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ANTI PD1 THERAPY ACTIVATES TUMORICIDIC PROPERTIES OF NKT CELLS AND CONTRIBUTES TO OVERALL DECELERATION OF TUMOR PROGRESSION IN A MODEL OF MURINE MAMMARY CARCINOMA

ANTI PD1 TERAPIJA AKTIVIRA TUMORICIDNA SVOJSTVA NKT ĆELIJA I DOPRINOSI UKUPNOM USPORAVANJU PROGRESIJE TUMORA U MODELU MIŠJEG KARCINOMA DOJKE

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Abstract

**Introduction:** Immune checkpoint therapy is well-established therapeutic approach in treatment of malignant disease and is thought to be mostly based on facilitating adaptive immune responses. However, cells of innate immune response, such as NKT cells, might also be important for successful anti-programmed cell death protein 1 therapy, as they initiate anti-tumor immune response.

**Materials and methods:** For tumor induction, 4T1 cells syngenic to BALB/c background were used after which mice underwent anti-programmed cell death protein 1 treatment. After the mice were sacrificed, NKT cells, dendritic cells and macrophages derived from spleen and primary tumor tissue were analyzed using flow cytometry.

**Results:** Anti-programmed cell death protein 1 therapy significantly decelerates tumor growth and enhanced expression of activating molecules CD69, NKp46, NKG2D in NKT cells of tumor and spleen. Anti-programmed cell death protein 1 therapy activates protumoricidic changes in dendritic cells and macrophages of primary tumor tissue.

**Conclusion:** Anti-programmed cell death protein 1 therapy activates NKT cell directly, and indirectly via DCs. Activated NKT cells provide tumoricidic properties directly, by secreting perforin, and indirectly by stimulating M1 macrophages polarization. Since anti-programmed cell death protein 1 therapy induces significant changes in NKT cells, dendritic cells and macrophages, efficacy of overall anti-programmed cell death protein 1 therapy is increased, contributing to more efficient anti-tumor immune response.

**Key words:** anti-PD1 therapy, breast cancer, NKT cells, dendritic cells, macrophages
Apstrakt

Uvod: Imunoterapija je danas dobro poznat terapeutski pristup lečenju malignih bolesti koji se temelji na stimulisancu stečenog imunskog odgovora. Međutim, ćelije urođenog imunskog odgovora, kao NKT ćelije, mogu biti takođe bitne za uspešnu anti-PD1 terapiju jer su ključne za započinjanje anti-tumorskog imunskog odgovora.

Materijal i metode: Za indukciju tumora korišćene su 4T1 ćelije, singene za BALB/c miševe, nakon čega su miševi tretirani anti-PD1 antitelom. Nakon žrtvovanja miševa, NKT ćelije, dendritske ćelije I makrofagi iz slezine I primarnog tumora su analizirani uz pomoć protočne citometrije.

Rezultati: Anti-PD1 terapija je značajno usporila rast tumora I povećala ekspresiju aktivirajućih molekula CD69, NKp46, NKG2D u NKT ćelijama slezine i tumora. Anti-PD1 terapija je podstakla anti-tumorske promene fenotipa dendritskih ćelija i makrofaga u primarnom tumorskom tkivu.

Zaključak: Anti-PD1 terapija aktivira NKT ćelije direktno i indirektno, preko dendritskih ćelija. Aktivirane NKT ćelije nakon anti-PD1 terapije zadobijaju tumoricidna svojstva direktno, preko povećanog stvaranja perforina, i indirektno, uz pomoć polarizacije makrofaga prema M1 fenotipu. Kako anti-PD1 terapija indukuje značajne promene u NKT ćelijama, dendritskim ćelijama i makrofagima, efikasnost sveukupnog anti-tumorskog odgovora je veća.

Ključne reči: anti-PD1 terapija, karcinom dojke, NKT ćelije, dendritske ćelije, makrofagi
Introduction

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

Underlying mechanism in anti PD1 therapy is blockage of PDL/PD1 axis. Under physiological circumstances, programmed death ligand (PDL) is found on many epithelial, endothelial and immune cells, such as dendritic cells and macrophages. The main role of this ligand is to limit over-reactive immune response, therefore restricting tissue damage due to unrestrained immune response, since activation PDL/PD1 axis potently hinders T-cell receptor activation. However, during a malignant disease, PDL is often found on cancer cells. PD1 molecule is mainly expressed on effector immune cells, such as T lymphocytes, NK cells and NKT cells. Given the expression on cancer cells and effector cells of immune response, activation of PDL/PD1 axis in these terms subsequently leads to deteriorating of immune response to malignant disease. Having in mind these assets of a PDL/PD1 axis, it is clear that its inhibition is important for treating many diseases, especially cancers. Until now, anti-PD1 therapy is approved for many types of solid cancers: metastatic melanoma, non-small cell lung cancer, renal cell carcinoma, bladder cancer and triple negative breast cancer with high PDL expression.

