4. MATERIAIS E MÉTODOS

4.1 Animais e grupos experimentais

Foram utilizadas 25 fêmeas adultas da linhagem Balb/c, com idades entre 06 e 08 semanas de vida (média de peso 23g±0,8g), oriundas do Biotério Central da Universidade Federal do Triângulo Mineiro, Uberaba, Minas Gerais, Brasil. Os animais foram mantidos em gaiolas plásticas, com espaço adequado para sua acomodação, em um sistema fechado de alojamento com ventilação e temperatura controlada (21±3°C), em ciclo claro-escuro de 12h, com comida e água disponíveis *ad libitum*.

Dos 25 animais, 22 foram separados em três grupos experimentais: Controle (n=7) com animais sem indução ao desenvolvimento tumoral pela linhagem de células 4T1 e sem vacinação profilática por células dendríticas; grupo Tumor (n=8), com animais submetidos apenas à indução tumoral com células 4T1; e grupo Vacina (n=7), com animais que receberam a profilaxia com vacina de células dendríticas e posterior indução tumoral com a linhagem celular 4T1 (Tabela 2). Os outros 3 animais foram eutanasiados para a confecção da vacina de células dendríticas. Este estudo foi previamente aprovado pelo Comitê de Ética no Uso de Animais (CEUA/UFTM) sob registro de número 379 e seguiu normativas e regulamentos do CONCEA e da Declaração de Basileia.

Tabela 2: distribuição dos animais nos grupos de estudo

Grupos	**Inoculação 4T1**	**Inoculação Vacina**	**Número de animais**
Controle	Não	Não	07
Tumor	Sim	Não	08
Vacina*	Sim	Sim	07

*****DC, Dcprev, Vacina DC

Após o período experimental (Figura 5A), os animais dos grupos experimentais foram eutanasiados, sendo retirados os baços, fígados, pulmões e tumores, estes últimos quando presentes, de todos os animais.

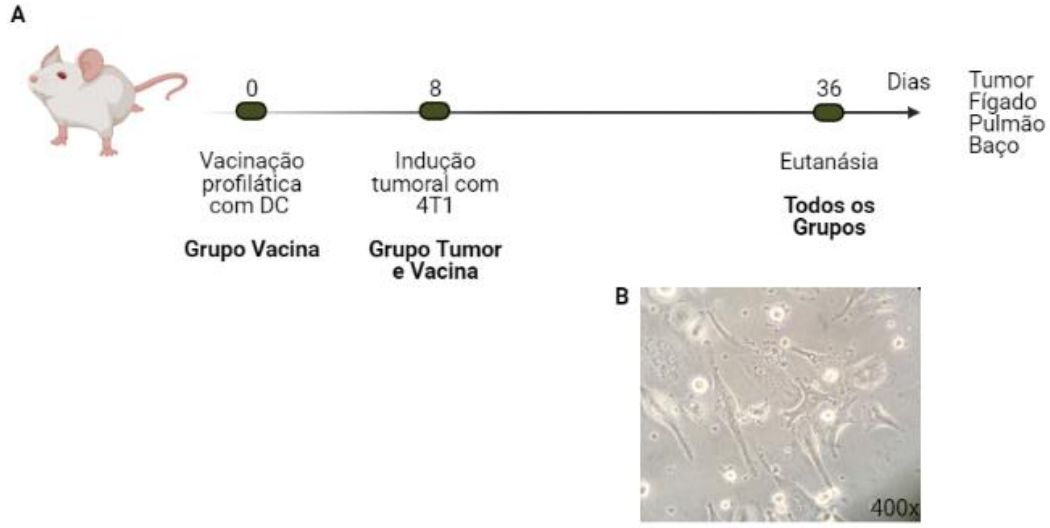


Figura 5. Delineamento experimental. A. Esquema demonstrando, em escala temporal, os procedimentos realizados no período experimental. **B.** Fotografia demonstrando o aspecto morfológico das células dendríticas, *in vitro*, diferenciados e maturadas com GM-CSF, IL-4, TNF-α e antígeno tumoral de 4T1. Aumento: 400x.

4.2 Indução tumoral de modelo de câncer de mama com células 4T1

Antes do início da experimentação, os animais foram randomizados para formação dos grupos experimentais. Os animais inoculados com a linhagem tumoral (Tumor e Vacina) receberam uma única dose de 2,0 x 10^5 células, em um volume de 50µL, injetadas na glândula mamária inferior esquerda. O volume tumoral foi mensurado a cada dois ou três dias e foi determinado pela fórmula [maior diâmetro^x(menor diâmetro)^2] x 0,5 (ROLAND et al., 2009).

A linhagem de células tumorais 4T1 são descritas como potentes indutoras de tumores de mama em camundongos da linhagem Balb/c. Seu crescimento tumoral e disseminação metastática assemelham-se muito ao câncer de mama humano (TNBC), sendo um modelo animal próximo ao estágio IV. Elas foram obtidas do Banco de Células do Rio de Janeiro. Até o momento da inoculação, as células foram mantidas em meio RPMI-1680 (Sigma-Aldrich®), suplementado com HEPES ($C_8H_{18}N_2O_4S$), Bicarbonato de Sódio ($NaHCO_3$), soro bovino fetal (SBF), Estreptomicina, L-Glutamina (200mM), Piruvato de Sódio ($C_3H_3NaO_3$), β-Mercaptoethanol (C_2H_6OS), em estufa a 5% de CO_2, a 37°C. No momento que antecede a aplicação, as células foram lavadas com solução fisiológica 0,9%, a 805xg, 4°C, por 10 min.

4.3 Vacina de Células Dendríticas e protocolo de vacinação

A vacina foi confeccionada a partir de células da medula óssea de fêmures e tíbias de 3 camundongos Balb/c. As medulas foram retiradas com auxílio de solução fisiológica 0,9% e uma seringa 13,4 x 5mm. As células medulares (multipotentes) foram então cultivadas em garrafas de cultura de 25cm^2, em meio IMDM (Sigma-Aldrich®) suplementado com 0,1mM de vitaminas, 2mM de l-glutamina, 100μg/mL de gentamicina, 1mM de Piruvato de Sódio ($C_3H_3NaO_3$) e 5% SBF, incubadas em estuda de CO_2 a 5% de umidade e a 37°C. Após plaqueamento, no dia 1, as células foram estimuladas com 10ng/μL de GM-CSF e 10ng/μL de IL-4. No dia 5 foram estimuladas com 10ng/μL de TNF-α e antígeno tumoral da linhagem de células 4T1 (obtidos pelo congelamento e descongelamento das células 4T1). No dia 07, as células dendríticas diferenciadas foram lavadas e ressuspensas em solução fisiológica 0,9%. Foram administradas 5,0 x 10^6 células, em um volume de 50μL de solução fisiológica, em dose única. A avaliação das células dendríticas diferenciadas foi feito qualitativamente, por controle visual através de microscópio óptico, já que análises anteriores (LOPES; MICHELIN; MURTA, 2017) demonstraram eficácia nesse processo de diferenciação (Figura 5B).

4.4 Retirada dos fígados, tumores, baços e pulmões

Após o período experimental, os fígados, baços, tumores e pulmões dos grupos estudados foram removidos após eutanásia e necropsia dos animais. Parte dos fígados, baços e tumores foram submetidos a ruptura mecânica para a realização do protocolo de citometria de fluxo e cultura de células (apenas baço). Outra parte dos fígados e os pulmões foram fixados em formalina 10%, seguindo em inclusão em parafina para análise de metástases por hematoxilina-eosina (HE).

4.5 Citometria de Fluxo

As amostras de baço, fígado e tumor, dos diferentes grupos experimentais foram analisadas por meio da técnica de citometria de fluxo, em citômetro FACS Calibur^{TM} (BD Biosciences, San Diego, CA, EUA). A técnica foi realizada de acordo com o protocolo de citometria sugerido pelo fabricante, com utilização de anticorpos *BD Pharmigen^{TM}* para marcação extracelular (CD3 FITC, CD4 PeCy 5.0, CD25 PE e CD8a APC para avaliação de linfócitos), e para marcação intracelular (Tbet A.F. 488, GATA-3 PE, RORγt PE, FoxP3 A. F. 488, IL-12 PE, TNF-α PE, IFN-γ FITC, IL-10 FITC e IL-17A PerCP.Cy 5.5), além dos

respetivos marcadores para isotipo. Todos os anticorpos utilizados no protocolo de citometria de fluxo foram obtidos da BD Biosciences, San Diego, CA, EUA.

As células leucocitárias hepáticas, esplênicas e intratumorais foram obtidas por centrifugação após uso de solução de lise (FACS Lysing Solution, BD Biosciences), na proporção de 1:20mL e incubação por 20 minutos a temperatura ambiente. Foram então lavadas por 3x em solução salina tamponada com fosfato (PBS), sendo que entre cada lavagem, era realizada centrifugação a 290xg, 10 minutos, 4°C, conservando sempre o precipitado celular ao término de cada lavagem. A esse precipitado celular foi acrescido 2,5mL de proteína transportadora inibitória (BD Golgistop^{TM}), seguindo de incubação a 4°C, 20 minutos. Posteriormente, as células foram submetidas a mais uma lavagem com PBS, sob mesmas especificações descritas logo acima, ficando o precipitado celular ressuspenso em 1mL de PBS. As células foram então distribuídas, após contagem em câmara de Neubauer, em 1 x 10^6 células por tubo e foram realizadas as marcações extracelulares com os anticorpos, seguindo de incubação a 4°C, 30 minutos, na ausência de luz. Em seguida, as células foram lavadas 2x com PBS, e incubadas com solução permeabilizadora e fixadora (BD Cytofix/Cytoperm^{TM}), por 20 minutos, 4°C, ao abrigo de luz. Então as células foram lavadas 2x com solução tampão de BD Perm/Wash^{TM} Buffer e marcadas intracelularmente com os anticorpos, seguindo com incubação por 4°C, 30 minutos, ao abrigo da luz. Após a incubação, as células foram novamente lavadas mais 2x com a solução tampão, ficando as células ressuspensas em 50 μL de PBS para leitura em citômetro.

4.5.1 Estratégia de gate

Linfócitos foram inicialmente selecionados com base no tamanho e granularidade (FSC X SSC). Nos linfócitos, uma gate para CD3+ foi desenhada para marcar as células T. A partir desta gate foi traçado um gráfico CD4 vs. CD8 para separar as células T CD4 + e CD8 +. Dentro da população de T CD4+, foi plotado um gráfico de T-bet, para marcar T auxiliares do tipo 1, outro plotando GATA-3, para marcar T auxiliares do tipo 2, RORγt, para marcar populações T auxiliares do tipo 17 e um gráfico plotando CD25 e FoxP3 foi utilizado para delinear células T reguladoras. Ainda, a partir da gate de populações T CD4+ (CD3+CD4+) foram plotados histogramas para as marcações intracelulares de TNF-α, IFN-γ, IL-12, IL-17 e IL-10.

