

## Prophylactic dendritic cell vaccination in antitumor immune response and tumor growth in a breast cancer mouse model

A vacinação profilática com células dendríticas na resposta imune antitumoral e no crescimento do tumor em um modelo de camundongo com câncer de mama

Vacunación profiláctica con células dendríticas sobre la respuesta imune antitumoral y el crecimiento tumoral en un modelo de ratón de cáncer de mama

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**Jéssica Ferreira Vieira**

ORCID: <https://orcid.org/0000-0003-3556-9849>  
Universidade Federal do Triângulo Mineiro, Brazil  
E-mail: [jessica.vieira@uftm.edu.br](mailto:jessica.vieira@uftm.edu.br)

**Eddie Fernando Cândido Murta**

ORCID: <https://orcid.org/0000-0003-4014-1345>  
Universidade Federal do Triângulo Mineiro, Brazil  
E-mail: [eddiemurta@mednet.com.br](mailto:eddiemurta@mednet.com.br)

**Márcia Antoniazi Michelin**

ORCID: <https://orcid.org/0000-0003-0842-8805>  
Universidade Federal do Triângulo Mineiro, Brazil  
E-mail: [marcia.michelin@uftm.edu.br](mailto:marcia.michelin@uftm.edu.br)

### Abstract

Dendritic cell vaccines have demonstrated promising results for poorly immunogenic tumors, which may promote the generation of better immune responses in the tumor microenvironment. However, the vaccine has little been evaluated as a prophylactic option. Therefore, this study evaluates the influence of prophylactic dendritic cell vaccination on the antitumor immune response in the tumor microenvironment and on tumor growth, in an experimental model with breast cancer induced by 4T1. Therefore, Balb/c mice were separated into a vaccinated group and an unvaccinated group. Dendritic cell vaccine was differentiated and matured *ex vivo* from bone marrow. During the experimental period, the tumor volumes were checked periodically. The tumors were evaluated for immune cells (helper T lymphocytes and cytotoxic T lymphocytes), helper T cells (Th1, Th2, Th17, and Treg), TNF- $\alpha$ , and IFN- $\gamma$  synthesis by Th1 and cytotoxic T lymphocytes. The vaccinated group had decreased tumor volume (14.0, 0-131.7) compared to the unvaccinated group (89.59, 0.1250-459.6) ( $p=0.0421$ ). The Th1, Th2, Th17, Treg, cytotoxic T subtypes, including TNF- $\alpha$  and IFN- $\gamma$  produced by Th1 and T cytotoxic, showed a significant increase in the vaccinated group, as did the balance of Th1/Th2 and Th1/Treg. The results showed that prophylactic vaccination with dendritic cells showed a considerable antitumor effect in the studied model by promoting an increase in the activation of important cells in the immune response and a reduction in tumor volume. The data provide evidence for timely activation of immune surveillance in the absence of tumor burden.

**Keywords:** Cancer; Dendritic cells; Vaccination; Immunotherapy; Tumor immunity.

### Resumo

As vacinas de células dendríticas têm demonstrado resultados promissores para tumores pouco imunogênicos, podendo promover a geração de melhores respostas imunológicas no microambiente tumoral. Portanto, este estudo avalia a influência da vacinação profilática de células dendríticas na resposta imune antitumoral no microambiente tumoral e no crescimento tumoral, em modelo experimental com câncer de mama induzido por 4T1. Portanto, camundongos Balb/c foram separados em um grupo vacinado e um grupo não vacinado. A vacina de células dendríticas foi diferenciada e maturada *ex vivo* da medula óssea. Durante o período experimental, os volumes do tumor foram verificados periodicamente. Os tumores foram avaliados quanto à células imunes (linfócitos T auxiliares e linfócitos T citotóxicos), perfil T auxiliar (Th1, Th2, Th17 e Treg), síntese de TNF- $\alpha$  e IFN- $\gamma$  por Th1 e linfócitos T citotóxicos. O grupo previamente vacinado apresentou diminuição do volume tumoral (14,0, 0-131,7) em comparação ao grupo não vacinado (89,59, 0,1250-459,6) ( $p = 0,0421$ ). Os subtipos Th1, Th2, Th17, Treg, T citotóxico, incluindo o TNF- $\alpha$  e IFN- $\gamma$  produzidos por Th1 e T citotóxicos, apresentaram aumento significativo no grupo vacinado, tanto quanto o balanço de Th1/Th2 e Th1/Treg. Os resultados demonstraram que a vacinação profilática com células dendríticas demonstrou considerável efeito antitumoral no modelo estudado, promovendo aumento da ativação de células importantes na resposta imune e redução do volume tumoral. Os dados fornecem evidências de uma ativação oportuna da vigilância imunológica na ausência de carga tumoral.

