupnom usporavanju progresije tumora u modelu mišjeg karcinoma dojke

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Abstract

Background/Aim. Immune checkpoint therapy is a well-established therapeutic approach in the treatment of malignant diseases and is thought to be mostly based on facilitating the adaptive immune response. However, the cells of the innate immune response, such as natural killer T (NKT) cells, might also be important for a successful anti-programmed cell death protein-1 (anti-PD-1) therapy, as they initiate the antitumor immune response. The aim of this study was to investigate the influence of anti-PD-1 therapy on the immune response against tumors. **Methods.** For tumor induction, 4T1 cells synergic to BALB/c background were used, after which mice underwent anti-PD-1 treatment. After the mice were sacrificed, NKT cells, dendritic cells (DCs), and macrophages derived from spleen and primary tumor tissue were analyzed using flow cytometry. **Results.** Anti-PD-1 therapy enhanced the expression of activating molecules CD69, NKp46, and NKG2D in NKT cells of the tumor and spleen. This therapy activated NKT cells directly and indirectly via DCs. Activated NKT cells acquired tumoricidic properties directly, by secreting perforin, and indirectly by stimulating M1 macrophages polarization. **Conclusion.** Anti-PD-1 therapy activates changes in DCs and macrophages of primary tumor tissue towards protumoricidic activity. Since anti-PD-1 therapy induces significant changes in NKT cells, DCs, and macrophages, the efficacy of the overall antitumor response is increased and has significantly decelerated tumor growth.

Key words:
antineoplastic agents; breast neoplasms; immunomodulation; killer cell, natural; macrophages; mice.

Apstrakt

Uvod/Cilj. Imunoterapija je danas dobro poznat terapijski pristup u lečenju malignih bolesti koji se temelji na stimulisanju stečenog imunskog odgovora. Međutim, ćelije urođenog imuskog odgovora, kao što su prirodne T ćelije ubice – *naturall killer T cells* (NKT), takođe mogu biti bitne za uspešnu terapiju i započinjanje antitumorskog imunskog odgovora delovanjem na protein 1 programirane ćelijske smrti (PD-1). Cilj rada bio je da se ispita uticaj anti-PD-1 terapije na antitumorski imunski odgovor. **Metode.** Za indukciju tumora korišćene su 4T1 ćelije, singene za BALB/c miševe, nakon čega su miševi tretirani anti-PD-1 antitelom. Nakon žrtvovanja miševa, NKT ćelije, dendritske ćelije (DC) i makrofagi iz slezine i primarnog tumora analizirani su uz pomoć protočne citometrije. **Rezultati.** Anti-PD-1 terapija je povećala ekspresiju aktivirajućih molekula CD69, NKp46, i NKG2D u NKT ćelijama slezine i tumora. Ova terapija aktivira NKT ćelije direktno i indirektno, preko DC. Aktivirane NKT ćelije nakon anti-PD-1 terapije stiču tumoricidna svojstva direktno, preko povećanog stvaranja perforina, i indirektno, putem polarizacije makrofaga u pravcu M1 fenotipa. **Zaključak.** Anti-PD-1 terapija je podstakla promene fenotipa DC i makrofaga u primarnom tumorskom tkivu u pravcu antitumorske aktivnosti. Kako

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anti-PD-1 terapija indukuje značajne promene u NKT ćelijama, DC i makrofagima, efikasnost sveukupnog antitumorskog odgovora je veća i značajno je usporila rast tumora.

Ključne reči:
antineoplastici; dojka, neoplazme; imunomodulacija; ćelije ubice, prirodne; makrofagi; miševi.

Introduction

Immunotherapy is an emerging approach to the treatment of many cancers nowadays [1]. Since their discovery, immune checkpoint inhibitors (anti-programmed cell death protein 1 (anti-PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibodies have been utilized in various diseases, such as autoimmune or even infectious diseases. However, they are predominantly used in malignant diseases, with evolving strategies in the management of the diseases [2].

The underlying mechanism of anti-PD-1 therapy is blockage of the programmed death ligand (PDL)/PD-1 axis. Under physiological circumstances, PDL is found on many epithelial, endothelial, and immune cells, such as dendritic cells (DCs) and macrophages [3]. The main role of this ligand is to limit over-reactive immune response, therefore restricting tissue damage due to unrestrained immune response, since the activation of the PDL/PD-1 axis potently hinders T-cell receptor activation [4, 5]. However, during a malignant disease, PDL is often found on cancer cells. PD-1 molecule is mainly expressed on effector immune cells, such as T lymphocytes, natural killer (NK) cells, and natural killer T (NKT) cells [6]. Given its expression on cancer cells and effector cells of the immune response, the activation of the PDL/PD-1 axis in these terms subsequently leads to a deteriorating immune response to malignant diseases [7]. Bearing in mind these assets of a PDL/PD-1 axis, it is clear that its inhibition is important for treating many diseases, especially cancers. Until now, anti-PD-1 therapy has been approved for many types of solid cancers, such as metastatic melanoma, non-small cell lung cancer, renal cell carcinoma, bladder cancer, and triple-negative breast cancer with high PDL expression [8–10].