4.6 Avaliação de metástases pulmonares e hepáticas (HE)

Para a avaliação da presença ou não de metástases foram confeccionadas lâminas de pulmão e fígado dos animais dos grupos Tumor e Vacina. Após a eutanásia, os órgãos foram excisados e fixados em solução de formalina 10%, seguido de inclusão em parafina. Os cortes de 5 μm de espessura foram feitos em micrótomo rotativo automatizado (Leica RM2255) e posteriormente corados pela técnica de coloração histológica de hematoxilina-eosina (HE). As lâminas foram observadas em microscópio óptico (Olympus BX41). Para cada órgão, foram preparadas três lâminas para que todo o órgão fosse amostrado. Em cada corte foi avaliado 15 campos às cegas. A quantificação das metástases foi realizada no microscópio Nikon Eclipse Ti2 com o Software Nikon Analyzes.

4.7 Níveis de citocinas e cinética da produção de citocinas por ELISA

A presença das citocinas IL-12, TGF-β, IL-4, IFN-γ, IL-10, IL-12 e TNF-α, no sobrenadante de culturas de células esplênicas, foi analisado por ELISA do tipo sanduíche utilizando anticorpos BD OptEIA (BD Biosciences). As células esplênicas foram distribuídas e incubadas em placas de 6 poços a 37°C, 5% CO_2, na ausência e presença de LPS, por 12h, 24h e 36h. Os sobrenadantes das culturas foram congelados a -80°C até o momento da dosagem

4.8 Análise Estatística

Gráficos e análise estatística foram realizados em GraphPad Prism 8.4 (GraphPad Software). Os resultados foram testados quanto à distribuição por *Kolmogorov-Smirnov test* ou *Shapiro-Wilk Normality Test*. Para distribuições paramétricas das variáveis, foi utilizado teste de *t-Student* para 2 comparações e *One-way Anova* com pós-teste de *Tukey* para mais de duas comparações/grupos, com resultados expressos em média±SD. Para variáveis não-paramétricas, foi utilizado *Mann-Whitney* para comparações entre dois grupos e *Kruskal-Wallis* com pós-teste de *Dunn* para comparações com mais de dois grupos, com os resultados expressos em mediana (mín-máx). Teste de *Fisher* e qui-quadrado (χ^2) foram utilizados para estabelecer as diferenças estatísticas nas proporções da vacinação e metástases. Teste de *Pearson* foi aplicado para determinar a correlação entre tamanho tumoral tipos de células imunes. Para todas as análises, $p < 0,05$ foi considerado significativo.

5. RESULTADOS

De alguns resultados obtidos foram confeccionados três artigos. Os dados que compõe cada um deles encontra-se disposto na figura 6.

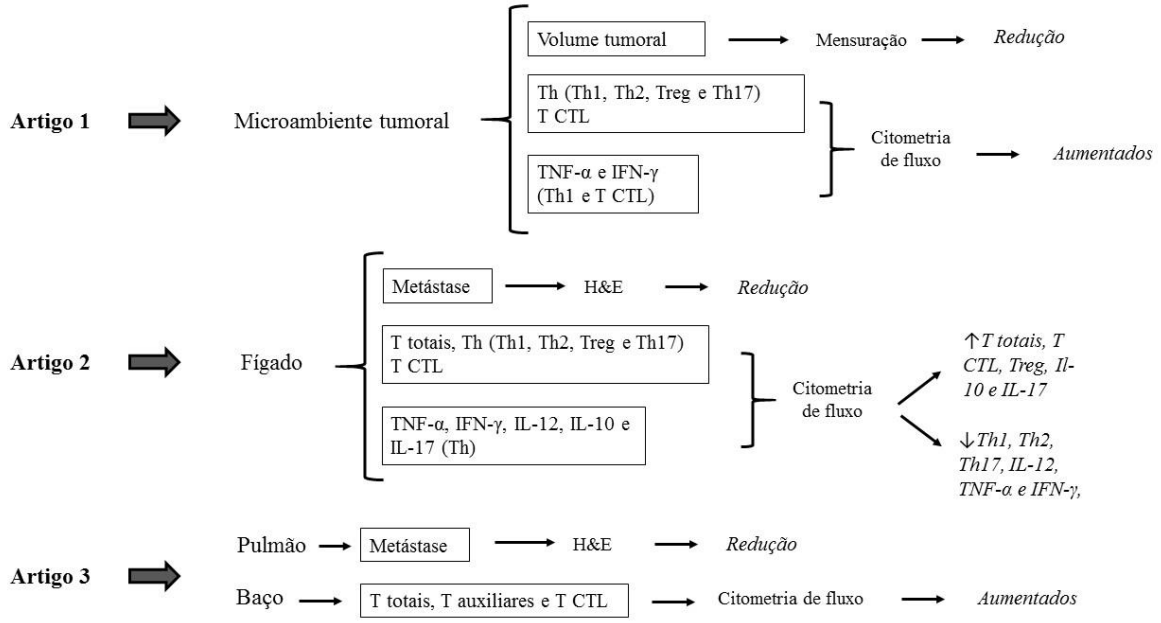


Figura 6. Distribuição resultados obtidos.

5.1 Artigo 1

Article title: Prophylactic dendritic cell vaccination mediates antitumor immune response and tumor growth in a breast cancer

Authors:
Jéssica Ferreira Vieira
Eddie Fernando Cândido Murta
Márcia Antoniazi Michelin*

Author affiliations: Instituto de Pesquisa em Oncologia, IPON, Universidade Federal do Triângulo Mineiro, UFTM, Uberaba, Minas Gerais, Brazil

***Author for correspondence:** Márcia A. Michelin, marcia.michelin@uftm.edu.br, IPON, UFTM, Avenida Guilherme Ferreira n°1940, 38022-200, São Benedito, Uberaba, Minas Gerais, Brazil.

Author Contributions: Conceptions and design of the study: JFV, MAM, and EFCM. Performed the experiments: JFV and MAM. Wrote the paper: JFV and MAM. All authors were involved with the conduction, collected the data, and interpret the data.

Acknowledgements: The authors grateful to CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais), and FUNEPU (Fundação de Ensino e Pesquisa de Uberaba).

Abstract

Background: This study evaluates the influence of prophylactic dendritic cell vaccination in antitumor immune response on the tumor microenvironment and in tumor growth, in an experimental model with breast cancer induced by 4T1. **Materials and methods:** Balb/c mice were separated into a vaccinated group and an unvaccinated group. Dendritic cell vaccine was differentiated and maturated *ex vivo* from bone marrow. During the experimental period, the tumor volumes were checked periodically. The tumors were evaluated to immune cells (helper T and cytotoxic T lymphocytes), helper T profile (Th1, Th2, Th17, and Treg), TNF-α and IFN-γ synthesis by Th1 and cytotoxic T lymphocytes. **Results:** The vaccinated group showed a decrease in tumor volume (14.0, 0-131.7) in comparison to the unvaccinated group (89.59, 0.1250-459.6) (p=0.0421). All subtypes evaluated, T helper, Th1, Th2, Th17, Treg, T cytotoxic, including the TNF-α and IFN-γ producing Th1 and CTL showed a significant increase in the vaccinated group, as considerably as the Th1/Th2 balance and Th1/Treg. **Conclusion:** Prophylactic dendritic cell vaccination demonstrated a considerable antitumor effect in the model studied by promoting increased activation of important cells in the immune response and reduction in tumor volume. The data provide evidence of a timely activation of immune surveillance in the absence of tumor burden.

Keywords: cancer, dendritic cells, vaccination, immunotherapy, tumor immunity

Introduction

Immunotherapies can be applied specifically 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).

Therefore, DCs have been used in studies models of cancer therapy, in addition to being widely investigated in more than 200 clinical trials (WCULEK et al., 2020) They have a high potential to stimulate T lymphocytes and they can establish a bridge between immune response mechanisms and can induce memory immune responses. Thus, they are an important instrument 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; LIU; WICHA, 2011). DCs can modulate the differentiation of T lymphocytes into subpopulations depending on the activating stimulus and the maturation process. Different parameters are usually used to distinguish Th lymphocytes, such as the transcriptional profile and the types of secreted cytokines. Thus, Tbet is described in the characterization of the Th1, GATA3 in Th2, RORγt for Th17, and FoxP3 for the regulatory T cells (Treg) (CHEMIN; GERSTNER; MALMSTRÖM, 2019; LIUDAHL; COUSSENS, 2017).

The main immune mechanism of tumor elimination occurs by CTL T cells (BURNET, 1970). However, the Th1 subtypes are interesting in the elimination of tumor cells, by characterized of synthesis IL-2, TNF-α, and IFN-γ, both regulatory cytokines of other leukocytes important for antitumor activity, including CTL, NK, and macrophages (Mo), other key 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 these group, it is possible to observe a relevant role of this vaccine on the immune mechanisms (DA CUNHA; ANTONIAZI MICHELIN; CÂNDIDO MURTA, 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-γ, TNF-α), when compared with the cytokines of the Th2 profile (IL-4, IL-10) and Treg (TGF-β) 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, generating low side effects and ensure better actions in the target neoplastic tissue.

This 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, although long-term benefits are rarely reported,(MACCALLI; PARMIANI; FERRONE, 2017; SABADO; BALAN; BHARDWAJ, 2017) the study of new antitumor therapies has supported by the use of these cells as a therapeutic instrument, associated with encouraging of 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).

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, accounting for long after its administration, 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.

Materials and Methods

Animals

For this study, eighteen animals, eight-week-old female Balb/c mice (mean of body weight 23g ± 0.8g) 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 CDs (cluster of differentiation).

Tumor induction

The animals were inoculated with the tumor line 4T1 with a single dose of 2.0×10^5 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 x smallest diameter^{2} x 0.5 (ROLAND et al., 2009). 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_2 and 37°C.

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_2 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. The single dose of 5.0×10^6 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; MICHELIN; MURTA, 2017; SALLUSTO; LANZAVECCHI, 1994).

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^x SSC). In lymphocytes, a gate for CD3+ was designed to mark T cells. From this gate, a CD4 vs. CD8 plot was drawn to separate CD4+ and CD8+ T cells. In the CD4+ 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+Tbet+ and T CD8+ populations, histograms for the intracellular markings of TNF-α and IFN-γ were plotted.

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.

Results

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). This demonstrates that the prophylactic DC vaccine promotes a smaller rate of tumor growth.

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.