**Palavras-chave:** Câncer; Células dendríticas; Vacinação; Imunoterapia; Imunidade tumoral.

### Resumen

Las vacunas de células dendríticas han mostrado resultados prometedores para tumores poco inmunogénicos y pueden promover la generación de mejores respuestas inmunitarias en el microambiente tumoral. Por lo tanto, este estudio evalúa la influencia de la vacunación profiláctica con células dendríticas sobre la respuesta inmune antitumoral en el microambiente tumoral y sobre el crecimiento tumoral, en un modelo experimental de cáncer de mama inducido por 4T1. Por tanto, los ratones Balb / c se separaron en un grupo vacunado y un grupo no vacunado. La vacuna de células dendríticas se diferenciaba y maduraba *ex vivo* a partir de la médula ósea. Durante el período experimental, se controlaron periódicamente los volúmenes tumorales. Los tumores fueron evaluados para células inmunes (linfocitos T colaboradores y linfocitos T citotóxicos), perfil T colaborador (Th1, Th2, Th17 y Treg), síntesis de TNF- $\alpha$  e IFN- $\gamma$  por linfocitos T citotóxicos y Th1. El grupo previamente vacunado tenía un volumen tumoral reducido (14,0, 0-131,7) en comparación con el grupo no vacunado (89,59, 0,1250-459,6) ( $p = 0,0421$ ). Los subtipos Th1, Th2, Th17, Treg, T citotóxicos, incluidos TNF- $\alpha$  e IFN- $\gamma$  producidos por Th1 y T citotóxico, mostraron un aumento significativo en el grupo vacunado, al igual que el equilibrio de Th1 / Th2 y Th1 / Treg. Los resultados mostraron que la vacunación profiláctica con células dendríticas mostró un efecto antitumoral considerable en el modelo estudiado, promoviendo un aumento en la activación de células importantes en la respuesta inmune y una reducción del volumen tumoral. Los datos proporcionan evidencia de una activación oportuna de la vigilancia inmunológica en ausencia de carga tumoral.

**Palabras clave:** Câncer; Células dendríticas; Vacunación; Imunoterapia; Inmunidad tumoral.

## 1. Introduction

Immunotherapies can be used to cancer, reaching the disease with minimal impact on normal tissue (Aly, 2012). And Dendritic Cell (DC)-based vaccines have shown satisfactory results by generating a tumor-specific active immune response, with specificity for tumor cells and lasting memory capacity to protect against possible recurrences in treatment studies (Palucka & Banchereau, 2013).

DCs are used in different cancer-therapy studies, with more than 200 clinical trials (Wculek et al., 2020). They have a high potential to stimulate T lymphocytes, bridging between immune response mechanisms and inducing memory immune responses. Thus, they are part of a crucial mechanism for the antitumor response, considering that cancer can lead to dysfunctional immune responses (Banchereau & Steinman, 1998; O'Neill & Pearce, 2016; Palucka & Banchereau, 2012; Zong et al., 2016).

In the process of tumor elimination, the immune system needs a connection acting reciprocally with DCs, mast cells, natural killer cells (NK), B and T lymphocytes, including subsets helper T lymphocytes (Th) and cytotoxic T lymphocytes (CTL) (Korkaya et al., 2011). DCs can modulate the differentiation of T lymphocytes into subpopulations depending on the activating stimulus and the maturation process. Different parameters have been used to distinguish Th lymphocytes, such as the transcriptional profile and the types of secreted cytokines. Thereby, Tbet is described in the characterization of the Th1, GATA3 in Th2, ROR $\gamma$ t for Th17, and FoxP3 for the regulatory T cells (Treg) (Chemin et al., 2019; Liudahl & Coussens, 2017).

The principal immune mechanism of tumor elimination occurs by CTL T cells (Burnet, 1970). However, the Th1 subtypes are critical in the elimination of tumor cells, by characterized of synthesis IL-2, TNF- $\alpha$ , and IFN- $\gamma$ , both regulatory cytokines of other leukocytes important for antitumor activity, including CTL, NK, and macrophages (Mo), other indispensable cells involved in the elimination of tumor cells (Corthay et al., 2005; Liudahl & Coussens, 2017; Pardoll & Topalian, 1998).

Considering the known mechanisms of DCs and the previous observations by this group, it is possible to observe a relevant role of this vaccine on the immune mechanisms (A. da Cunha et al., 2016; Matias et al., 2013; Rodrigues et al., 2011). These findings showed, for example, that the targets treated with the DC vaccine stimulate immune responses, with a higher concentration of cytokines of the Th1 profile (IL-2, IL-12, IFN- $\gamma$ , TNF- $\alpha$ ), when compared with the cytokines of the Th2 profile (IL-4, IL-10) and Treg (TGF- $\beta$ ) and an increase in the total percentage of T lymphocytes (CD3+) after immunotherapy.

Other studies have shown that the immune mechanisms of antitumor responses provided by the effector T lymphocytes generated by the dendritic cell vaccination, result in low side effects and ensure better actions in the target neoplastic tissue. That allows tumor regression and the production of memory cells, which protect in cases of recurrence (Anguille et al., 2014; Matheoud et al., 2010; Palucka & Banchereau, 2012, 2013).