Although therapeutic PDL/PD-1 blockage is thought to be mainly carried through blockage on T lymphocytes, there is emerging evidence that other effector cells, such as NK and NKT cells, take part in the beneficial effects of PDL/PD-1 axis blockage [11]. Until now, it has been well known that in some malignant diseases, the PD-1 molecule is more expressed in NK cells, which suggests damaged NK cell function [12]. Since it is well known that anti-PD-1 therapy increases cytokine production, especially in T lymphocytes, it remains unclear whether anti-PD-1 therapy acts directly on NK cells or indirectly via secretion of activating molecules, such as interferon (IFN) -γ [13]. Data are very modest when it comes to NKT cells and anti-PD-1 therapy. These cells play an important role in the interplay between innate and acquired immune responses [14]. Moreover, it is known that NKT cells produce cytokines that can activate macrophages and DCs and, therefore, coordinate immune response [15]. However, the effect of anti-PD-1 therapy on NKT cells is yet to be elucidated. Our data imply that NKT cells might also be important for more effective anti-PD-1 therapy in malignancies and might contribute to the overall effective immune response to mammary carcinoma, as anti-PD-1 therapy induces phenotype changes in NKT cells.

Methods

Mice

Female, six to eight weeks old, BALB/C wild type (WT) mice were used in all experiments. Experiments were conducted at the Center for Molecular Medicine and Stem Cell Research of the Faculty of Medical Sciences, University of Kragujevac, Serbia. The mice were housed under standard laboratory conditions (22 ± 2 °C, relative humidity 51 ± 5%, and a 12-hour light-dark cycle) throughout the whole experiment. All experiments were approved by the Animal Ethics Board of the Faculty of Medical Sciences, University of Kragujevac, Serbia (01-12188). Mice were divided into two experimental groups, each group consisting of six mice per group: 1) wild type (WT) BALB/C untreated mice and 2) WT anti-PD-1 treated mice – treated with the anti-PD-1 antibody on the third, sixth, ninth, and eleventh day after tumor induction.

Induction of tumor

Murine mammary carcinoma-4T1, syngenic to the BALB/c background, was purchased from the American Type Culture Collection (ATCC, USA). 4T1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, 1 mmol/L penicillin-streptomycin, and 1 mmol/L mixed nonessential amino acids (Sigma-Aldrich). Cultured 4T1 cells were harvested by brief treatment with 0.25% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS, PAA Laboratories GmbH, Etobicoke, Canada) and washed three times in serum-free PBS before use in all *in vivo* and *in vitro* experiments. The viable cell number was determined by trypan blue exclusion. Suspensions only with > 95% viable cells were used in experiments. Each mouse was inoculated with 5×10^3 4T1 cells into the 4th mammary fat pad. The dosage of 5×10^3 4T1 cells per mouse was determined based on our preceding experiments.

Administration of anti-PD-1 antibody

Murine anti-PD-1 antibody was purchased from BioXcell. Antibody was administered intraperitoneally to mice on

the third, sixth, ninth, and eleventh day, beginning from the day of tumor induction, at 150 μg per mouse of anti-PD-1 antibody dissolved in 150 μL of PBS, as previously described by Qin et al. [16] and Shimizu et al. [17]. WT mice that did not receive anti-PD-1 therapy were injected with 150 μL of PBS only, on the same days, according to the model from the study by Vo et al. [18].

Evaluation of tumor growth

The appearance of the primary tumor was monitored daily after the induction by palpation. After tumor appearance, the diameter of the primary tumor was measured three times per week using a caliper. On the 40th day after tumor induction, mice were sacrificed; the primary tumor and spleen were surgically removed.

Flow cytometric analysis of splenocytes and tumor-infiltrating leukocytes

We analyzed the spleen for the assessment of systemic antitumor immune response as our previous results illustrated that phenotype changes in splenocytes are more likely to resemble phenotype changes occurring in tumor-infiltrating leukocytes [19]. Single-cell suspensions of the spleen were obtained by mechanical dispersion, while single-cell suspensions of primary tumors were obtained by enzymatic digestion. Primary 4T1 tumors were minced and placed in 5 mL of DMEM containing 1 mg/mL collagenase I, 1 mM EDTA, and 2% FBS (all from Sigma-Aldrich) for enzymatic digestion. After incubation for 2 hrs at 37 °C, 10 mL of 0.25% trypsin was added and incubated for 3 min, followed by DNase I (Sigma-Aldrich) solution for 1 min, and the digests were filtered through a 40 mm nylon cell strainer (BD Biosciences).