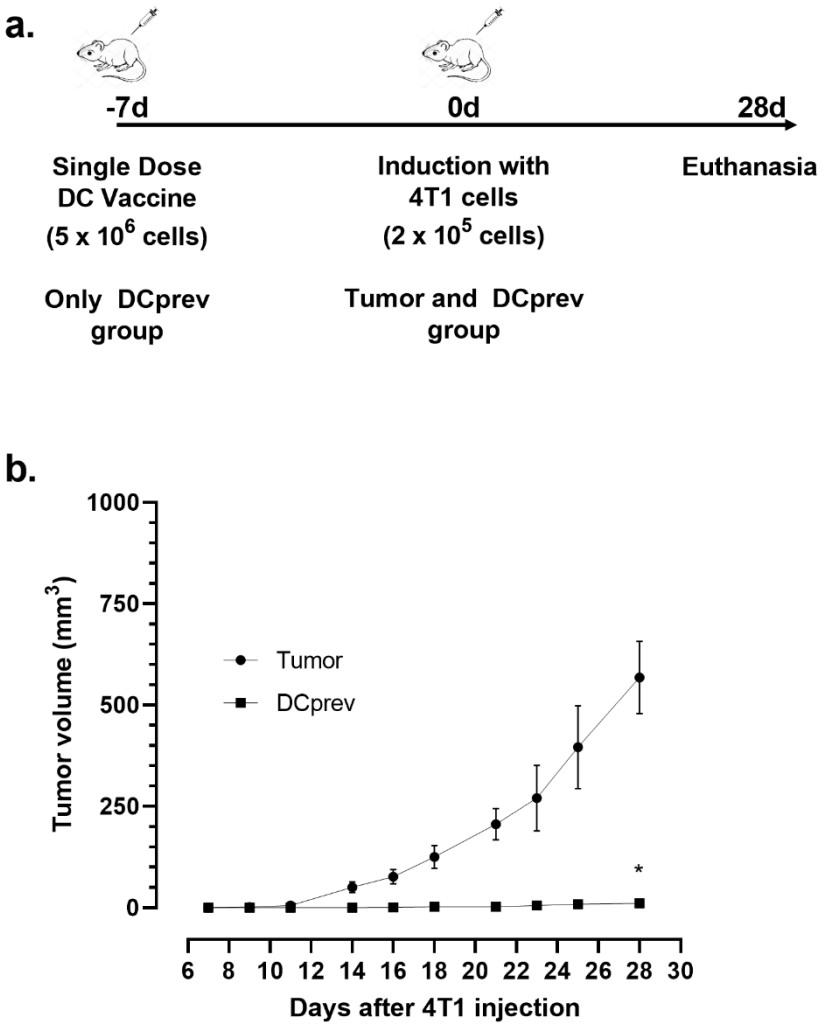


Figure 1. Evaluation of tumor growth in experimental groups demonstrates that prophylactic dendritic cells vaccination controls tumor growth. a. Representation of the study design showing on a timescale the vaccination with dendritic cells, tumor induction and euthanasia. The single dose of vaccine preceded 1 week of the 4T1 cells injection. The animals had their tumor volumes measured every two-three days until the day of euthanasia. b. The tumor volume measured by D x d^2 x 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 days until the day of euthanasia. Tumor volumes represented by mean±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.

A significant increase in the percentage of Th and T CTL cells was observed in the DCprev group (CD4: 80.54±2.160; CD8: 42.18±2.163) compared to the Tumor group (CD4: 36.92±2.449; CD8: 26.57±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, RORγt and Foxp3 were significantly higher in DCprev group (Tbet: 6074±2.160; GATA3: 2069±2.160; RORγt: 4634±2.160; FoxP3: 4929±2.160) compared to Tumor group (Tbet: 2213±2.449; GATA3 1320±2.449; RORγt: 623.1±2.449; FoxP3: 4532±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±0.002020) compared to the Tumor group (1.677±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±0.0001018) compared to the Tumor group (0.48884±0.0002765), an increase of 2.52x ($p < 0.0001$, Figure 2d). In both situations, polarization occurred in the Th1 domain.

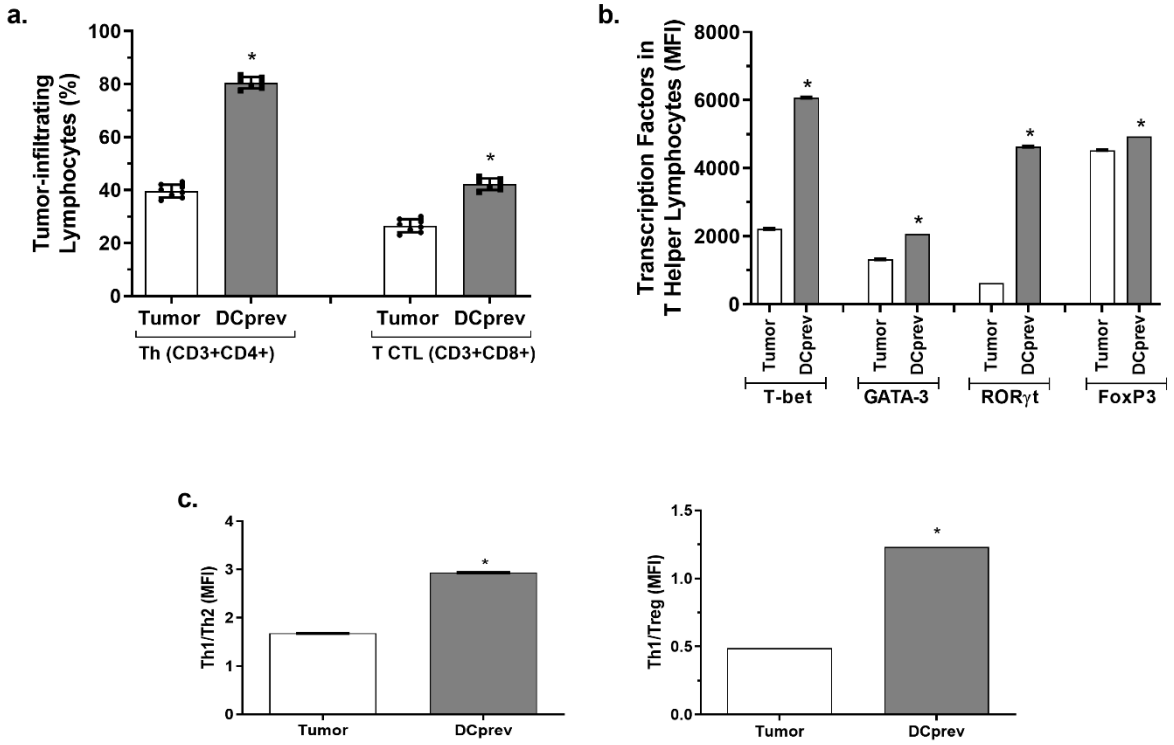


Figure 2. Flow cytometry of different subsets of intratumoral T cells. Evaluation of the influence of the prophylactic vaccine on T cell phenotypes by flow cytometry in tumor samples from different experimental groups. (a.) Gate percentage of Th and T CTL (Tcit) cells in tumor samples (Th *p< 0.0001; Tcit *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± standard deviation (SD). Unpaired t test was used to determine statistical significance. MFI: Mean Fluorescence Intensity; Th: Helper T lymphocytes; Tcit: 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.

Evaluation of TNF-α and IFN-γ cytokine production by helper T and cytotoxic T lymphocytes

Still using flow cytometry, we evaluate the impact of DC vaccine in TNF-α and IFN-γ 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.

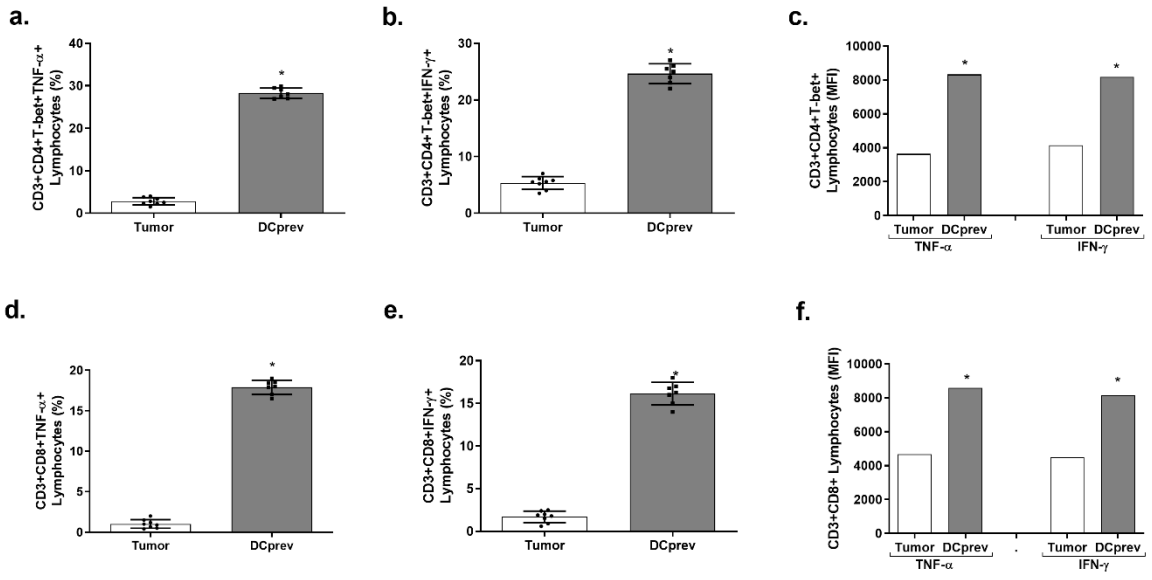


Figure 3. Evaluation of TNF-α and IFN-γ production in Th and T CTL lymphocytes. Evaluation of the influence of the prophylactic vaccine on the expression of TNF-α and IFN-γ in Th1 and T CTL cells by flow cytometry. (a.) Gate percentage of TNF-α-producing Th1 lymphocytes (*p<0.0001). (b.) Gate percentage of IFN-γ-producing Th1 lymphocytes (*p <0.0001). (c.) MFI of TNF-α and IFN-γ in Th1 lymphocytes (*p<0.0001). (d.) TNF-α production by T CTL (*p<0.0001). (e.) IFN-γ production by T CTL lymphocytes (*p<0.0001). (f.) MFI of TNF-α and IFN-γ in T CTL lymphocytes (*p<0.0001). Results represented as mean±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.