Thus, based on the ability of dendritic cells to initiate a specific, robust, long-lasting, and intense antitumor immune response, the study of new antitumor therapies has been supported by the use of these cells as a therapeutic instrument. Also, the strategy could be associated with encouraging the use of non-pharmacological interventions that can activate the immune system and improve the quality of life of patients with chronic diseases (Koido et al., 2000), although long-term benefits are rarely reported (Maccalli et al., 2017; Sabado et al., 2017).

Therefore, the DC vaccination can be promising, not only in treatment, but also in the prophylaxis by generating T-mediated antitumor response, prevention of metastasis, long-lasting antitumor effects, and prevention of tumor recurrence, which could prevent tumor growth and tumor recurrence. Thus, the purpose of this work is to analyze the influence and efficacy of prophylactic dendritic cell vaccination on the tumor growth rate and the populations of helper T and cytotoxic T lymphocytes, as well as the relationship between cell subtypes and tumor size.

## **2. Methodology**

### **2.1 Animals**

For this study, eighteen female Balb/C mice, eight-week-old (mean of body weight  $23\text{g} \pm 0.8\text{g}$ ), were obtained from the Biotério Central of the Universidade Federal do Triângulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil. The study was performed following the principles of the Helsinki declaration and Basel Declaration and the number of animals was sufficient to perform statistical analyzes and obtain significant data. It was approved by the Committee on Ethics in the Use of Animals by UFTM, protocol number 379.

The animals were separated into two experimental groups, randomly, the Tumor group ( $n=8$ ), submitted to tumor induction with 4T1 cells; and the DCprev group ( $n=7$ ), that received prophylaxis with DC vaccine and subsequent tumor induction. The other three animals were euthanized to make the DC vaccine. After the experimental period (Figure 1a), the tumors of the studied groups were removed after euthanasia and necropsy of the animals. The tumors were submitted to mechanical rupture and the cells were homogenized in physiological solution, to perform the flow cytometry protocol. The use of enzymes has been discarded because there are some cellular markers that could be cleaved by the same enzymes/collagenases, such as CD<sub>s</sub> (cluster of differentiation).

### **2.2 Tumor induction**

The animals were inoculated with the tumor line 4T1 with a single dose of  $2.0 \times 10^5$  cells, injected into the lower-left mammary gland. Tumor size measurements were made every two or three days and the tumor volume was measured using largest diameter  $\times$  smallest diameter $^2 \times 0.5$  (Roland et al., 2009). The 4T1 tumor cell line is described as potent inducer of breast tumors in mice of the Balb/c line. Its tumor growth and metastatic spread are very similar to human breast cancer (TNBC), being an animal model close to stage IV. 4T1 cells were obtained from the Rio de Janeiro Cell Bank. Until inoculation, the cells were kept in RPMI medium (Sigma-Aldrich®, St. Louis, MO, USA), at 5% CO<sub>2</sub> and 37°C.

### **2.3 Dendritic Cell Vaccine**

The vaccine was made from the cells of the bone marrow of 3 Balb/c mice. The cells were cultured in IMDM medium

(Sigma-Aldrich®, St. Louis, MO, USA) supplemented with 0.1 mM vitamins, 2 mM L-glutamine, 100µg/mL gentamicin, 1 mM sodium pyruvate, and 5% fetal bovine serum, incubated in a 5% CO<sub>2</sub> and 37°C, in the presence of 10ng/µL of GM-CSF and 10ng/µL of IL-4. On day 5, the differentiated cells were placed with 10ng/µL of TNF-α and tumor antigen from the 4T1 cells, obtained by thawing and refreezing. All antibodies were obtained from BD Pharmigen™, San Diego, CA, USA. On day 07, the differentiated dendritic cells were washed and resuspended in 0.9% saline (sterile physiological solution). The single dose of 5.0×10<sup>6</sup> dendritic cells was applied 7 days before tumor induction. The evaluation of the differentiated DCs was made qualitatively by visual control through an optical microscope since previous analyzes demonstrated effectiveness in this differentiation process (Lopes et al., 2017; Sallusto & Lanzavecchi, 1994).

#### **2.4 Flow cytometry protocol**

The tumor samples from the different experimental groups were analyzed using the flow cytometry technique, in a FACS Calibur™ cytometer (BD Biosciences, San Diego, CA, USA). The technique was performed according to the cytometry protocol suggested by the manufacturer. We used BD Pharmigen™ antibodies for extracellular labeling (CD3 #cat 553062, CD4 #cat 553050, CD25 #cat 553075, and CD8a #cat 553035) and for intracellular labeling (Tbet #cat 561266, GATA-3 #cat 560074, RORγt #cat 562607, FoxP3 #cat 560407, TNF-α #cat 554419, and IFN-γ #cat 554411) in addition to the respective markers for isotype. Leukocyte cells were obtained by centrifugation after using lysis solution (FACS Lysing Solution, BD Biosciences). All antibodies used in the flow cytometry protocol were obtained from BD Biosciences, San Diego, CA, USA.