Although therapeutic PDL/PD1 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 beneficial effects of PDL/PD1 axis blockage. Until now, it is well known that in some malignant diseases PD-1 molecule is more expressed in NK cells, which suggests damaged NK cell function. Since it is well known that anti-PD1 therapy increases cytokine production, especially in T lymphocytes, it remains unclear whether anti-PD1 therapy acts directly on NK cells or indirectly, via secretion of activating molecules, such as IFNγ. Data are very modest when it comes to NKT cells and anti-PD1 therapy. These cells play an important role in interplay between innate and acquired immune response. Also, it is known that NKT cells produce cytokines that can activate macrophages and dendritic cells and therefore coordinate immune response. However, the
effect of anti-PD1 therapy on NKT cells is yet to be elucidated. Our data imply that NKT cells might be also important for more effective anti-PD1 therapy in malignancies, and might contribute to overall effective immune response to mammary carcinoma, as anti-PD1 therapy induces phenotype changes in NKT cells.

Material and Methods

Mice

Female, six to eight weeks old, BALB/C wild type (WT) mice were used in all experiments. Experiments were conducted in the Center for Molecular Medicine and Stem Cell Research of 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 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 BALB/C untreated mice (WT) and 2) wild type anti-PD1 treated (WT anti-PD1 treated), that were treated with anti-PD1 antibody on the third, the sixth, the ninth and the eleventh day after induction of tumor.

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). 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. Viable cells number was determined by trypan blue exclusion. Suspensions only with >95% viable cells were used in experiments. Each mouse was inoculated with 5 x 10^3 4T1 cells into the 4th mammary fat pad. The dosage of 5 x 10^3 of 4T1 cells per mice was determined based on our preceding experiments.
Administration of anti-PD1 antibody

Murine anti-PD1 antibody was purchased from BioXcell. Antibody was administered intraperitoneally to mice on the third, the sixth, the ninth and the eleventh day, beginning from the day of tumor induction, at 150 μg per mice of anti-PD1 antibody dissolved in 150 μl of PBS, as previously described by Qun et al and Shmizu et al\textsuperscript{16,17}. WT mice that didn’t receive anti-PD1 therapy were injected with 150 μl of PBS only, on the same days, according to the model from the study of Vo et al\textsuperscript{18}.

Evaluation of tumor growth

Appearance of primary tumor was monitored daily after induction of tumor, by palpation. After tumor appearance, diameter of primary tumor was measured three times per week using caliper. On the 40\textsuperscript{th} 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 spleen for assessment of systemic anti-tumor immune response, as our previous results illustrate that phenotype changes in splenocytes are more likely to resemble to phenotype changes occurring in tumor-infiltrating leukocytes\textsuperscript{19}. Single-cell suspensions of 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 hr 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 digests filtered through 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, NJ/Invitrogen, Carlsbad, 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 hr and stained with fluorochrome-labeled anti-
mouse mAbs specific for perforin (eBio0MAK-D), granzyme (16g6; NGZB), Foxp3 (MF23), IFN-γ (XMG1.2), IL-10 (JES5-16E3), TNF-α (MP6-XT22), (PharMingen/BioLegend/eBiosciences). 20,000 to 50,000 cells were acquired for FACS analysis. 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-PD1 therapy activates splenic NKT cells and skews its phenotype towards more tumoricidal one**
Administration of anti-PD1 therapy significantly decelerated tumor growth, in comparison to untreated WT mice. The significant difference between tumor diameter was detected on 14th day after tumor induction (WT vs. WT + anti-PD1: 1.57 mm vs. 0.50 mm; p<0.05) and remained until 40th day when mice were sacrificed (WT vs. WT + anti-PD1: 11.93 mm vs. 9.37 mm; p<0.05). Further, we analyzed NKT cells in spleen of tumor bearing WT mice and WT mice treated with anti PD1 antibody. There was no difference in percentage of CD3+CD49b+ NKT cells between experimental and control group (Fig 1A). Expression of activation marker CD69 was significantly elevated in WT anti-PD1 treated mice in comparison to WT untreated mice (p<0.05; Fig 1B). The percentage of IFNγ+ and perforin+ CD3+CD49b+ NKT cells was significantly higher, while percentage of FoxP3+ CD3+CD49b+ NKT cells was significantly lower in WT anti-PD1 treated mice in comparison to WT untreated mice (p<0.05; Fig 1C-E). There were no significant changes in percentage and phenotype changes in macrophages and dendritic cells in spleen (data not shown).