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
Tumor	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
DCprev	CD4	0.4082	0.3632
	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

Discussion

Dendritic cells vaccines have studied for pre-surgery and post-surgery treatment of several types of cancers and vaccination strategies have been developed considering these special coordination properties of immune responses (BAUER et al., 2011; MARKOV et al., 2015; SHANGGUAN et al., 2020; SIMON; FONTENEAU; GRÉGOIRE, 2009). Therefore, the identification of protocols that result in a potent, robust, and lasting immune response that promote regression and/or eradication of tumors, has been the focus of several studies (LOPES; MICHELIN; MURTA, 2017; PALUCKA; BANCHEREAU, 2013; PEREZ; DE PALMA, 2019). However, as a stand-alone therapy or as a prophylactic therapy, DC's vaccines have not been much investigated. Understanding the idea of vaccination strategy, as well as the biology, function and metabolism of DCs, would help us to better explore this tool, supporting the ideia 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, as 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 the future.

In the tumor microenvironment, thus as in the carcinogenesis process, some mechanisms make the DCs into disfunction and tolerogenic cells (FU; JIANG, 2018; LIN et al., 2020; LURJE; HAMMERICH; TACKE, 2020; SELEDTSOV; GONCHAROV; SELEDTSOVA, 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; NAJAFI; MORTEZAEE, 2019; MAHMOUD et al., 2012, 2011; MARTÍNEZ-LOSTAO; ANEL; PARDO, 2015).

However, prophylaxis in our study promoted an increase not only in T CTL and in Th1. Also 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 (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). This 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. This is partly due to its robust IFN-γ 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-γ and TNF-α by cells of the Th1 and T CTL cells and observed an increase in both cells in both cells. TNF-α is a pleiotropic cytokine capable of engaging both promoter and suppressor responses in tumors and an important 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 (BALKWILL, 2011, 2012; EGBERTS et al., 2008; MERCOGLIANO et al., 2020).

Therefore, the reduction in tumor volume that observed in the vaccinated group may be associated with an increase in the Th1 and T CTL cells, as well as the production of IFN-γ and TNF-α by these cells. Moreover, this interaction raises a crucial point in establishing that response and in the role played by prophylactic vaccination with DCs: immune memory. Some of the T cells generated from the antigen-specific response induced by DC vaccination survive as memory cells and are maintained for periods in peripheral tissues. This allows them to be broadly 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; GEGINAT; LANZAVECCHIA, 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. This 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; KANDALAFT 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.

In summary, this work demonstrated 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.

Future perspective

Immunotherapy based on dendritic cells has shown to be a promising approach for treatment, by promote protection in cases of recurrence. A tool takes advantage of the immune system itself to eliminate tumor cells and metastatic processes. However, there are frequent questions about the vaccine's effectiveness, such as the ideal method of preparing the vaccine. It is still premature to affirm the most efficient method of using and differentiating dendritic cells, which maintain robust and long-lasting responses, and which overcome the evasive immune mechanisms of the tumor microenvironment. However, previous and baseline studies, like this one, provide important information that contributes to the knowledge of dendritic cells and tumor biology. Furthermore, 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.

Summary points

Dendritic cells have been used as a treatment for some subtypes of cancer, but the role in the prevention or control of cancer is not commonly evaluated.

The development and use of strategies that activate specific, robust, and long-lasting antitumor immunity are crucial, in the near future, to improve combinatory approaches in the clinic of patients with cancer.

The safety of dendritic cells has been established in vivo trials and presents minimal side effects.

Prophylactic dendritic cell vaccination works by reducing tumor growth.

Dendritic cell vaccine may inhibit 4T1 tumor growth recurrence, by inducing antitumor immunity and maintained a lower rate of growth.

The vaccination promotes an increase in the antitumor immune response.

The dendritic cell vaccine induces an increase of Th1 and cytotoxic T lymphocytes; and an increase of TNF-α and IFN-γ by both cells.

The antitumor immunity induced by the dendritic cell vaccination promotes the lower growth in tumors.

5.2 Artigo 2

Artigo aceito na *Anticancer Research* (ISSN 1791-7530; Fator de impacto: 1.994).

Prophylactic Dendritic Cell Vaccination in Experimental Breast Cancer Controls Immunity and Hepatic Metastases

Jéssica Ferreira Vieira[a], Ana Paula Peixoto[a], Eddie Fernando Cândido Murta[a, b] and Márcia Antoniazi Michelin[a, c,@]

[a] *Oncology Research Institute (Instituto de Pesquisa em Oncologia, IPON), Federal University of the Triangulo Mineiro (Universidade Federal do Triângulo Mineiro, UFTM), Uberaba, Minas Gerais, Brazil.*
[b] *Discipline of Gynecology and Obstetrics, UFTM, Uberaba, Minas Gerais, Brazil*
[c] *Titular Professor/Discipline of Immunology, UFTM, Uberaba, Minas Gerais, Brazil*

[@] Correspondence: Oncology Research Institute, Federal University of Triangulo Mineiro, Avenida Guilherme Ferreira n° 1940, CEP 38022-200, São Benedito, Uberaba, Minas Gerais, Brazil. E-mail: marcia.michelin@uftm.edu.br (M. A. Michelin).

Keywords: dendritic cell vacines, prevention, antitumoral imune response, vaccination, immunotherapy, cancer, liver

Abstract

Background/Aim: Liver metastases are among the principal mortality causes in cancer patients. Dendritic cell immunotherapies have shown promising results in some tumors by mediate immunological mechanisms that could be involved in liver metastases during primary tumor growth. Materials and Methods: We analyzed the liver of adult female mice of the Balb/c submitted or not to vaccination with dendritic cell before the induction of 4T1 tumor lineage by flow cytometry (markers CD3, CD4, CD8, CD25, IL-10, IL- 12, IL-17, TNF-α, IFN-γ, T-bet, GATA3, RORγt, and FoxP3) and HE. The dendritic cell vaccine was differentiated and matured ex vivo from the bone marrow. Results: Prophylactic vaccination reduced areas of liver metastases ($p=0.0049$), promoted an increase in the percentage of total T and cytotoxic T lymphocytes ($p <0.0001$), as well as FoxP3 ($p <0.0001$). It also increased the cytokines IL-10 and IL-17 in helper T lymphocytes ($p <0.0001$). Conclusion: The prophylactic dendritic cell vaccine changes the cell phenotype in the liver's immune response, and it was able to reduce metastases. Cytotoxic T cells and Treg are more present, likewise, the production of IL-10 and IL-17 simultaneously, demonstrating that the vaccine can induce a state of control of pro-inflammatory responses, which can favor a less favorable environment for installation metastatic.

Introduction

Although liver is not considered a classic secondary lymphoid organ, it represents a unique site for the development of adaptive immune responses. The responses are mediated by a diverse repertoire of immune cells and other non-hematopoietic cells. In addition to hepatocytes, there is a considerable wealth of antigen-presenting cells (APCs), natural killer cell (NK), natural killer T cell (NKT), T and B lymphocytes, neutrophils, eosinophils, and components of the complement system (KUBES; JENNE, 2018). However, the full spectrum of immune cells that resides in the liver and their role in different pathophysiological processes is still unclear.

In the liver, the generation of an integrated adaptive immune response may not occur as efficiently and directly as in a lymph node or spleen. Some authors considered this to be immune paralysis. However, it could occur due to patterns related to the organ's anatomy, physiology, and location, which directly affect the way the organ will articulate its immune mechanisms (HINES; SON; KREMER, 2010).

The liver can activate a robust immune response via T cells in baseline conditions; however, it needs to have an immune-tolerogenic response pattern that leads to the expansion of cognate T lymphocytes, without supporting the effectiveness of cytotoxic effector functions (DIEHL et al., 2008; DURAND; FRANCOZ, 2017). The organ can still provide a hypoimmune environment, with an easing in the processes of immune tolerance, which could justify the success of transplants (KUBES; JENNE, 2018).

Liver is often a target organ for metastases from different types of primary tumors (5). Metastatic development is a multi-step process, supported by both the primary tumor site and the hepatic metastatic microenvironment. Growth factors, cytokines, and other soluble factors are involved in the interactions between the disseminated cancer cells and the resident hepatic cells that control liver metastasis (CLARK et al., 2016; KORKAYA; LIU; WICHA, 2011; WU et al., 2017). The presence of liver metastases represents a poor prognosis, affecting the overall survival of patients (HE et al., 2017).

In the fight against neoplasms, immunotherapies can be applied to treat the disease with minimal impact on normal tissues. The cell-based vaccines can be of a prophylactic or therapeutic type. Both types have been shown to generate a specific immune response against the tumor, and in parallel have the capacity to generate cellular immune memory, aiming to protect against possible recurrences (ALY, 2012).

Dendritic cells (DCs) have a fundamental role in antitumor vaccine models for the treatment of cancer, as they have a high potential for stimulation of T lymphocytes

(PALUCKA; BANCHEREAU, 2013). In the literature, there are studies demonstrating that the antitumor immune response provided by the DC vaccine-activated effector T lymphocytes ensures an efficient antitumor mechanism. This is due to the fact of DCs being the best antigen-presenting cells, performing the processes of recognition, processing, and presentation of tumor cell antigens, as well as of their potential to stimulate T lymphocytes and establish a bridge between immune mechanisms. Therefore, DC vaccination allows tumor regression and remaining memory cells, hence, protecting recurrences (PALUCKA; BANCHEREAU, 2013).

Murine breast carcinoma with 4T1 cells is a highly tumorigenic and invasive model, with a very aggressive profile, with rapid evolution, high risk of recurrence, mostly visceral metastases, high death rates, and significant resistance to immunotherapies (BOYLE, 2012). Therefore, it is important to have alternatives that control its growth, preventing its accelerated dissemination, focusing on detection and prognosis.

In this work, the aim was to evaluate the impact of prophylactic dendritic cell vaccination on the phenotypic profile of T lymphocytes and metastases in the liver of mice with 4T1 mouse breast carcinoma, submitted to prophylactic vaccine with dendritic cells.

Materials and Methods

Animals. A total of 25 adult female Balb/c mice (6-8-weeks-old, an average weight of 23g±0.8 g) from Biotério Central of Universidade Federal do Triângulo Mineiro-UFTM were used. The animals were kept in plastic cages, in a closed housing system with ventilation and controlled temperature (21±3°C), in a 12-h light-dark cycle, with access to food and water *ad libitum*.

The animals were randomized into three experimental groups: C (Control; n=7) animals without induction of 4T1 mouse breast tumor cells and without prophylactic vaccination by dendritic cells; group T (Tumor; n=8), with animals submitted to tumor induction with 4T1 cells; and group DC (Dendritic cell vaccine; n=7), with animals that received dendritic cell vaccine and subsequent tumor induction (Figure 1). Three animals were euthanized for the preparation of the dendritic cell vaccine. This study was approved by the Ethics Committee on the Use of Animals - CEUA of the UFTM (registration number 379) and followed the regulations of the Basel Declaration.