Gate strategy: lymphocytes were initially selected based on size and granularity (FSC×SSC). In lymphocytes, a gate for CD3<sup>+</sup> was designed to mark T cells. From this gate, a CD4 vs. CD8 plot was drawn to separate CD4<sup>+</sup> and CD8<sup>+</sup> T cells. In the CD4<sup>+</sup> T population, a Tbet graph was plotted to mark T helper type 1 (Th1), GATA3 to mark T helper type 2 (Th2), RORγt to mark T helper populations of type 17 (Th17) and a graph plotting CD25 and FoxP3 was used to outline Treg cells. Also from the gate of T CD4<sup>+</sup>Tbet<sup>+</sup> and T CD8<sup>+</sup> populations, histograms for the intracellular markings of TNF-α and IFN-γ were plotted.

#### **2.5 Statistical analysis**

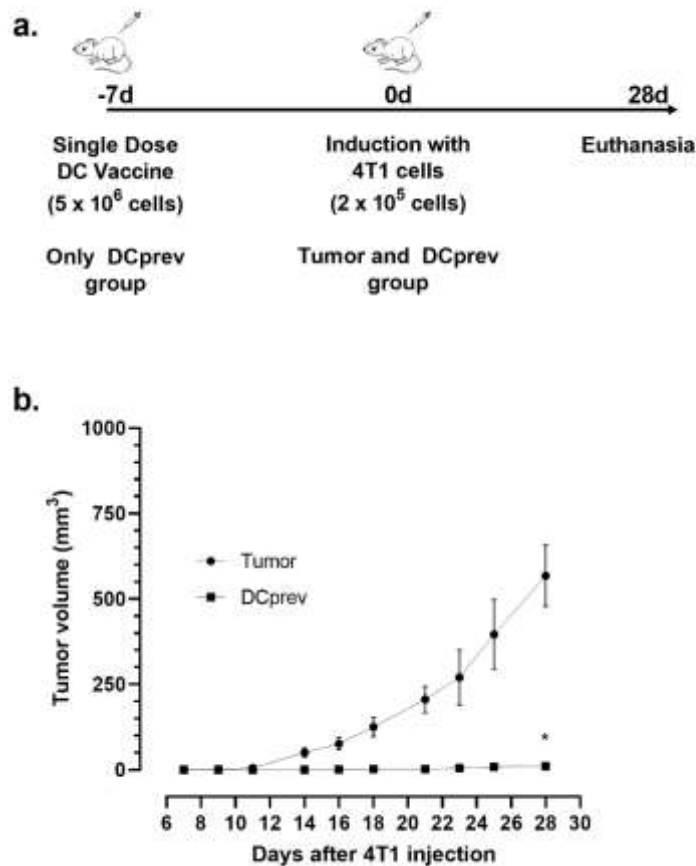
All statistical analyses were performed using GraphPad Prism 8.4.3 Software (GraphPad Software, Inc). Student's t-test was used to compare Tumor and DCprev groups about aspects of immune cells, with results expressed as mean±SD (standard deviation) and tumor volume growth, with results expressed as mean±SEM (standard error of the mean). A correlation test was applied to determine the correlation between tumor size and percentage of cells (types) using the Pearson test. Values of p<0.05 were considered statistically significant.

### **3. Results**

#### **3.1 Prophylactic DC vaccination promotes smaller tumoral growth**

To evaluate the antitumor effects of the prophylactic DC vaccination in tumor development, the animals had tumor volumes measured every two or three days from the seventh day after the injection with tumor cells until the moment of euthanasia. The group vaccinated with DCs activated by the tumor antigen (170.1±60.59) showed less tumor growth compared to the unvaccinated group/Tumor group (3.179±1.250), (p=0.0131; Figure 1b). The prophylactic DC vaccine promotes less rate of tumor growth.

**Figure 1.** Evaluation of tumor growth in experimental groups demonstrates that prophylactic dendritic cells vaccination controls tumor growth. a. Representation of the study design showing on a timescale the vaccination with dendritic cells, tumor induction and euthanasia. The single dose of vaccine preceded 1 week of the 4T1 cells injection. The animals had their tumor volumes measured every two-three day until the day of euthanasia. b. The tumor volume measured by  $D \times d^2 \times 0.5$ , where  $D$  represents the largest diameter and  $d$  the smallest diameter (\* $p=0.0131$ ). The animals had tumor volumes measured every two-three day until the day of euthanasia. Tumor volumes represented by mean $\pm$ SEM (standard error of the mean). Experimental groups: Tumor (n=08) with animals that received the injection of 4T1 cells; DCprev (n=07) with animals that received preventive vaccination with dendritic cells and subsequent injection of 4T1 cells.



Source: Authors.

### 3.2 Intratumoral lymphocyte subtypes and the profile of the immune response after prophylactic DC vaccination

Some specific cell subtypes of the antitumor immune response may be associated with tumor regression. Thus, the immune response triggered by the prophylactic vaccine was characterized by flow cytometry through the analysis of the presence of some subtypes of lymphocytes in tumor samples.

A significant increase in the percentage of Th and T CTL cells was observed in the DCprev group (CD4:  $80.54 \pm 2.160$ ; CD8:  $42.18 \pm 2.163$ ) compared to the Tumor group (CD4:  $36.92 \pm 2.449$ ; CD8:  $26.57 \pm 2.449$ ), ( $p < 0.0001$ ; Figure 2a).