Fluorochrome-labeled anti-mouse mAbs specific for CD3 (145-2C11), CD49b (HMa2), NKp46 (29A1.4), CD69 (H1.2F3), CD11c (N418), F4/80 (T45-2342), NKG2D (CX5), KLRG-1 (2F1), or isotype-matched controls (BD Pharmingen, New Jersey (NJ)/Invitrogen, Carlsbad, California (CA)) were used. For intracellular staining, cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL, Sigma-Aldrich), ionomycin (500 ng/mL, Sigma-Aldrich), and GolgyStop (BD Pharmingen, NJ) for 4 hrs and stained with fluorochrome-labeled anti-mouse mAbs specific for perforin (eBio0MAK-D), granzyme (16g6; NGZB), Foxp3 (MF23), IFN-γ (XMG1.2), interleukin (IL)-10 (JES5-16E3), tumor necrosis factor (TNF)-α (MP6-XT22), (Pharmingen/BioLegend/eBiosciences). For the purpose of flow cytometry (FACS) analysis, 20,000 to 50,000 cells were acquired. Flow cytometry was conducted on FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA), and the data were analyzed using FlowJo (Tree Star).

Statistical analysis

The data were analyzed using commercially available software (SPSS version 23.0). All results were analyzed using the Student's *t*-test, Mann-Whitney *U* test, ANOVA, or Kruskal-Wallis test where appropriate. Data are presented as mean ± SEM. Statistical significance was set at $p < 0.05$.

Results

Anti-PD-1 therapy activates splenic NKT cells and skews its phenotype towards a more tumoricidic one

Administration of anti-PD-1 therapy significantly decelerated tumor growth compared to untreated WT mice. The significant difference between tumor diameters was detected on the 14th day after tumor induction (WT vs. WT + anti-PD-1: 1.57 mm vs. 0.50 mm; $p < 0.05$) and remained until the 40th day when mice were sacrificed (WT vs. WT + anti-PD-1: 11.93 mm vs. 9.37 mm; $p < 0.05$). Further, we analyzed NKT cells in the spleen of tumor-bearing WT mice and WT mice treated with anti-PD-1 antibodies. There was no difference in the percentage of $CD3^+CD49b^+$ NKT cells between the experimental and control groups (Figure 1A). Expression of the activation marker CD69 was significantly elevated in WT anti-PD-1 treated mice compared to WT untreated mice ($p < 0.05$; Figure 1B). The percentage of IFN-γ^+ and perforin$^+$ $CD3^+CD49b^+$ NKT cells was significantly higher, while percentage of $FoxP3^+$ $CD3^+CD49b^+$ NKT cells was significantly lower in WT anti-PD-1 treated mice in comparison to WT untreated mice ($p < 0.05$; Figures 1C–E). There were no significant changes in percentage and phenotype changes in macrophages and DC in the spleen (data not shown).

Enhanced accumulation and alteration toward tumoricidal phenotype of NKT cells in the tumor microenvironment

Within primary tumor tissue, the percentage of $CD3^+CD49b^+$ NKT cells was significantly higher in anti-PD-1 treated mice compared to the untreated group ($p < 0.05$; Figure 2A). Percentage of $NKp46^+$ ($p < 0.05$; Figure 2B) and $NKG2D^+$ ($p < 0.05$; Figure 2C), as well as IFN-γ^+ $CD3^+CD49b^+$ NKT cells ($p < 0.01$; Figure 2D) was significantly higher in WT anti-PD-1 treated mice. Percentage of $FoxP3^+$ ($p < 0.05$; Figure 2E) and $KLRG1^+$ $CD3^+CD49b^+$ NKT cells ($p < 0.05$; Figure 2F) was significantly lower in WT anti-PD-1 treated mice in comparison to WT untreated mice.

Anti-PD-1 therapy facilitates the accumulation and polarization of macrophages in the tumor microenvironment

Anti-PD-1 treatment significantly increased the percentage of $F4/80^+$ cells within primary tumor tissue compared to WT untreated mice ($p < 0.05$; Figure 3A). In addition, the expression of TNF-α in $F4/80^+$ cells was significantly higher in anti-PD-1 treated mice compared to untreated mice ($p < 0.05$; Figure 3B).