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

Within primary tumor tissue, the percentage of CD3+CD49b+ NKT cells was significantly higher in anti-PD1 treated mice in comparison to untreated group (p<0.05; Fig 2A). Percentage of NKp46+ (p<0.05; Fig 2B) and NKG2D+ (p<0.05; Fig 2C), as well as IFNγ+ CD3+CD49b+ NKT cells (p<0.01; Fig 2D) was significantly higher in WT anti-PD1 treated mice. Percentage of FoxP3+ (p<0.05; Fig 2E) and KLRG1+ CD3+CD49b+ NKT cells (p<0.05; Fig 2F) was significantly lower in WT anti-PD1 treated mice in comparison to WT untreated mice.

Anti-PD1 therapy facilitates accumulation and polarisation of macrophages in tumor microenvironment

Anti-PD1 treatment significantly increased the percentage of F4/80+ cells within primary tumor tissue in comparison to WT untreated mice (p<0.05; Fig 3A). Also, expression of TNF-α in F4/80+ cells was significantly higher in anti-PD1 treated mice in comparison to untreated mice (p<0.05; Fig 3B).

Anti PD1 therapy diminishes expression of immunosuppressive molecules in dendritic cells within primary tumor

There was no statistical difference in the percentage of CD11c+ cells in tumor microenvironment between groups (Fig 4A), however percentage of IL-10 producing CD11c+ cells was significantly lower in tumor microenvironment of anti-PD1 treated mice (p<0.05; Fig 4B).

Discussion

As it is well known, checkpoint inhibitors are currently taking an important role in management of malignant diseases. More specifically, anti PD1 antibody has been and is yet to be investigated in numerous oncological diseases, such as melanoma, lung, head and neck and genitourinary cancers. As it prolongs half-life of effector immune cells, anti-PD1 therapy efficiently modulates and stimulates more efficient immune response. Many studies have shown beneficial effects on T lymphocytes. It has been shown that anti-PD1 therapy efficiently increases the percentage of cytotoxic T within tumor tissue. Also,
there is some evidence that anti-PD1 therapy elevates the percentage of CD4+ cells in peripheral blood of patients undergoing anti-PD1 therapy24-28. Even though T lymphocytes are rather important for compliant anti-PD1 therapy, other cells, such as NK and NKT cells might contribute to more potent effects of anti-PD1 therapy. Until now, the anti-PD1 therapy has been thoroughly studied in terms of adaptive immune response25,27, but is yet to be studied in innate immunity during antitumor immune response, especially in regard to NKT cells. It is of great significance to elucidate effects of immune checkpoint therapy on NK and NKT cells, as these cells might be the key of initiating successful anti-PD1 therapy when function of T lymphocytes is impaired29,30. NKT cells have important role in antitumor immunity. As these cells possess tremendous capacity to rapidly secrete IFNγ, IL-2, TNF-α, and IL-4 after antigen stimulation on one hand, and possibility of specific recognition of antigens on the other hand, NKT cells might be one of the first cells to instigate antitumor immune response31. Also, malignancies have potential to disrupt 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 antigens32. Also, interaction of NKT cells with other innate cells, such as antigen-presenting cells, stimulates antitumor immune response altogether33,34,35.