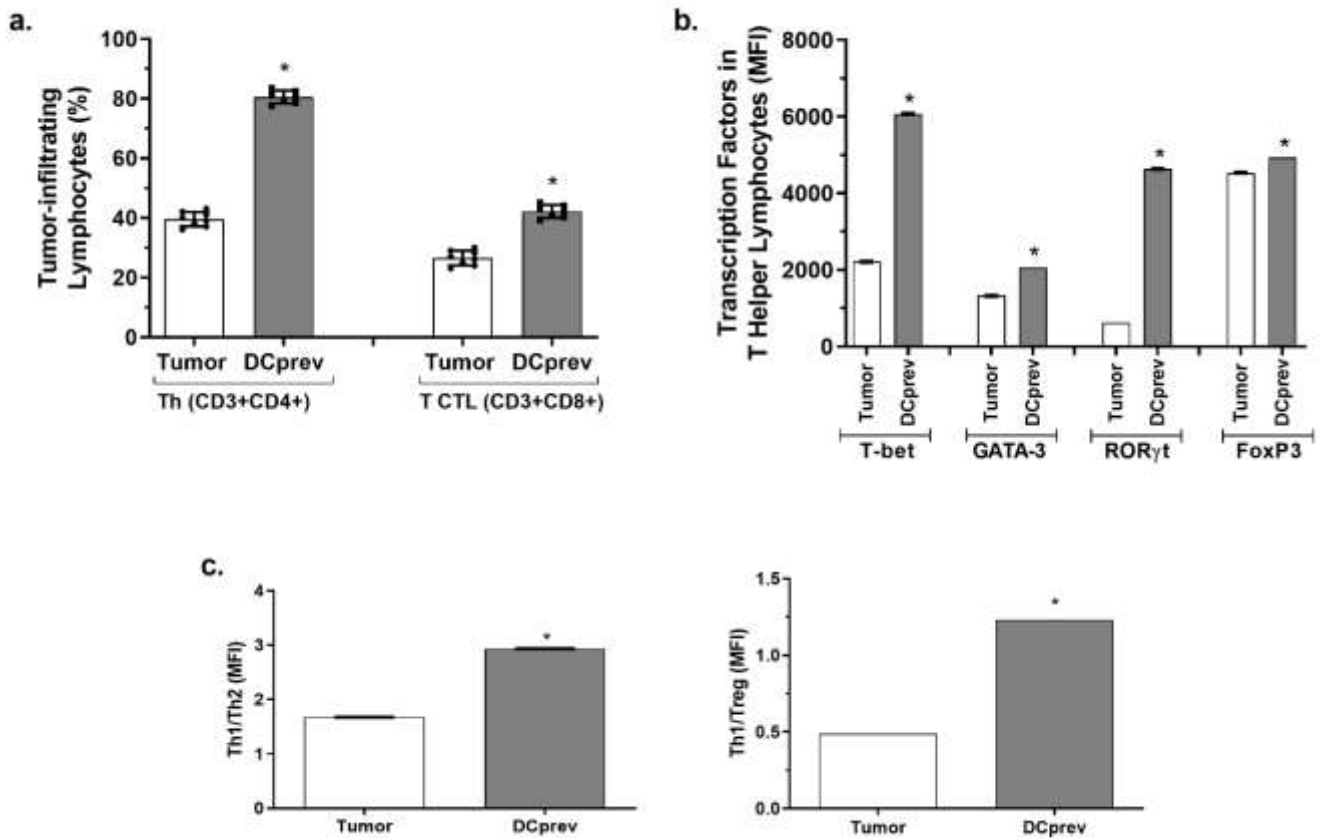
Flow cytometry also evaluated the profile of helper T lymphocyte subtypes, Th1, Th2, Th17, and Treg, in tumor samples, using the mean fluorescence intensity (MFI) of key transcription factors that participate in the process of differentiation of subtypes.

Thus, it was observed that Tbet, GATA3, ROR $\gamma$ t and Foxp3 were significantly higher in DCprev group (Tbet:  $6074 \pm 2.160$ ; GATA3:  $2069 \pm 2.160$ ; ROR $\gamma$ t:  $4634 \pm 2.160$ ; FoxP3:  $4929 \pm 2.160$ ) compared to Tumor group (Tbet:  $2213 \pm 2.449$ ; GATA3:  $1320 \pm 2.449$ ; ROR $\gamma$ t:  $623.1 \pm 2.449$ ; FoxP3:  $4532 \pm 2.449$ ), ( $p < 0.0001$ , Figure 2b). Furthermore, the Tbet factor is

significantly increased about the other transcription factors evaluated in the vaccinated group ( $p < 0.0001$ ).

The Th1/Th2 and Th1/Treg balance was evaluated and it was observed that Th1/Th2 dynamics had increased in the DCprev ( $2.935 \pm 0.002020$ ) compared to the Tumor group ( $1.677 \pm 0.001256$ ), with an increase of 1.75x ( $p < 0.0001$ , Figure 2c). The same pattern was observed in the Th1/Treg dynamics with a significant increase in the DCprev group ( $1.232 \pm 0.0001018$ ) compared to the Tumor group ( $0.48884 \pm 0.0002765$ ), an increase of 2.52x ( $p < 0.0001$ , Figure 2d). In both situations, polarization occurred in the Th1 domain.