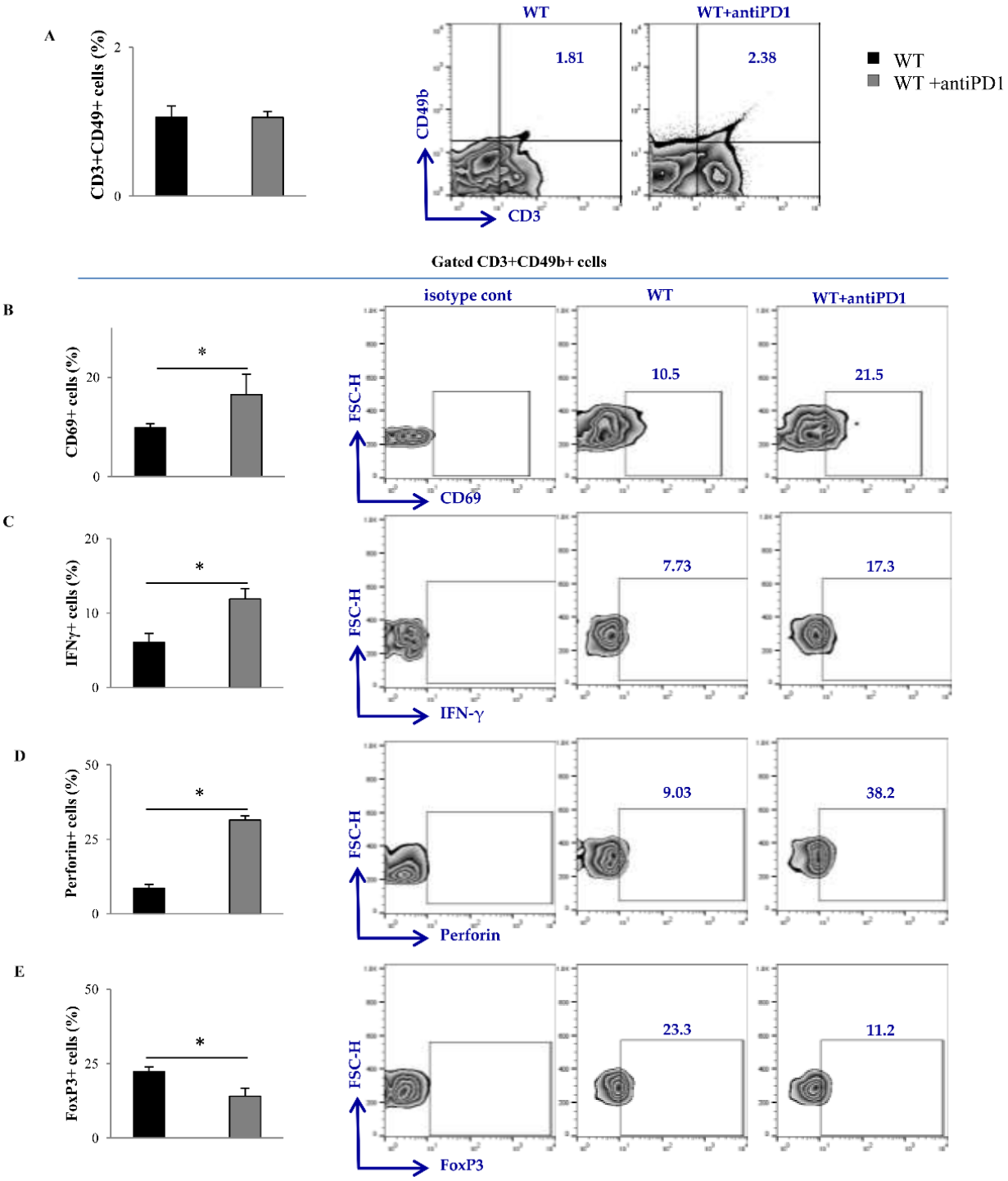


Fig. 1 – Altered phenotype of splenic natural killer T (NKT) cells in anti-programmed cell death protein-1 (anti-PD-1) treated mice. The graphs and representative flow cytometry (FACS) plots display the percentage of $CD3^+CD49b^+$ cells (A), $CD69^+$ (B), interferon (IFN) γ^+ (C), $perforin^+$ (D), and $FoxP3^+$ (E) $CD3^+CD49b^+$ NKT in spleens of wild type (WT) and WT anti-PD-1 treated mice, acquired by FACS. Data are shown as mean ± SEM of six mice per group and are representative of three separate experiments. Statistical significance was tested by the Mann-Whitney Rank Sum test or Student's unpaired *t*-test, where appropriate.