Tumor induction of breast cancer model. The animals of T and DC groups were inoculated with 4T1 mouse breast tumor cells (model of syngeneic transplantable tumors) (MILLER; MILLER; HEPPNER, 1983; TAO et al., 2008); in a single dose of 2.0×10^5 cells, injected into the lower-left mammary gland. This lineage has tumor growth and metastatic spread similar to stage IV

human breast cancer. The strain was obtained by the Rio de Janeiro Cell Bank and maintained in RPMI-1640 medium (Sigma-Aldrich®, St. Louis, MO, USA), supplemented with 0.24% HEPES ($C_8H_{18}N_2O_4S$), sodium bicarbonate ($NaHCO_3$), 10% fetal bovine serum (FBS), 1% streptomycin, 1% L-glutamine (200 mM), 0,1% sodium pyruvate ($C_3H_3NaO_3$), 0.1% β-mercaptoethanol (C_2H_6OS), at 5% CO_2 and 37°C. All products used for supplementing the RPMI-1640 medium were obtained from Sigma-Aldrich®, St. Louis, MO, USA.

Dendritic cell vaccine and vaccination protocol. The dendritic cell vaccine was made from the bone marrow of the femur and tibia of the three mice Balb/c, grown in IMDM medium (Sigma-Aldrich®, St. Louis, MO, USA), supplemented with 0.1 mM of vitamins, 2 mM L-Glutamine, 100 µg/ml Gentamicin, 1 mM Sodium Pyruvate and 5% FBS, incubated in a 5% CO_2 and 37°C. All products used for supplementing the IMDM medium were obtained from Sigma-Aldrich®.

On the first day, the cells were stimulated with 10 ng/µl of Granulocyte-macrophage Colony-stimulating factor (GM-CSF) and 10 ng/µl of IL-4. In day five, cells were stimulated with TNF-α and tumor lysate from 4T1 cells. GM-CSF, IL-4 and TNF- α was obtained from BD Pharmigen™, BD Biosciences, San Diego, CA, USA. After 48 h of incubation, the differentiated dendritic cells were washed and resuspended in 0.9% saline solution. A single dose of 5.0×10^6 cells was administered in the animals of the DC group, 7 days before tumor inductions with 4T1 tumor cell line (Figure 1).

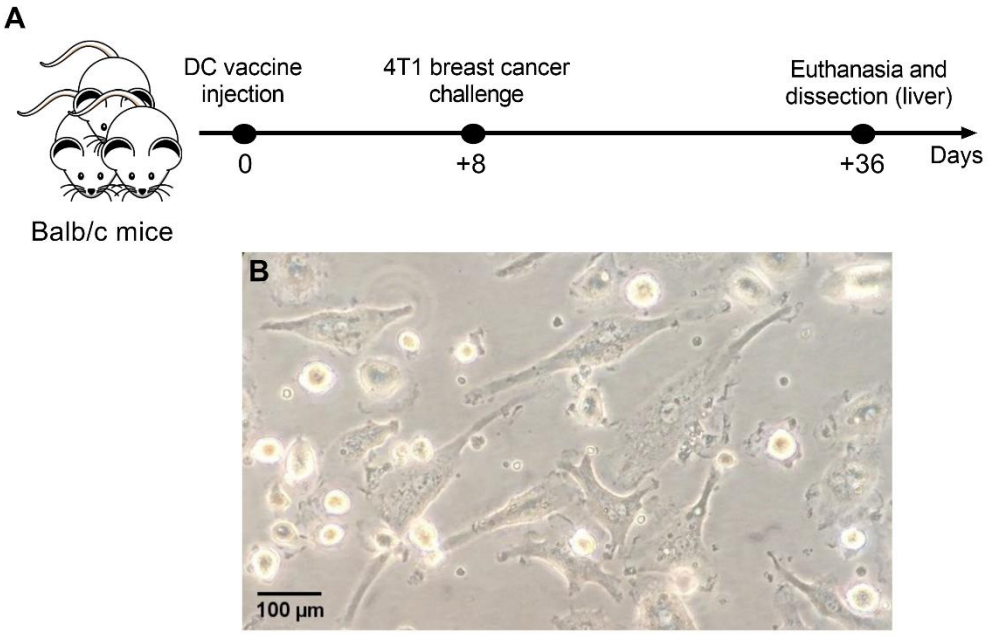


Figure 1. Representation of the experimental design. (A) Experiment period showing vaccination, tumor induction, and euthanasia on a temporal scale. (B) Representation of dendritic cell morphology after differentiation with granulocyte-macrophage colony-

stimulating factor (GM-CSF), interleukin (IL)-4, tumor necrosis factor (TNF)-α, and lysate tumor (scale bar 100 μm).

Isolation of liver cells and flow cytometry. At the end of experimental period, the livers were removed after euthanasia and necropsy of the animals. Part of the organs was subjected to mechanical rupture, and the cells followed a flow cytometry protocol. All material for flow cytometry analysis was obtained from BD Biosciences, San Diego, CA, USA. The leukocyte cells of liver were obtained by centrifugation after using lysis solution FACS Lysing Solution. The technique was performed according to the protocol suggested by the manufacturer, using *BD Pharmigen*^{TM} antibodies for extracellular labeling (CD3, CD4, CD25, and CD8 for lymphocyte evaluation) and for intracellular marking (T-bet, GATA3, RORγt, FoxP3, IL-12, TNF-α, IFN-γ, IL-10, and IL-17A). The liver samples from the three experimental groups were analyzed using FACS Calibur^{TM} cytometer.

Gate strategy: lymphocytes were initially selected based on size and granularity (FSC x SSC). In lymphocytes, a gate for $CD3^+$ was designed to mark T cells. From this gate, a CD4 *vs*. CD8 graph was plotted to separate $CD4^+$ and $CD8^+$ T lymphocytes. Within the $CD4^+$ T lymphocytes, a T-bet graph was plotted to mark helper T lymphocytes type 1 (Th1), another plotting GATA-3 to mark helper T lymphocytes type 2 (Th2), RORγt, to mark helper t lymphocytes type 17 (Th17) and a graph plotting CD25 and FoxP3 was used to outline regulatory T lymphocytes (Treg). Also from the helper T lymphocytes gate (CD3+CD4+), histograms for the intracellular TNF-α, IFN-γ, IL-12, IL-17, and IL-10 markings were plotted.
Analysis of metastases in the liver (H&E) – histology. The livers of T and DC group were fixed in a 10% formalin solution, followed by inclusion in paraffin. Tissue was cut in 5-μm sections in an automated rotating microtome (Leica RM2255) and the sections were stained with the hematoxylin and eosin (H&E). The slides were observed under an optical microscope (Olympus BX41). Qualitative and quantitative measurements were made by blinded researchers. For each organ, three slides were prepared so that the entire organ could be sampled. In each slide, 15 blind fields were evaluated (45 fields per animal of each group). Quantification of metastases was performed using a Nikon Eclipse Ti2 microscope with Nikon Analyzes Software.

Statistical analyses. Graphs and statistical analyses were performed using GraphPad Prism 8.4 (GraphPad Software). One-way ANOVA with Tukey post-test and t-Student was performed with results expressed as mean±SD. Fisher's exact test was used to establish statistical

differences in the association of vaccination and metastasis. For all analyses, $p<0.05$ was considered statistically significant.

Results

The prophylactic vaccine with dendritic cells reduced the number of liver metastases. Metastatic liver areas were observed in all animals in the two groups, T and DC (Figure 2). In general, the foci of metastases were smaller and more rare in DC than in T group.

First, we evaluated the association between vaccination and the number of fields that were positive or negative for metastases. A number of 45 fields were evaluated per animal (15 fields per slide, 3 slides per animal, totaling 360 fields evaluated for the T-group, and 315 fields for the DC). A reduction in the positive fields was observed in DC (95 fields) compared to the T group (171 fields) ($p<0.0001$; Figure 2A). Thus, the data showed that approximately 70% of the total evaluated fields were negative in the DC group. In addition, when the total metastasis area was measured, a statistically significant reduction was observed in DC ($0.7 \times 10^6 \pm 0.41 \times 10^6$) compared to T ($3.8 \times 10^6 \pm 2.3 \times 10^6$) ($p=0.0049$; Figure 2B). Figures 2C and 2D illustrate metastatic foci found in animals of the T and DC group, respectively. Therefore, prophylactic vaccination with dendritic cells appeared to reduce total number of metastases and total metastasis area in the liver of mice with breast cancer.

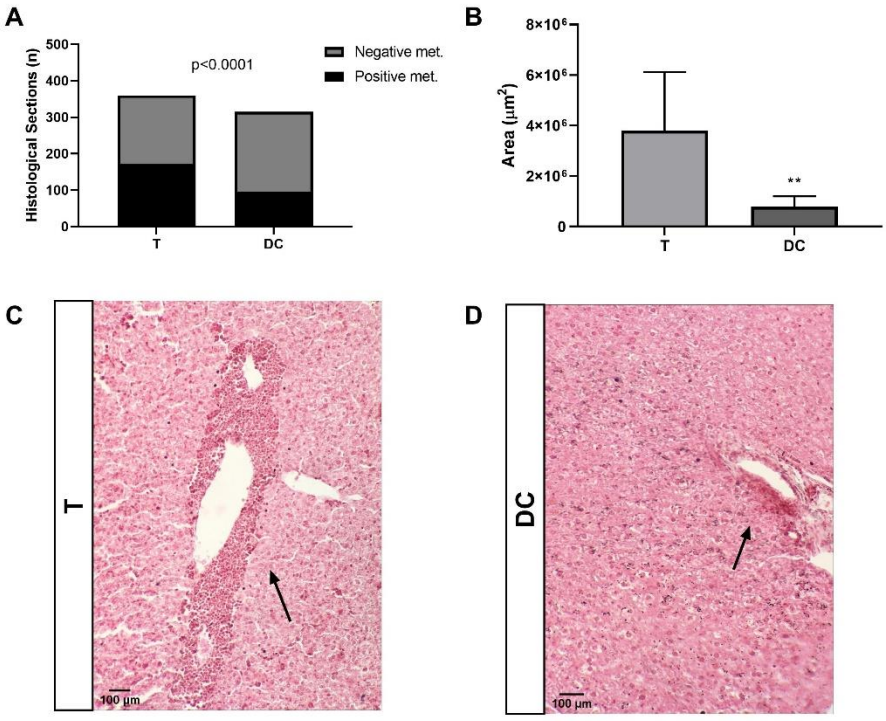


Figure 2. Prophylactic dendritic cell vaccination reduced the metastatic area in the liver of Balb/c mice induced to breast cancer with 4T1. **(**A) Quantification of metastasis-positive fields

in the two experimental groups; p<0.0001, according to Fisher's exact test. (B) Quantification of metastases. Statistical test: independent sample t-test. The results are expressed as mean±SD. **p=0.0049. (C) Representation of a metastatic area (arrow) in an animal in the Tumor group. (D) Representation of a metastatic area (arrow) in an animal in the DC group. Scale bar: 100 µm. T: Tumor group; DC: dendritic cell vaccine group.