**Figure 2.** Flow cytometry of different subsets of intratumoral T cells. Evaluation of the influence of the prophylactic vaccine on T cell phenotypes by flow cytometry in tumor samples from different experimental groups. (a.) Gate percentage of Th and T CTL (Tc1t) cells in tumor samples (Th  $*p < 0.0001$ ; Tc1t  $*p < 0.0001$ ). (b.) MFI of transcription factors in Th cells ( $*p < 0.0001$ ). (c.) Th1/Th2 balance representation in the studied groups. (d.) Th1/Treg balance representation in the groups studied. Results represented as a mean  $\pm$  standard deviation (SD). Unpaired t test was used to determine statistical significance. MFI: Mean Fluorescence Intensity; Th: Helper T lymphocytes; Tc1t: Cytotoxic T lymphocytes. Experimental groups: Tumor (n=08), animals that received the injection of 4T1 cells; DCprev (n=07), animals that received preventive vaccination with dendritic cells and subsequent injection of 4T1 cells.

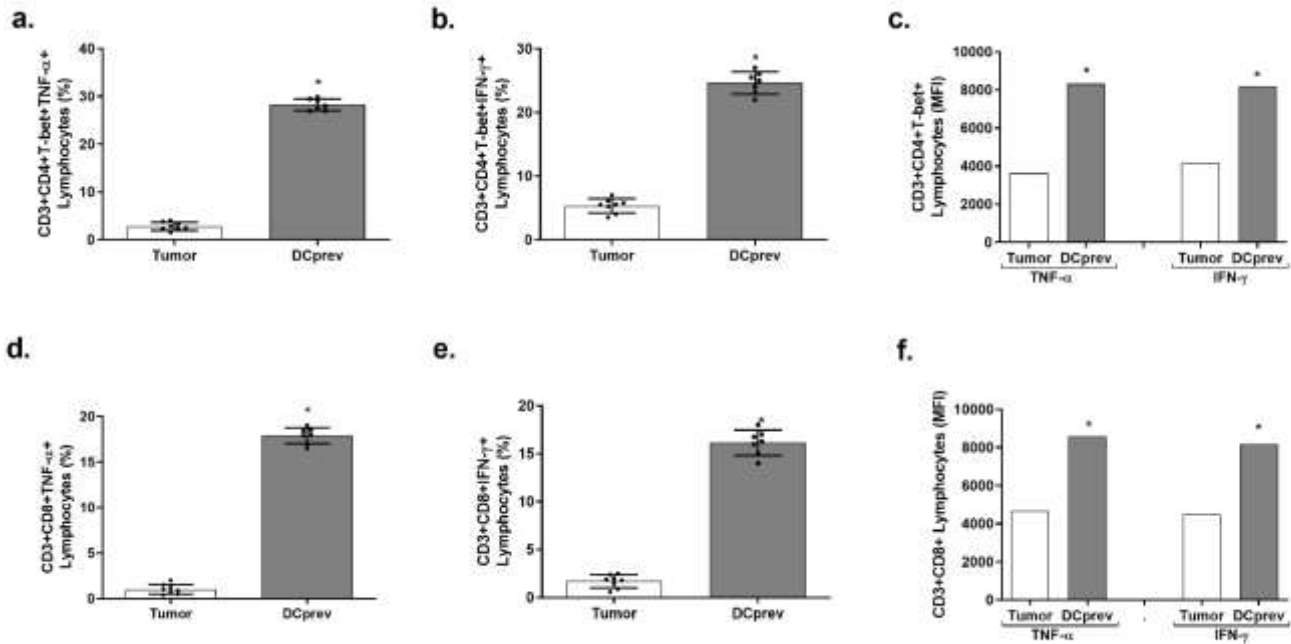


Source: Authors.

### 3.3 Evaluation of TNF- $\alpha$ and IFN- $\gamma$ cytokine production by helper T and cytotoxic T lymphocytes

Still using flow cytometry, we evaluate the impact of DC vaccine in TNF- $\alpha$  and IFN- $\gamma$  expression cytokines on helper T and cytotoxic T lymphocytes. Figure 3a-f demonstrates this expression through the percentage of the gate and by MFI. It is observed a significant increase in the two cytokines in the vaccinated group, both in Th1 and T CTL. The vaccine influenced the production levels of these cytokines compared to the non-vaccinated group.