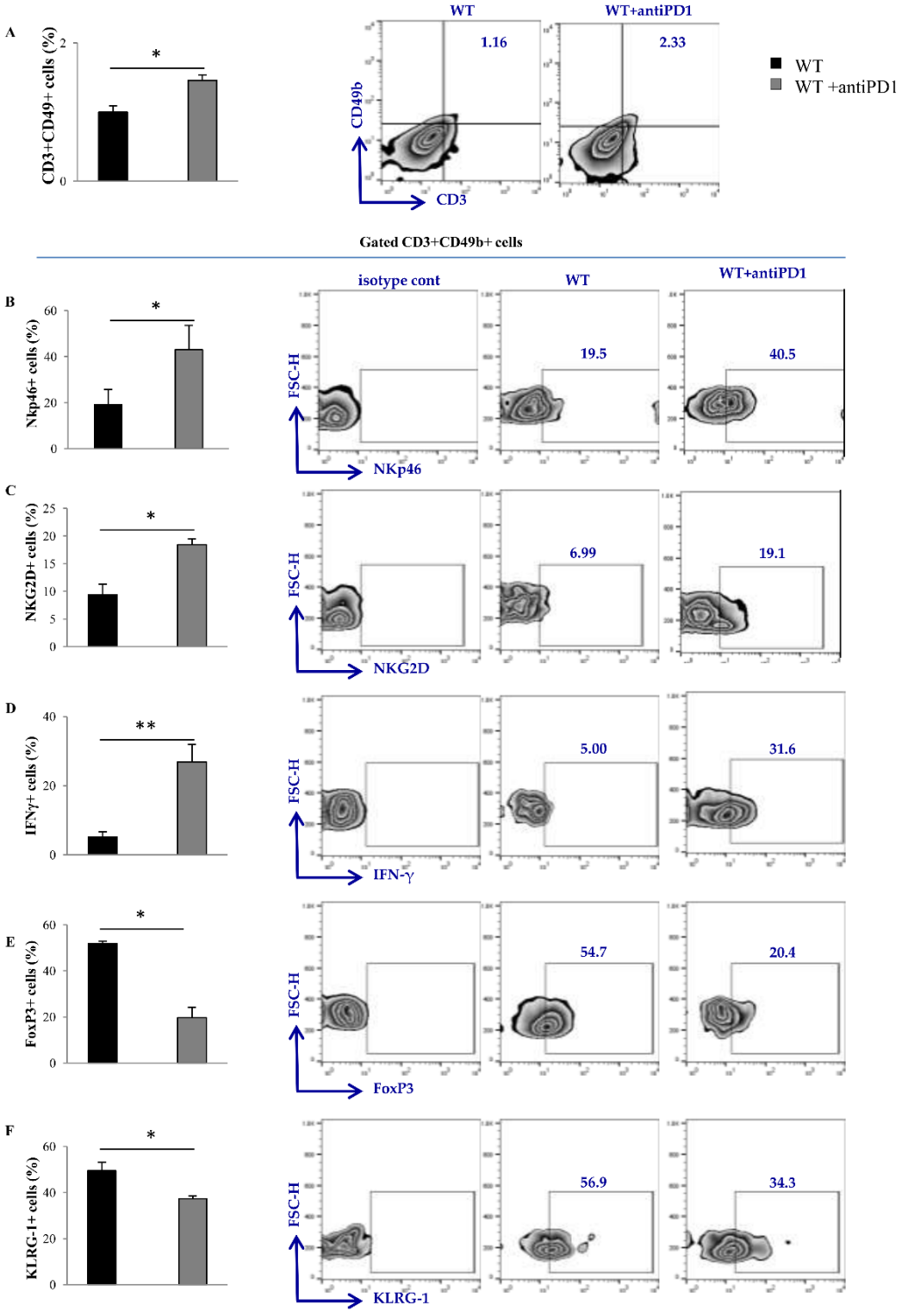


Fig. 2 – Enhanced tumoricidic phenotype of natural killer T (NKT) cells in the tumor microenvironment. The graphs and representative flow cytometry (FACS) plots display the percentage of $CD3^+CD49b^+$ cells (A), $NKp46^+$ (B), $NKG2D^+$(C), interferon (IFN) γ^+ (D), $FoxP3^+$ (E) and $KLRG1^+$ (F) $CD3^+CD49b^+$ NKT in primary tumor of wild type (WT) and WT anti-PD-1 treated mice, acquired by FACS. Data are shown as mean ± SEM of six mice per group and are representative of three separate experiments. Statistical significance was tested by the Mann-Whitney Rank Sum test or Student's unpaired *t*-test, where appropriate.

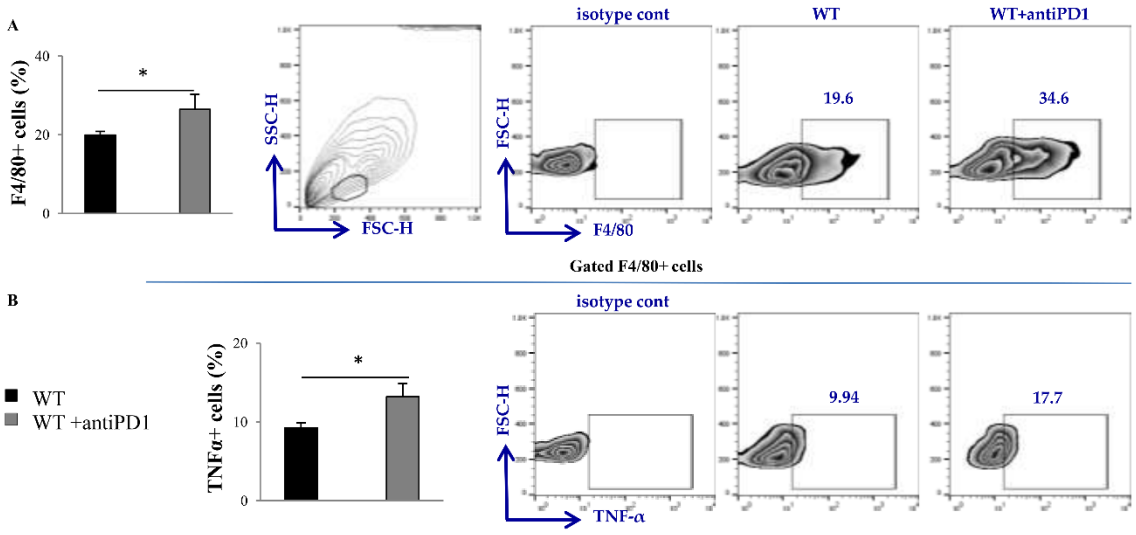


Fig. 3 – Macrophage activation in primary tumor tissue of anti-programmed cell death protein-1 (anti-PD-1) treated mice. The graphs and representative flow cytometry (FACS) plots display the percentage of F4/80$^+$ cells (A) as well as the percentage of tumor necrosis factor (TNF) α$^+$ F4/80$^+$ cells (B) in primary tumor tissue. Data are shown as mean ± SEM of six mice per group and are representative of three separate experiments. Statistical significance was tested by the Mann-Whitney Rank Sum test or Student's unpaired *t*-test, where appropriate.