Subtypes of intrahepatic lymphocytes and cytokine production by helper T lymphocytes. The characterization of the phenotypic profile of immune cells in the livers of animals was performed by flow cytometry, right after euthanasia. The percentage of total T lymphocytes ($CD3^+$) was higher in the DC group (97.03±1.83) compared to the group T (85.09±2.38) (p<0.0001) (Figure 3A). The percentage of cytotoxic T lymphocytes ($CD3^+CD8^+$) was also significantly higher in the same group (DC) compared to T (p<0.0001) (Figure 3C). However, the percentage of helper T lymphocytes ($CD3^+CD4^+$) did not show significant changes (p=0.2756; Figure 3B) between the two groups (T and DC).

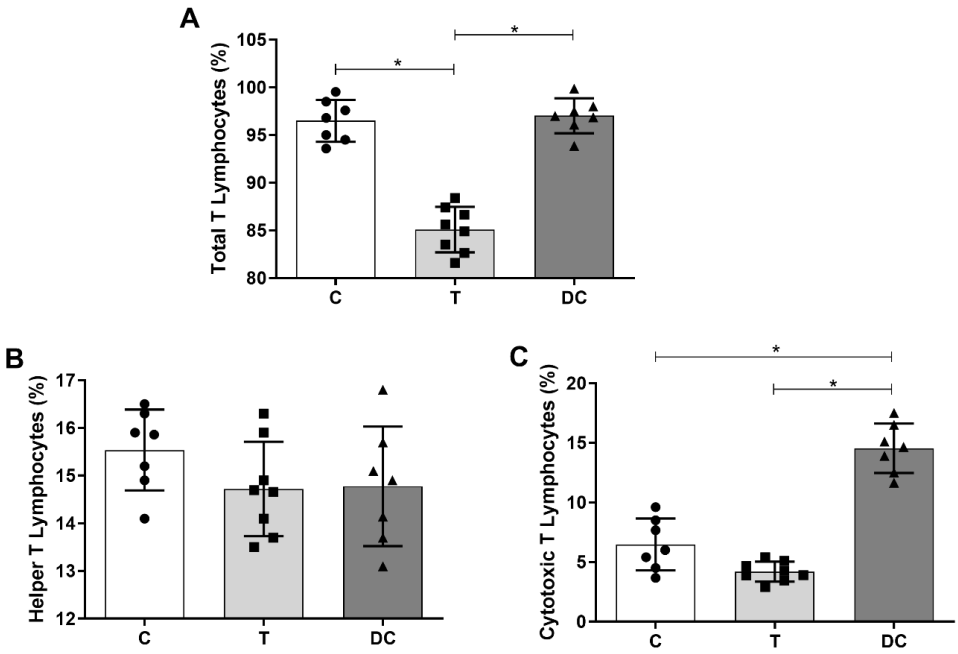


Figure 3. Percentage changes of T lymphocytes in the liver of mice with breast cancer, submitted or not to prophylactic DC vaccination. (A) Percentages of total T lymphocytes ($CD3^+$) (p<0.0001). (B) Percentages of helper T lymphocytes ($CD4^+$) in each experimental group (p=0.2756). (C) Percentages of cytotoxic T lymphocytes ($CD8^+$) in each experimental group (p<0.0001). Statistical test: One-way ANOVA with Tukey's multiple comparison test. The results are expresses as mean±SD. *p<0.0001. C: Control group; T: tumor group; DC: dendritic cell vaccine group.

Regarding lymphocyte populations, the profile of T helper lymphocyte subtypes was also evaluated using the mean fluorescence intensity (MFI) of the key transcription factors of the differentiation process. The transcription factors T-bet, GATA3, and RORγt were

significantly reduced in the vaccinated group (p<0.0001), compared to the T group (Figure 4A-C). However, FoxP3 was increased in the DC group compared to the tumor group (p<0.0001), while was decreased compared to the control group (p<0.0001; Figure 4D).

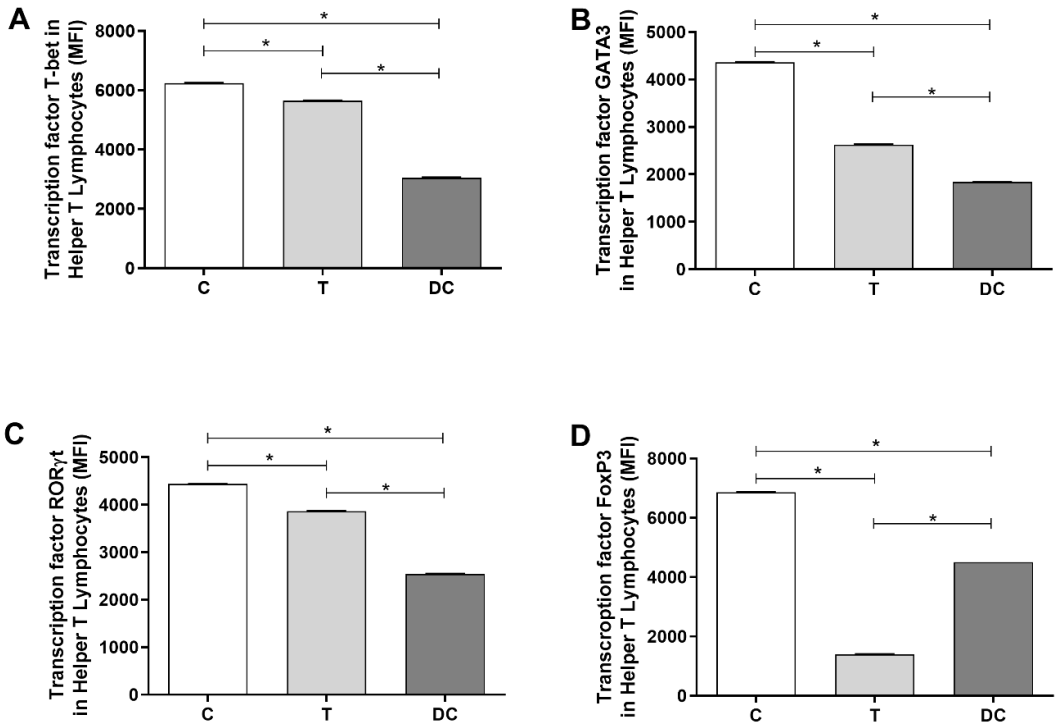


Figure 4. Transcription factors of hepatic T helper lymphocytes in the liver of mice with breast cancer, submitted or not to prophylactic DC vaccination. The graphs represent the mean fluorescence intensity (MFI) of T-bet$^+$ (A), GATA3$^+$ (B), RORγt$^+$ (C), and FoxP3$^+$ (D) hepatic T helper lymphocytes in each experimental group. The results are represented as mean±SD. *p<0.0001, according to one-way ANOVA with Turkey's multiple comparisons test. T-bet: T-box expressed in T-cells; GATA3: GATA binding protein 3; RORγt: retinoic acid-related orphan receptor gamma t; FoxP3: forkhead box P3; C: control group; T: tumor group DC: dendritic cell vaccine group.

We also evaluated the impact of prophylaxis on the expression of cytokines IL-12, IFN-γ, TNF-α, IL-10, and IL-17 in helper T lymphocytes. IL-12, IFN-γ, and TNF-α were significantly reduced in the vaccinated group, compared to the T group (p<0.0001). The cytokines IL-10 and IL-17 were significantly increased in the DC group compared to the T group (p<0.0001; Figure 5).

Discussion

Liver is a target organ for metastases in several types of cancers. In breast cancer, metastases could target various organs, such as lymph nodes, liver, lungs, brain, and bones

(TSUJI; PLOCK, 2017). The hepatic metastases represent poor prognosis in breast cancer, affecting survival of patients (HE et al., 2017; PENTHEROUDAKIS et al., 2006).

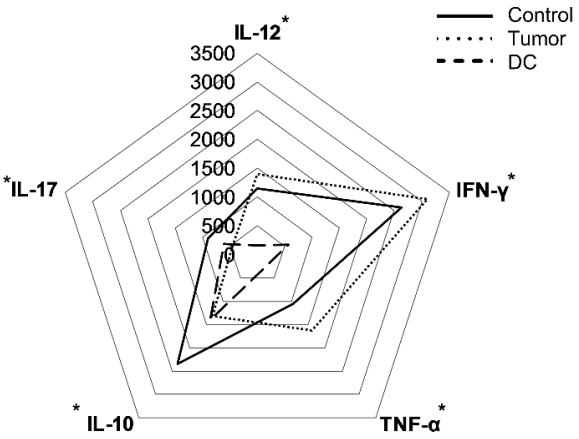


Figure 5. Expression of cytokines (IL-12, IFN-γ, TNF-α, IL-17, and IL-10) in T helper lymphocytes obtained from the liver of mice with breast cancer, submitted or not to DC vaccination. The cytokine levels were quantified in T helper lymphocytes ($CD3^+CD4^+$) by flow cytometry. The values represent the MFI. IL-12, IFN-γ and TNF-α were significantly reduced in DC compared to T; while IL-10 and IL-17 were significantly increased in DC compared to T; *$p<0.0001$, according to one-way ANOVA with Turkey's multiple comparisons test. C: Control group; T: tumor group: DC: dendritic cell vaccine group; MFI: mean fluorescence intensity.

Considering the immune context, liver represents a fundamental spot for adaptive immune responses, even though it is not considered a typically secondary lymphoid organ, as spleen, for example (BOGDANOS; GAO; GERSHWIN, 2013; KUBES; JENNE, 2018). Furthermore, it is known that the immune response, depending on its mechanisms, could mediate the progression or regression of cancer, facilitating or not the implantation of metastatic. This demonstrate the complex modulations of the immune system (DOUGAN; DRANOFF, 2009). Thus, this study sought to assess the impact of prophylactic DC vaccination, evaluating the metastatic area and profile of T lymphocytes in the liver of animals with breast cancer.

Our data showed reduced metastatic areas in the livers from vaccinated animals, suggesting that DC-vaccination may be an important instrument to manipulate the antitumor and metastatic response. Previous studies have also demonstrated the antitumor and antimetastatic effect of prophylactic DC vaccination in melanoma and pancreatic cancer (MARKOV et al., 2015; ODA et al., 2012; SHANGGUAN et al., 2020).; however, there is little data on its effectiveness in target organs for metastases, such as liver.