As NKT cells present important player in antitumor immunity, we focused our research on effects of anti-PD1 therapy on NKT cells, dendritic cells and macrophages in a model of murine mammary carcinoma. Initially, prior to analysis of immune cells phenotype, tumor growth and progression was significantly slower in anti-PD1 treated mice when compared to untreated mice. This finding is in line with many previous clinical trials revealing beneficial effect of anti-PD1 therapy on decelerating tumor growth and progression, including lung cancer, renal cancer, and especially melanoma3,10,11. Further on, we analyzed phenotype of NKT cells in spleen and tumor microenvironment. Although the percentage of CD3+CD49+ NKT cells in spleens of anti-PD1 treated mice remains unchanged (Fig 1A), phenotype of CD3+CD49+ NKT cells is remolded towards a more active one. There was significantly higher percentage of CD69+CD3+CD49+ cells in anti-PD1 treated mice, which implies that anti-PD1 therapy might enforce activation of CD3+CD49+ cells in spleen (Fig 1B). In line with this finding, it has been shown that in highly immunosuppressive tumors, such as head and neck carcinomas, ligands for PD1 in tumor tissue potentially inhibit expression of CD69, and consequently dampen down
activation of immune cells\textsuperscript{36}. Anti-PD1 therapy also significantly raised the percentage of IFNγ\textsuperscript{+} CD3\textsuperscript{+}CD49\textsuperscript{+} cells in spleen (Fig 2C). Available data suggests that anti-PD1 therapy increases expression of IFNγ and inhibits progression of aggressive tumors such as NK/T lymohomas\textsuperscript{37}. Also, it has been shown that augmented IFNγ production in NKT cells stimulates cytotoxic T lymphocyte (CTL) mediated antitumor immunity in a model of highly immunogenic T cell lymphoma\textsuperscript{38}. On the other hand, anti-PD1 therapy also enhanced production of perforin in CD3\textsuperscript{+}CD49\textsuperscript{+} cells, suggesting that anti-PD1 therapy can directly enhance cytotoxic potential of NKT cells (Fig 2D). In addition, expression of immunosuppressive marker FoxP3 was significantly lower in CD3\textsuperscript{+}CD49\textsuperscript{+} cells of anti-PD1 treated mice (Fig 2E). This indicates that anti-PD1 therapy, besides directly enhancing IFNγ production, simultaneously weakens immunosuppressive assets of NKT cells, therefore contributing to more tumoricidal phenotype of NKT cell altogether.

When it comes to tumor microenvironment, the percentage of CD3\textsuperscript{+}CD49\textsuperscript{+} cells was significantly higher in anti-PD1 treated mice (Fig 2A), implicating intensive accumulation of NKT cells in primary tumor tissue due to anti-PD1 therapy. As it is already known, the presence of NKT cells within primary tumor tissue modifies tumor microenvironment by secreting IFNγ, that activates effector cells, and suppresses immunosuppressive populations, therefore enabling more fluent antitumor immune response\textsuperscript{39,40,41}. Our results imply that anti-PD1 therapy might stimulate these beneficial properties of NKT cells. Also, the percentage of NKp46\textsuperscript{+} and NKG2D\textsuperscript{+} cells was also significantly increased in anti-PD1 treated mice (Fig 2 B, C), which reflects a more dexterous phenotype of NKT cell in tumor microenvironment. Similarly as in spleen, anti-PD1 therapy also raised the percentage of IFNγ-producing CD3\textsuperscript{+}CD49\textsuperscript{+} cells within primary tumor tissue (Fig 2D). Furthermore, the expression of FoxP3 and KLRG-1 markers in CD3\textsuperscript{+}CD49\textsuperscript{+} NKT cells was significantly diminished in tumor microenvironment, which is indicative of NKT cell phenotype that is less prone to anergy\textsuperscript{39}.