**Figure 3.** Evaluation of TNF- $\alpha$  and IFN- $\gamma$  production in Th and T CTL lymphocytes. Evaluation of the influence of the prophylactic vaccine on the expression of TNF- $\alpha$  and IFN- $\gamma$  in Th1 and T CTL cells by flow cytometry. (a.) Gate percentage of TNF- $\alpha$ -producing Th1 lymphocytes (\*p<0.0001). (b.) Gate percentage of IFN- $\gamma$ -producing Th1 lymphocytes (\*p <0.0001). (c.) MFI of TNF- $\alpha$  and IFN- $\gamma$  in Th1 lymphocytes (\*p<0.0001). (d.) TNF- $\alpha$  production by T CTL (\*p<0.0001). (e.) IFN- $\gamma$  production by T CTL lymphocytes (\*p<0.0001). (f.) MFI of TNF- $\alpha$  and IFN- $\gamma$  in T CTL lymphocytes (\*p<0.0001). Results represented as mean $\pm$ standard deviation (SD). Unpaired t test was used to determine statistical significance. MFI: Mean Fluorescence Intensity. Experimental groups: Tumor (n=08) with animals that received the injection of 4T1 cells; DCprev (n=07), animals that received preventive vaccination with dendritic cells and subsequent injection of 4T1 cells.



Source: Authors.

### 3.4 Correlation between tumor volume and immune cells

We have shown that immune response cells are increased in the previously vaccinated group and the tumor volume in the same animals was showed a lower growth rate compared to the unvaccinated group. However, when we performed the correlation analysis between the immune cells and the tumor volume, it is not possible verified a significant relation (Table 1).

It is worth noting that even not though the correlation, the animals that received the vaccine presented a higher percentage of Th and T CTL cells to the Tumor group and this data can modify the clinical impact of the antitumor immune response in the animal.

**Table 1.** Correlation between the T cells and tumor volume.

Group studied	Variable studied	Correlation Coefficient	p
<b>Tumor</b>	CD4	0.1230	0.7716
	CD8	0.1230	0.7716
	Th1	0.1230	0.7716
	Th2	0.1231	0.7717
	Th17	0.1232	0.7718
	Treg	0.1233	0.7719
	<b>DCprev</b>	CD4	0.4082
CD8		0.4075	0.3642
Th1		0.4082	0.6332
Th2		0.4083	0.6333
Th17		0.4084	0.6334
Treg		0.4085	0.6335

Source: Authors.

#### 4. Discussion

Dendritic cells vaccines have been studied for pre-surgery and post-surgery treatment of several types of cancers. Vaccination strategies have been developed considering these particular coordination properties of immune responses (Bauer et al., 2011; Markov et al., 2015; Shangguan et al., 2020; Simon et al., 2009). Therefore, the identification of protocols that result in a potent, robust, and lasting immune response that promote regression or eradication of tumors, has been the focus of several studies (Lopes et al., 2017; Palucka & Banchereau, 2013; Perez & De Palma, 2019). However, as a stand-alone therapy or as a prophylactic therapy, the DC vaccine has not been much investigated. Understanding the vaccination strategy, as well as the biology, function, and metabolism of DCs would assist explore this tool. And thus, supporting the idea of its use in the delay of tumor development.

In this study, we evaluated the role of the prophylactic autonomous DC vaccine in antitumor immune response in a murine model for breast cancer. It was observed that prophylactic DC vaccination inhibited tumor growth in mice. The same was observed in a prophylactic vaccination study in a pancreatic cancer model (pancreatic ductal adenocarcinoma) (Shangguan et al., 2020). These data suggest that vaccination alone used autonomously can be an efficient adjunct tool in the clinical setting. Other perspectives need to be verified to understand the ideal strategy for vaccination and the best way for this tool.

Another study demonstrated the efficiency of the DCs vaccine in the process of inhibiting metastases, and not specifically their role in the primary tumor (Markov et al., 2015). This perspective showed the prophylactic role of a DC vaccine is quite broad, and it could be the difference in the future.

Some mechanisms in the carcinogenesis and tumor microenvironment turn the DCs into disfunction and tolerogenic cells (Fu & Jiang, 2018; Lin et al., 2020; Lurje et al., 2020; Seledtsov et al., 2015). So, using a DC-based vaccine at any time of this process could show satisfactory results that have a relation with DC's role: generation a specific immune response by T CTL and Th1 cells (Ahrends et al., 2019; Ott et al., 2019).

The T TCL is an important cell to antitumor response as a result of acting on the death of tumor cells by several mechanisms. Including, its presence is associated with better responses and good predictors of the immune response (Durgeau et al., 2018; Farhood et al., 2019; S. Mahmoud et al., 2012; S. M. A. Mahmoud et al., 2011; Martínez-Lostao et al., 2015).

However, prophylaxis in our study promoted an increase not only in T CTL and in Th1. Besides in other lymphocytes,



as Th2 and Treg, subtypes associated with immunosuppression of antitumor immune mechanisms, and in Th17, which has different roles depending on the context evaluated (L. L. Cunha et al., 2020; Kachler et al., 2018; Stanton & Disis, 2016; Tindemans et al., 2014; Tosolini et al., 2011),

Due to the increase in all subtypes, we evaluated the Th1/Th2 and Th1/Treg. This type of data provides us with information on the dynamics of this balance (exchange of responses) since this balance is integrated into the immune regulation in a very dynamic way and linked to other patterns of immune responses, such as DCs. Th1/Th2 cell differentiation is counter-regulatory and self-reinforcing. When this balance changes to a Th1 domain, the results are favorable to the antitumor immune response as seen in the vaccinated group. The tumor microenvironment transferred to the Th1 dominance may have beneficial effects on tumor regression (Kachler et al., 2018; Lee et al., 2019; Ott et al., 2019; Stanton & Disis, 2016; Tosolini et al., 2011). That corroborates with our data, in which the prevalence of Th1 occurs over the other subtypes.