Some studies have shown that the DC vaccine could act on both growth of the tumor and antimetastatic response by mechanisms of the antitumor immune response. They demonstrated that autologous DCs loaded with tumor antigens increased the polarization of Th1 immune responses and reduced Treg, in addition to the cytotoxicity generated by DC. Moreover, the vaccine induced responses and cytotoxic T lymphocytes, both *in vitro* and *in vivo*. Thus, they suggested that immunotherapy with DC in the context of breast cancer not only is safe, but it also reduces the risk of relapse and improves immune parameters, and can be a strategic approach to combating metastasis. Generally, this activity may be associated with immune surveillance generated by vaccination, specifically prepared for antitumor activity. Th1 cells and an increase of cytotoxic T lymphocytes mediate this antitumor mechanism. However, these studies did not used the DC vaccine as a prophylactic intervention (SHEVCHENKO et al., 2020; TOMASICCHIO et al., 2019).

The liver, as a secondary lymphoid organ, activates T cells locally and this activation occurs in an environment that is biased towards immune tolerance (CRISPE, 2014). Immune tolerance could be attributed to the low expression of the major histocompatibility complex (MHC) and IL-10 expression (BERTOLINO; MCCAUGHAN; BOWEN, 2002; PILLARISETTY et al., 2004; SANA et al., 2014). Even though the liver is a tolerogenic organ, its immune function can generate rapid and controlled responses to tumor cells or pathogenic organisms that have the liver as their target (PROTZER; MAINI; KNOLLE, 2012).

Despite the metastatic reduction due to prophylactic vaccination, our data indicated an increase in cytotoxic T lymphocytes and Treg in the liver tissue. The liver is rich in lymphocytes. Even though they are populations similar to those present in the peripheral circulation, these lymphocytes differ in proportions and performance standards, and their roles remain uncertain (KUBES; JENNE, 2018). The composition and location of cell populations can change liver inflammatory conditions. Associated with anatomy and the vasculature liver, these conditions can exchange immunological information continuously due to communication with splenic venous system and inferior vena cava, lymphatic drainage (BOGDANOS; GAO; GERSHWIN, 2013; CHAUDHRY; EMOND; GRIESEMER, 2019).

In the liver, T helper lymphocytes play a crucial role in the production of cytokines that act on hepatocytes and other immune response cells. Cytotoxic T lymphocytes, activated or memory cells, are also present in the liver and their activity is associated with apoptosis and exclusion of T cells (ROBINSON; HARMON; O'FARRELLY, 2016). Some studies have pointed out liver-mechanisms that actively prevent the generation of cytotoxic T lymphocytes, preventing immunity mediated by functional T cells and inducing T cell tolerance (BERG et

al., 2006; DIEHL et al., 2008). However, this information is still uncertain. The liver T helper lymphocyte subtypes are in balance, Th1/Th2 and Th17/Treg. The Th1 cells are involved in pro-inflammatory cell-mediated responses, while the Th2 cells promote tolerance (JAESCHKE et al., 2002). An imbalance, however small, leads to the production of dominant cytokines for one of these profiles.

Th17 are closely related to Treg; the reciprocal relationship between these two represents a delicate balance between tolerance and induction of inflammatory responses or immune tolerance (NOACK; MIOSSEC, 2014). A Th17/Treg imbalance may be responsible for liver-pathological processes (WANG et al., 2014). In other words, it seems that these two cells populations make it possible to maintain a subtle balance in liver homeostasis, while a slight imbalance is associated with diseases and injuries (ZHANG; JIANG; ZHANG, 2019).

In hepatocellular cancer, an imbalance of Th17 and Treg seems to be associated with the process of carcinogenesis. The isolated increase in intratumoral Th17 is indicative of tumor activity and angiogenesis. It also affects the prognosis and is associated with low survival rates (YAN et al., 2014; ZHANG et al., 2009). Thus, Th17 and Treg and their cytokines play a double role in the liver. The lack of control of the immune system in this organ can generate favorable conditions for metastatic development. Therefore, considering that our data showed that there is an association between an increase in cytotoxic T lymphocyte and Treg and an increase in IL-10 and IL-17, the combined effects seem important for the hepatic immune responses acting on metastasis. Anyway, the presence of Treg is fundamental in regulating a balance on hepatic inflammatory responses and seems to have substantial effects that need to be further evaluated (ZHANG; JIANG; ZHANG, 2019).

Another point that leads us to assume a relative tolerance is the low levels of IL-12 observed in the vaccinated group. This environment promotes change in Th1 to Th2 responses and maintenance of Treg (JOMANTAITE et al., 2004), which would justify the increase of this phenotype in our study. At the same time, other cells, such as the myeloid lineages, could create new modulations of the immune responses (CALMEIRO et al., 2020).

In addition to the increase in IL-10, we also found that IL-17 was increased in the vaccinated group. IL-10 is an important anti-inflammatory and immunosuppressive cytokine that acts by inhibiting the synthesis of other cytokines, such as IL-12, IFN-γ, and TNF-α, as well as inhibiting proliferation and Th1 at the expense of Th2. Simultaneously, it can also be an important differentiating factor for cytotoxic T lymphocytes, even if it is not its primary function (THOMPSON-SNIPES et al., 1991).

It is worth mentioning that the healthy adult liver tends to have an active and complex environment composed of cytokines, either pro-inflammatory, such as IL-3, IL-7, IL-12, IL-15, and IFN-γ, or anti-inflammatory, such as IL-10, IL-13, and TGF-β (KELLY et al., 2006). This cytokine environment exists in the absence of any pathogen or pathological inflammation and arises through habitual and physiological processes in the liver (TU et al., 2007), which emphasizes and supports the idea of why healthy liver is described as immunologically tolerogenic. However, it is still evident that rapid and robust immune responses are successfully generated in this environment (CRISPE, 2014; PROTZER; MAINI; KNOLLE, 2012).

Our study allowed a description of immune cell populations in the liver tissue involved in the antitumor immune response. We also demonstrated the importance of prophylactic immunotherapy beyond the tumor microenvironment. Other phenotyping and genotyping methods could provide a better knowledge of the possible interactions in this tissue.

The prophylactic DC vaccine altered the cell phenotype in the liver immune response, suggesting that the liver functions as an important regulator of systemic immunity. Our data reveal that the liver mediates distinct functional processes to the preventive response induced by the vaccine, acting as sentinel, which can lead to the interference of metastatic processes. These findings demonstrate that the DC vaccine controls the pro-inflammatory environment, promoting the increase of cytotoxic T cells and regulatory T lymphocytes and increases the production of IL-10 and IL-17, simultaneously. However, additional research is needed in order to comprehend these mechanisms and their relationships.

The literature has mentioned the DC vaccine as a mechanism to control cancer recurrence and as an enhancer of the immune response. Therefore, these data provide evidence of timely activation of immune surveillance in the absence of tumor burden, as well as new perspectives about the immunotherapies scenario. Besides, it can be associated in the future with the study of control metastases cancer.

5.3 Artigo 3

Artigo publicado na *Brazilian Journal of Development* (ISSN 2525-8761; Qualis CAPES B2).

Imunoterapia profilática com células dendríticas reduz metástases pulmonares em modelo de câncer de mama experimental

Prophylactic immunotherapy with dendritic cells reduces lung metastases in an experimental breast cancer model

Jéssica Ferreira Vieira
Mestre em Ciências da Saúde pela Universidade Federal do Triângulo Mineiro (UFTM)
Instituição: Instituto de Pesquisa em Oncologia – UFTM
Endereço: Avenida Guilherme Ferreira, n°1940, 38022-200, São Benedito, Uberaba – MG, Brasil. E-mail: jessica.vieira@uftm.edu.br

Ana Paula Peixoto
Bacharela em Biomedicina pela Universidade Federal do Triângulo Mineiro (UFTM)
Instituição: Instituto de Pesquisa em Oncologia – UFTM
Endereço: Avenida Guilherme Ferreira, n°1940, 38022-200, São Benedito, Uberaba – MG, Brasil. E-mail: a.ppeixoto@hotmail.com

Taissa Nayara Lemos de Abreu
Bacharela em Biomedicina pela Faculdade de Talentos Humanos (FACTHUS)
Instituição: Instituto de Pesquisa em Oncologia – UFTM
Endereço: Avenida Guilherme Ferreira, n°1940, 38022-200, São Benedito, Uberaba – MG, Brasil. E-mail: taissanayarabio@gmail.com

Eddie Fernando Cândido Murta
Doutor em Medicina pela Universidade de São Paulo
Instituição: Disciplina de Ginecologia e da Universidade Federal do Triângulo Mineiro e Instituto de Pesquisa em Oncologia - UFTM
Endereço: Avenida Getúlio Guaritá, s/n, 38025-440, Abadia, Uberaba – MG, Brasil. E-mail: eddiemurta@mednet.com.br

Márcia Antoniazi Michelin
Doutora em Imunologia Básica e Aplicada pela Universidade de São Paulo
Instituição: Disciplina de Imunologia da Universidade Federal do Triângulo Mineiro e Instituto de Pesquisa em Oncologia – UFTM
Endereço: Avenida Guilherme Ferreira, n°1940, 38022-200, São Benedito, Uberaba – MG, Brasil. E-mail: marcia.michelin@uftm.edu.br

RESUMO
Introdução e objetivo: As metástases pulmonares são uma das causas de mortalidade em pacientes com câncer de mama. A imunidade antitumoral induzida por células dendríticas têm mostrado resultados promissores em alguns tumores por mediar os mecanismos imunológicos. Portanto, o objetivo desse trabalho foi avaliar o papel da profilaxia com vacina de células dendríticas em metástases pulmonares e em linfócitos T esplênicos de camundongos submetidos à indução tumoral com carcinoma mamário com células 4T1. Materiais e Métodos: Utilizamos 25 camundongos fêmeas adultas da linhagem Balb/c, separadas em três grupos experimentais: Controle (n=07), Tumor (n=08) e Vacina DC (n=07). Os animais do último grupo receberam uma dose única de vacina de células dendríticas, antes da indução tumoral com linhagem celular de carcinoma mamário de células 4T1. Os pulmões foram avaliados por meio da técnica de coloração hematoxilina-eosina para avaliação de focos metastáticos. Os leucócitos esplênicos foram avaliados por citometria de fluxo para linfócitos T totais (CD3+),

linfócitos T auxiliares (CD3+CD4+) e linfócitos T citotóxicos (CD3+CD8+). A vacina de células dendríticas foi diferenciada e maturada *ex vivo* a partir da medula óssea (n=03). Resultados: A vacinação profilática reduziu as os focos (p<0,0001) e áreas (p=0,0106) de metástases pulmonares. Promoveu aumento no percentual de linfócitos T totais, T auxiliares e T citotóxicos (p<0,0001) no tecido esplênico dos animais vacinados em relação ao grupo Tumor. Conclusão: Novas investigações são necessárias para elucidar os mecanismos induzidos pela vacinação profilática em outros nichos e contextos. A imunoterapia profilática com células dendríticas deu indicativos de promoção da resposta contra tumores, por promover redução metastática pulmonar; e na avaliação sistêmica, por avaliação de células T esplênicas, a imunoterapia promoveu aumento de células T totais, T auxiliares e T citotóxicas.
Palavras-chave: Câncer. Imunoterapia. Imunologia Celular. Célula dendrítica.