As NKT cells are known to interact with many immune cells, such as T cells, dendritic cells and macrophages\textsuperscript{42,43}, and the fact that 4T1 mammary carcinoma presents low immunogenic tumor, we further analyzed dendritic cells and macrophages within the primary tumor. Tumor-associated macrophages (TAMs) are one of the most abundant cells within primary tissue of the tumor\textsuperscript{44}. Unfortunately, TAMs that reside in tumor microenvironment are mostly those of an immunosuppressive M2 phenotype, thus allowing
immune escape of the tumor\textsuperscript{45}. Given the vast range of macrophage immunomodulatory properties, facilitating these cells might be of a great significance when it comes to revealing more potent therapeutic strategies in a malignancy. As it is known, TAMs might stimulate anti tumor immune response, by secreting TNF\textalpha, and also suppress anti-tumor immune response, by secreting IL-10 that induces overall immunospuppression\textsuperscript{46,47}. Our results showed that anti-PD1 therapy significantly enhanced the percentage of F4/80\textsuperscript{+} macrophages within tumor microenvironment (Fig 3A), and, in addition, significantly increased production of TNF-\textalpha in F4/80\textsuperscript{+} macrophages (Fig 3B), which is a hallmark of a M1 phenotype\textsuperscript{48}. When it comes to dendritic cells, anti-PD1 therapy didn’t alter the percentage of resident CD11c\textsuperscript{+} dendriticcells within tumor microenvironment (Fig 4A). Yet, the percentage of IL-10 producing CD11c\textsuperscript{+} dendritic cells was significantly decreased in anti-PD1 treated group (Fig 4B). Dendritic cells, as professional antigen-presenting cells, are constantly circulating throughout tumor microenvironment where are continuously exposed to immunosuppressive molecules produced by cancer cells\textsuperscript{49}. As such, dendritic cell might become tolerogenic, and in fact, stimulate further immunosuppression by secreting molecules such as IL-10\textsuperscript{50}. According to their role in immune responses, dendritic cells are traditionally divided in two groups: conventional or classical dendritic cells and plasmacytoid dendritic cells\textsuperscript{51}. Conventional dendritic cells express high levels of MHC molecules, thus stimulating anti-tumor immunity, while plasmacytoid dendritic cells are mainly involved in interferon secretion. Apart from classification, during anti-tumor immune response, dendritic cells can switch between tolerogenic and effector phenotypes\textsuperscript{50,52}. As our results showed markedly lowered expression of IL-10 in dendritic cells, we believe that anti-PD1 therapy, at least in part, might abrogate polarization of dendritic cells towards tolerogenic phenotype. Dendritic cells, 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, TNF\textalpha\textsuperscript{53}. Given our result, that dendritic cells of anti-PD1 treated mice have more immunogenic phenotype than those in untreated mice, dendritic cells of anti-PD1 treated mice might be even more potent in triggering NKT cell activation, in addition to already shown direct activation of NKT cells by anti-PD1 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-\gamma in NKT cells upon anti-PD1 therapy, and, on the
other hand, IFN\(\gamma\) strongly facilitates macrophages towards M1 phenotype\(^{54}\), we speculate that anti-PD1 driven NKT cells to polarize macrophages towards anti-tumorigenic, M1 phenotype.

Taking into consideration presented results, we believe that anti PD1 therapy activates NKT cell directly, and indirectly via DCs. Activated NKT cells provide tumoricidic properties directly, by secreting perforin, and indirectly by polarizing macrophages towards M1 phenotype, as illustrated in Figure 5. Lastly, further studies are needed to clarify interplay between NKT cells and other immune cells in context of anti-PD1 therapy, shedding a new light to various beneficial aspects of immune checkpoint therapy.

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Figure legends

1. **Altered phenotype of splenic NKT cells in anti-PD1 treated mice.** The graphs and representative FACS plots displaying the percentage of CD3⁺CD49b⁺ cells (A), CD69⁺ (B), IFNγ⁺ (C), perforin⁺ (D), and FoxP3⁺ (E) CD3⁺CD49b⁺ NKT in spleens of WT and WT anti-PD1 treated mice, acquired by flow cytometry. Data are shown as mean±SEM of six mice per group and are representative of three separate experiments. Statistical significance was tested by Mann–Whitney Rank Sum test or Student’s unpaired t-test, where appropriate.

2. **Enhanced tumoricidal phenotype of NKT cells in tumor microenvironment.** The graphs and representative FACS plots displaying the percentage of CD3⁺CD49b⁺ cells (A), NKp46⁺ (B), NKG2D⁺ (C), IFNγ⁺ (D), FoxP3⁺ (E) and KLRG1⁺ (F) CD3⁺CD49b⁺ NKT in primary tumor of WT and WT anti-PD1 treated mice, acquired by flow cytometry. Data are shown as mean±SEM of six mice per group and are representative of three separate experiments. Statistical significance was tested by Mann–Whitney Rank Sum test or Student’s unpaired t-test, where appropriate.

3. **Macrophage activation in primary tumor tissue of anti PD1 treated mice.** The graphs and representative FACS plots displaying the percentage of F4/80⁺ cells (A) as well as the percentage of TNFα⁺ 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 Mann–Whitney Rank Sum test or Student’s unpaired t-test, where appropriate.

4. **Altered phenotype of dendritic cells in tumor microenvironment of anti–PD1 treated mice.** The graphs and representative FACS plots displaying the percentage of CD11c⁺ cells (A) as well as the percentage of 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 Mann–Whitney Rank Sum test or Student’s unpaired t-test, where appropriate.

5. **Effects of anti-PD1 therapy on NKT, dendritic cells and macrophages.** Anti PD1 therapy acts directly on NKT cell by facilitating its pro-tumoricidic phenotype, which further polarize macrophages towards M1 phenotype via augmented IFNγ secretion. In addition, anti-PD1 therapy lowers IL-10 production in dendritic cells, making them less tolerogenic and more efficient in activating NKT cells.