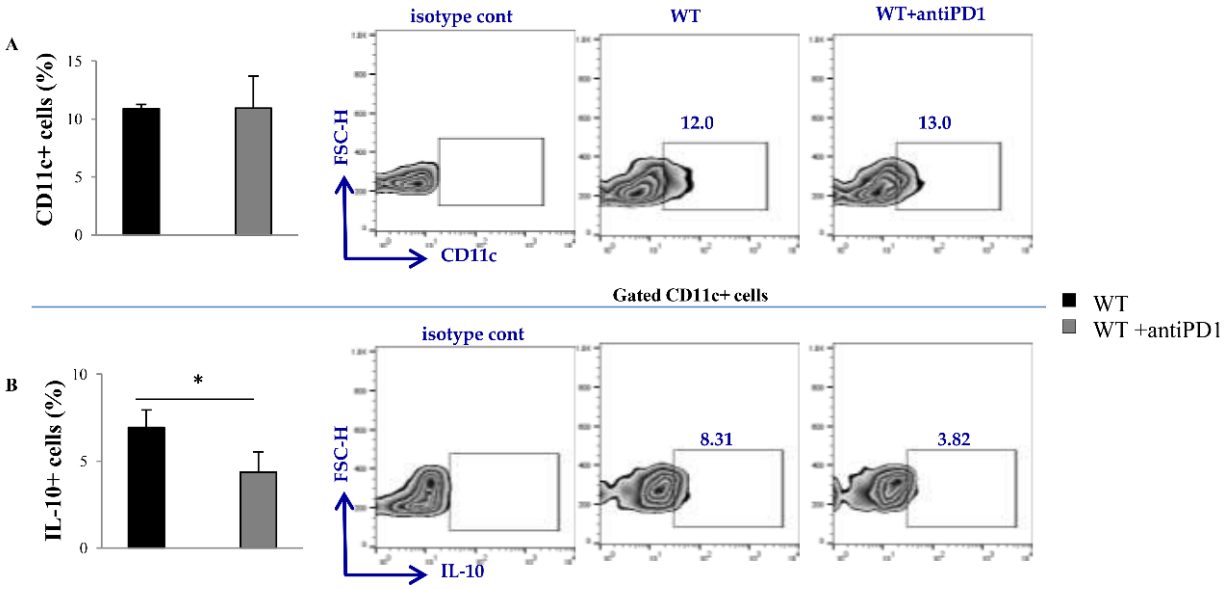


Fig. 4 – Altered phenotype of dendritic cells in the tumor microenvironment of anti-programmed cell death protein-1 (anti-PD-1) treated mice. The graphs and representative flow cytometry (FACS) plots display the percentage of CD11c$^+$ cells (A) as well as the percentage of interleukin (IL) -10$^+$ CD11c$^+$ cells (B) in primary tumor tissue. Data are shown as mean ± SEM of six mice per group and are representative of three separate experiments. Statistical significance was tested by the Mann-Whitney Rank Sum test or Student's unpaired *t*-test, where appropriate.

Anti-PD-1 therapy diminishes the expression of immunosuppressive molecules in dendritic cells within the primary tumor

There was no statistical difference in the percentage of CD11c$^+$ cells in the tumor microenvironment between groups (Figure 4A). However, the percentage of IL-10 producing CD11c$^+$ cells was significantly lower in the tumor microenvironment of anti-PD-1 treated mice ($p < 0.05$; Figure 4B).

Presumed mechanism of action of anti-PD-1 therapy on NKT, dendritic cells, and macrophages is given in Figure 5.