Currently, Th1 cells correlate with the best disease outcome in a broad spectrum of solid tumors. That is partly due to its robust IFN- $\gamma$  production and its downstream pleiotropic effects (Burke & Young, 2019; Fong et al., 2001; Jorgovanovic et al., 2020). We further evaluated the production of cytokines IFN- $\gamma$  and TNF- $\alpha$  by cells of the Th1 and T CTL cells and observed an increase in both cells. TNF- $\alpha$  is a pleiotropic cytokine capable of engaging both promoter and suppressor responses in tumors and a crucial mediator of inflammatory responses, depending on the concentration and context. But a more comprehensive understanding of the central role of this cytokine for better clinical interventions is still important (F. Balkwill, 2011; F. R. Balkwill, 2012; Egberts et al., 2008; Mercogliano et al., 2020).

Although the correlation tests have no statistical relevance, the reduction in tumor volume may be associated with an increase in antitumor immune cells. That interaction between antitumor immune cells and tumor raises a crucial point in the role played by prophylactic vaccination with DCs: immune memory. Some T cells generated from the antigen-specific response induced by DC vaccination, survive as memory cells. They could be maintained in peripheral tissues, divided into subsets of central memory and effective memory. Nevertheless, it is not yet clear what stimulates this division and its frequency (Lanzavecchia & Sallusto, 2005; Sallusto et al., 2004).

The existence of these subdivisions leads us to infer that prophylactic vaccination can induce mechanisms of immune memory as a result of a decrease in tumor volume observed. It may be associated with the presence of the already mentioned immune T cells and the maintenance of this response throughout the analyzed time scale. It is worth mentioning that the mechanisms that encourage this division are not yet clear. That is an important point of DC vaccine: the ability to activate T lymphocytes and initiation of the specific immune response depends on the state of maturity, and also the maintenance of the long-term response (Lokhov & Balashova, 2010; Mathis & Benoist, 2004).

However, assessing the immune and clinical efficacy of the DC vaccine is a complex task. Studies find difficulties in how to assess the clinical efficacy and how to define biomarkers to assess that efficacy. The association of clinical and immunological parameters as a tool for the analysis of the therapeutic of this vaccine on inhibiting primary tumor growth is demonstrated in some trials (Hong et al., 2013; Kandalaf et al., 2013; Koido et al., 2000; Phuphanich et al., 2013; Vik-Mo et al., 2013), but data on the prophylactic form are still scarce. The first FDA approval for a therapeutic vaccine based on dendritic cells was Sipuleucel-T (APC 8015), an APC-based cellular product of enriched peripheral blood (with a prostatic acid phosphatase fusion protein and GM-CSF). It showed satisfactory results of the patients' prolonged average survival (Drake, 2011; Kantoff et al., 2010).

The use of DCs as a prophylactic anti-tumor vaccine can be used in people with a genetic predisposition to cancer with a high risk of malignancy or in patients with levels of tumor markers in the blood (indicative of the risk of tumor development), and in situations in which that the tumor has not discovered but there is a high probability of development (Markov et al., 2015). Prophylactic vaccination provides timely activation of antitumor immune surveillance in the absence of

tumor burden and induction of T CTL cells specifically initiated with a positive effect on the humoral immunity (Töpfer et al., 2011).

In future studies, *in vitro* cytotoxicity analyzes can be included to track the activity of cytokines secreted by T CTL cells and determine the ability to induce cell death. Furthermore, it is interesting to include memory markers. Consideration should be given to the inclusion of new vaccination protocols with DC as well as different vaccination routes, analyzing whether this can alter the pattern of the immune response for the experimental design proposed in our study.

## 5. Conclusion

Immunotherapy based on dendritic cells has shown to be a promising approach by promoting protection in cases of recurrence. That tool could take advantage of the immune system itself to eliminate tumor cells and metastatic processes. However, it is still premature to affirm the most efficient method of using and differentiating dendritic cells, which maintain robust and long-lasting responses. And the most crucial: which overcome the evasive immune mechanisms of the tumor microenvironment. In the future, the dendritic cell vaccine must be thought of as a combinatorial tool with other established approaches. Furthermore, questions such as the ideal moment for vaccination and the role of combinations have yet to be assessed.

Thus, this work demonstrated the significant efficacy of the prophylactic DC vaccine (pre-exposure situation). It can act on tumor growth in the model studied and improve the antitumor immune response by promoting an increased activation of cells and can provide a timely activation of antitumor immune surveillance in the absence of tumor burden: the vaccination promotes an increase in the antitumor immune response, inducing an increase of Th1 and cytotoxic T lymphocytes; and an increase of TNF- $\alpha$  and IFN- $\gamma$  by both cells.

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