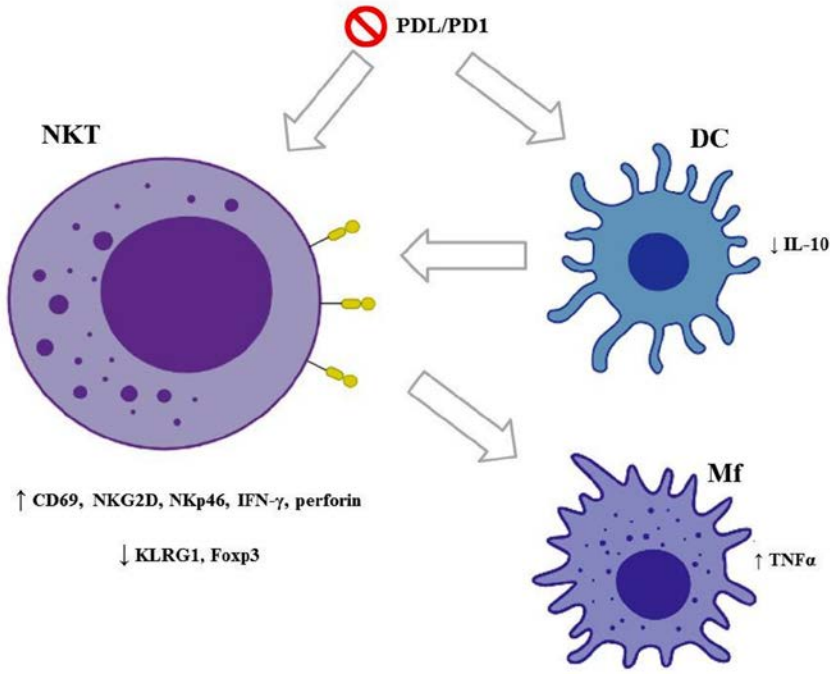


Fig. 5 – Effects of anti-programmed cell death protein-1 (anti-PD-1) therapy on natural killer T (NKT), dendritic cells (DC), and macrophages (Mf). Anti-PD-1 therapy acts directly on NKT cells by facilitating their pro-tumoricidic phenotype, which further polarizes Mf towards the M1 phenotype via augmented interferon (IFN) γ secretion. In addition, anti-PD-1 therapy lowers interleukin (IL)-10 production in DC, making them less tolerogenic and more efficient in activating NKT cells.
PDL – programmed death ligand.

Discussion

As it is well known, checkpoint inhibitors are currently taking an important role in the management of malignant diseases [20, 21]. More specifically, anti-PD-1 antibody has been and is yet to be investigated in numerous oncological diseases, such as melanoma, lung, head and neck, and genitourinary cancers [11, 22]. As it prolongs the half-life of effector immune cells, anti-PD-1 therapy efficiently modulates and stimulates a more efficient immune response [23]. Many studies have shown beneficial effects on T lymphocytes. It has been shown that anti-PD-1 therapy efficiently increases the percentage of cytotoxic T lymphocytes (CTL) within tumor tissue. Moreover, there is some evidence that anti-PD-1 therapy elevates the percentage of $CD4^+$ cells in the peripheral blood of patients undergoing anti-PD-1 therapy [24–28]. Even though T lymphocytes are rather important for compliant anti-PD-1 therapy, other cells, such as NK and NKT cells might contribute to more potent effects of anti-PD-1 therapy. Until now, the anti-PD-1 therapy has been thoroughly studied in terms of adaptive immune response [25, 27], but is yet to be studied in innate immunity during an antitumor immune response, especially regarding NKT cells. It is of great significance to elucidate the effects of immune checkpoint therapy on NK and NKT cells, as these cells might be the key to initiating successful anti-PD-1 therapy when the function of T lymphocytes is impaired [6, 29]. NKT cells have an important role in antitumor immunity. As these cells possess the tremendous capacity to rapidly secrete IFN-γ, IL-2, TNF-α, and IL-4 after antigen stimulation on the one hand and the possibility of specific recognition of antigens on the other, NKT cells might be one of the first cells to instigate antitumor immune response [30]. Additionally, malignancies have the potential to disrupt the metabolism of fatty acids and use them as a source for tumor expansion, while NKT cells that are mostly targeted to lipid antigens might suppress tumor progression by being aimed at altered lipid antigens [31]. In addition, the interaction of NKT cells with other innate immunity cells, such as antigen-presenting cells, stimulates antitumor immune response altogether [32–34].

As NKT cells present an important player in antitumor immunity, we focused our research on the effects of anti-PD-1 therapy on NKT cells, DCs, and macrophages in a model of murine mammary carcinoma. Initially, prior to analysis of immune cells phenotype, tumor growth and progression were significantly slower in anti-PD-1 treated mice when compared to untreated mice. This finding is in line with many previous clinical trials revealing the beneficial effect of anti-PD-1 therapy on decelerating tumor growth and progression, including lung cancer, renal cancer, and especially melanoma [3, 10, 11]. Further on, we analyzed the phenotype of NKT cells in the spleen and tumor microenvironment. Although the percentage of $CD3^+CD49^+$ NKT cells in spleens of anti-PD-1 treated mice remains unchanged (Figure 1A), the phenotype of $CD3^+CD49^+$ NKT cells is remolded towards a more active one. There was a significantly higher percentage of $CD69^+CD3^+CD49^+$ cells in

anti-PD-1 treated mice, which implies that therapy might enforce activation of $CD3^+CD49^+$ cells in the spleen (Figure 1B). In line with this finding, it has been shown that in highly immunosuppressive tumors, such as head and neck carcinomas, ligands for PD-1 in tumor tissue potentially inhibit expression of CD69 and consequently dampen down activation of immune cells [35]. Anti-PD-1 therapy also significantly raised the percentage of IFN-γ^+ $CD3^+CD49^+$ cells in the spleen (Figure 2C). Available data suggest that anti-PD-1 therapy increases the expression of IFN-γ and inhibits the progression of aggressive tumors such as NK/T lymphomas [36]. Furthermore, it has been shown that augmented IFN-γ production in NKT cells stimulates CTL- mediated antitumor immunity in a model of highly immunogenic T cell lymphoma [37]. On the other hand, anti-PD-1 therapy also enhanced the production of perforin in $CD3^+CD49^+$ cells, suggesting that anti-PD-1 therapy can directly enhance the cytotoxic potential of NKT cells (Figure 2D). In addition, the expression of immunosuppressive marker FoxP3 was significantly lower in $CD3^+CD49^+$ cells of anti-PD-1 treated mice (Figure 2E). This indicates that anti-PD-1 therapy, besides directly enhancing IFN-γ production, simultaneously weakens immunosuppressive assets of NKT cells, therefore contributing to the more tumoricidic phenotype of NKT cells altogether.

When it comes to the tumor microenvironment, the percentage of $CD3^+CD49^+$ cells was significantly higher in anti-PD-1 treated mice (Figure 2A), implicating intensive accumulation of NKT cells in primary tumor tissue due to anti-PD-1 therapy. As it is already known, the presence of NKT cells within primary tumor tissue modifies the tumor microenvironment by secreting IFN-γ that activates effector cells and suppresses immunosuppressive populations, therefore enabling a more fluent antitumor immune response [38–40]. Our results imply that anti-PD-1 therapy might stimulate these beneficial properties of NKT cells. Moreover, the percentage of $NKp46^+$ and $NKG2D^+$ cells was also significantly increased in anti-PD-1 treated mice (Figures 2B and 2C), which reflects a more dexterous phenotype of NKT cells in the tumor microenvironment. Similarly, as in the spleen, anti-PD-1 therapy also raised the percentage of IFN-γ-producing $CD3^+CD49^+$ cells within primary tumor tissue (Figure 2D). Furthermore, the expression of FoxP3 and KLRG-1 markers in $CD3^+CD49^+$ NKT cells was significantly diminished in the tumor microenvironment, which is indicative of the NKT cell phenotype that is less prone to anergy [38].

As NKT cells are known to interact with many immune cells, such as T cells, DCs, and macrophages [41, 42], and the fact that 4T1 mammary carcinoma presents a low immunogenic tumor, we further analyzed DCs and macrophages within the primary tumor. Tumor-associated macrophages (TAMs) are one of the most abundant cells within the primary tissue of the tumor [43]. Unfortunately, TAMs that reside in the tumor microenvironment are mostly those of an immunosuppressive M2 phenotype, thus allowing the immune escape of the tumor [44]. Given the vast range of macrophage immunomodulatory properties, facilitating these cells might be of great significance when it comes to revealing more potent therapeutic strategies in malignancy. As it is known, TAMs might stimulate an antitumor immune response by secreting TNF-α and also suppress the antitumor immune response by secreting IL-10 inducing overall immunosuppression [45, 46]. Our results showed that anti-PD-1 therapy significantly enhanced the percentage of $F4/80^+$ macrophages within the tumor microenvironment (Figure 3A) and, in addition, significantly increased the production of TNF-α in $F4/80^+$ macrophages (Figure 3B), which is a hallmark of the M1 phenotype [47]. When it comes to DCs, anti-PD-1 therapy did not alter the percentage of resident $CD11c^+$ DCs within the tumor microenvironment (Figure 4A). Yet, the percentage of IL-10 producing $CD11c^+$ DCs was significantly decreased in anti-PD-1 treated group (Figure 4B). As professional antigen-presenting cells, DCs are constantly circulating throughout the tumor microenvironment, where they are continuously exposed to immunosuppressive molecules produced by cancer cells [48]. As such, DCs might become tolerogenic and stimulate further immunosuppression by secreting molecules such as IL-10 [49]. According to their role in immune responses, DCs are traditionally divided into two groups: conventional or classical DCs and plasmacytoid DCs [50]. Conventional DCs express high levels of major histocompatibility complex (MHC) molecules, thus stimulating antitumor immunity, while plasmacytoid DCs are mainly involved in interferon secretion. Apart from classification, during an antitumor immune response, DCs can switch between tolerogenic and effector phenotypes [49, 51]. As our results showed markedly lowered expression of IL-10 in DCs, we believe that anti-PD-1 therapy, at least in part, might abrogate polarization of DCs towards tolerogenic phenotype. DCs, as it is known, interact with NKT cells via direct contact or indirectly by expressing and secreting modulating molecules, such as CD40, type I and II interferons, IL-10, and TNF-α [52]. Given our result, that DCs of anti-PD-1 treated mice have a more immunogenic phenotype than those in untreated mice, DCs of anti-PD-1 treated mice might be even more potent in triggering NKT cell activation besides the already shown direct activation of NKT cells by anti-PD-1 therapy. As mentioned before, upon activation, NKT cells rapidly secrete activating molecules that stimulate other immune cells, such as macrophages. Since our results imply enhanced secretion of IFN-γ in NKT cells upon anti-PD-1 therapy, and, on the other hand, IFN-γ strongly facilitates macrophages towards the M1 phenotype [53], we speculate that anti-PD-1 driven NKT cells polarize macrophages towards the antitumorigenic, M1 phenotype (Figure 5).

Conclusion

Anti-PD-1 therapy activates NKT cells directly and indirectly via DCs. Activated NKT cells provide tumoricidic properties directly by secreting perforin and indirectly by polarizing macrophages towards the M1 phenotype. Further studies are needed to clarify the interplay between NKT cells and other immune cells in the context of anti-PD-1 therapy, shedding new light on various beneficial aspects of immune checkpoint therapy.

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