ABSTRACT
Background and aim: Lung metastases are one of the causes of mortality in patients with breast cancer. The antitumor immunity induced by dendritic cells has shown promising results in some tumors by mediating the immunological mechanisms. Therefore, the objective of this work was to evaluate the role of prophylaxis with dendritic cell vaccine in lung metastases and in splenic T lymphocytes of mice submitted to tumor induction with 4T1 cell mammary carcinoma. Materials and Methods: We used 25 adult female Balb/c mice, separated into three experimental groups: Controle (n = 07), Tumor (n = 08), and Vacina DC (n = 07). The animals in the last group received a single dose of dendritic cell vaccine, before tumor induction with 4T1 cell mammary carcinoma cell line. The lungs were evaluated using the hematoxylin-eosin staining technique to assess metastasis. Splenic leukocytes were evaluated by flow cytometry for total T lymphocytes (CD3+), helper T lymphocytes (CD3+CD4+), and cytotoxic T lymphocytes (CD3+CD8+). The dendritic cell vaccine was differentiated and matured ex vivo from the bone marrow (n=03). Results: Prophylactic vaccination reduced the foci (p<0.0001) and areas (p = 0.0106) of lung metastases. It promoted an increase in the percentage of total T, helper T, and cytotoxic T lymphocytes (p<0.0001) in the splenic tissue of the vaccinated animals in relation to the Tumor group. Conclusion: Further investigations are needed to elucidate the mechanisms induced by prophylactic vaccination in other niches and contexts. Prophylactic immunotherapy with dendritic cells gave indications of promoting the response against tumors, by promoting metastatic lung reduction; and in the systemic evaluation, by evaluation of splenic T cells, immunotherapy promoted an increase in total T cells, helper T cells, and cytotoxic T cells.
Keywords: Cancer. Immunotherapy. Cellular Immunology. Dendritic cell.

Introdução

O câncer de mama é o mais frequente entre as mulheres do Brasil e do mundo, sendo a maior causa de mortalidade entre os tipos de cânceres (INCA, 2019; INTERNATIONAL AGENCY FOR RESEARCH ON CANCER (IARC), 2018). Cerca de 1/3 das mulheres com câncer de mama desenvolvem a doença metastática, que é fator central da sobrevida global e qualidade de vida (PENTHEROUDAKIS et al., 2006). Nesse contexto, as metástases pulmonares são indicativos de mau prognóstico, incidindo diretamente na sobrevida global das pacientes, por prejudicarem a função deste órgão. Isso ocorre devido à localização anatômica dos pulmões, o seu grande leito capilar, e por serem nicho propício com componentes que sustentam as metástases (HE et al., 2017; WU et al., 2017).

Na prevenção, desenvolvimento e eliminação do desenvolvimento tumoral e metastático, o sistema imunológico apresenta papel relevante. Ele identifica essas células transformadas como não-próprias e, as elimina por mecanismos de detecção de suas alterações. Em conjunto, elementos da imunidade inata e adquirida medeiam a resposta imune antitumoral, de forma local e sistêmica (BALKWILL; MANTOVANI, 2012; BURNET, 1970).

Porém, as metástases podem ocorrer por meio de ação cooperativa entre células imunológicas, epiteliais, mesenquimais e as próprias células tumorais, numa associação que promove evasão dos mecanismos imunes contra células tumorais. E qualquer diferença fenotípica ou genotípica antes, durante e após a instalação metastática são importantes para determinar suas relações com as respostas imunes (CHEUNG; EWALD, 2016).

No combate às neoplasias, as imunoterapias têm sido utilizadas como formas terapêuticas, atingindo a doença com o mínimo de impacto nos tecidos normais. E as vacinas baseadas em células dendríticas, têm se apresentado como uma ferramenta capaz de induzir respostas imunes específicas contra as massas tumorais e/ou metastáticas, podendo gerar proteção contra possíveis casos de reincidência (GARDNER; RUFFELL, 2016).

Isso ocorre pelo fato de que as células dendríticas possuem características que as classificam como as melhores células apresentadores de antígenos. Elas conseguem reconhecer as células neoplásicas por mecanismos de reconhecimentos antigênico, internalizá-las por fagocitose, processá-las, e apresentar antígenos aos linfócitos T naive, os ativando. Ou seja, as células dendríticas fazem um elo entre as funções efetoras do sistema imune, podendo iniciar respostas robustas, eficientes e específicas ao tumor (PALUCKA; BANCHEREAU, 2013).

Desta forma, a vacina de células dendríticas tem sido utilizada em diversos modelos de tratamento ao câncer, por todo esse potencial na estimulação de linfócitos T e por atuar na redução de massas tumorais primárias. A literatura menciona efeitos colaterais mínimos, além de fortalecimentos de respostas antitumorais com regressão tumoral e produção de células de memória protetivas para os casos de recidiva (ANGUILLE et al., 2014). Entretanto, ainda são escassos os dados que demonstrem a vacina de células dendríticas como medida protetiva contra tumores primários e/ou metástases. Por isso a importância de elucidar efeitos sobre os mecanismos imunes sistêmicos recrutados para minimizar o desenvolvimento tumoral e contribuir para essa discussão.

Assim, este trabalho teve como objetivo avaliar o papel da imunoterapia profilática com células dendríticas em metástases pulmonares e linfócitos T esplênicos de camundongos submetidos à vacina profilática com células dendríticas e induzidos ao carcinoma mamário com células 4T1.

Materiais e métodos

Animais e grupos experimentais

Este estudo foi previamente aprovado pelo Comitê de Ética no Uso de Animais da Universidade Federal do Triângulo Mineiro (CEUA/UFTM), sob registro de número 379 e seguiu normativas e regulamentos da Declaração de Basileia. Foram utilizadas 25 fêmeas, linhagem Balb/c, idade entre 06 a 08 semanas de vida (peso médio de 23g). Os animais foram mantidos em espaço adequado (gaiolas plásticas), sistema fechado com ventilação e temperatura controlada (em torno de 21°C), ciclo claro-escuro de 12h, sem restrição hídrica e alimentar por todo o período experimental.

Dos 25 animais, 22 foram randomizados e separados em três grupos experimentais, como consta na tabela 1. Os outros 3 animais foram eutanasiados para uso de suas células medulares para a preparação da vacina de células dendríticas.

Tabela 1 - distribuição dos animais nos grupos de estudo

Grupos	Inoculação 4T1	Inoculação Vacina	Número de animais
Controle	Não	Não	07
Tumor	Sim	Não	08
Vacina DC	Sim	Sim	07

Após o período experimental, os animais dos grupos experimentais foram eutanasiados de acordo com protocolos específicos do CEUA/UFTM, sendo retirados os baços e pulmões. Parte dos pulmões foram fixados em formalina 10%, seguindo inclusão em parafina para análise de metástases por hematoxilina-eosina. Parte dos baços foram submetidos a ruptura mecânica para realização de protocolo de citometria de fluxo. O delineamento do estudo consta na figura 1.

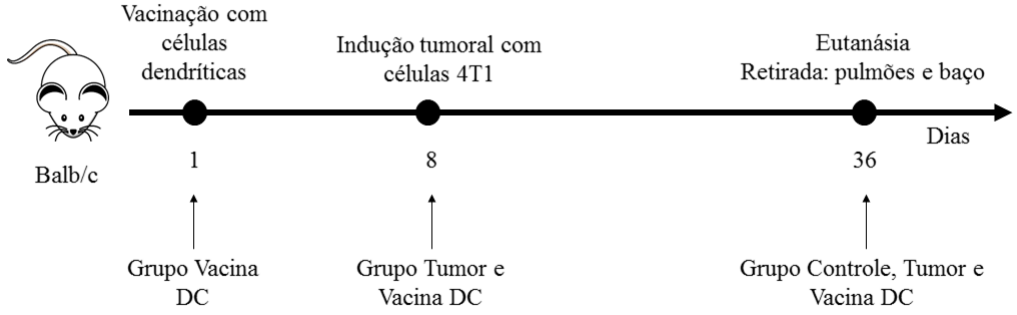
Indução tumoral

A indução tumoral foi realizada com a linhagem de carcinoma mamário de células 4T1. Essa linhagem apresenta alto poder tumorigênico, invasivo, proliferação epitelial maligna, poder metastático para pulmão, fígado, cérebro e ossos, com a doença metastática se desenvolvendo de forma espontânea e simultânea ao crescimento da massa primária, além de atingir os últimos estágios de estadiamento tumoral (TAO et al., 2008). Desta forma, elas são potentes indutoras de tumores de mama em camundongos da linhagem Balb/c. Seu crescimento

tumoral e disseminação metastática assemelham-se muito ao câncer de mama humano (comportando-se similarmente ao câncer de mama triplo-negativo).

A linhagem utilizada nesse trabalho foi obtida do Banco de Células do Rio de Janeiro. Até o momento da inoculação, as células foram mantidas em meio RPMI-1680 (Sigma-Aldrich®), suplementado com HEPES ($C_8H_{18}N_2O_4S$), Bicarbonato de Sódio ($NaHCO_3$), soro bovino fetal (SBF), Estreptomicina, L-Glutamina (200mM), Piruvato de Sódio ($C_3H_3NaO_3$), β-Mercaptoethanol (C_2H_6OS), em estufa a 5% de CO_2, a 37°C. No momento que antecede a aplicação, as células foram lavadas com solução fisiológica 0,9%, a 805xg, 4°C, por 10 min. Os animais inoculados com a linhagem tumoral (Tumor e Vacina DC) receberam uma única dose de 2,0 x 10^5 células, em um volume de 50µL, injetadas na glândula mamária inferior esquerda.

Figura 1: delineamento experimental.



Linha do tempo demonstrando o desenho e os procedimentos realizados no período experimental. **Fonte:** Autor.

Imunoterapia com vacina de células dendríticas

A vacina de células dendríticas foi confeccionada a partir de células medulares de fêmures e tíbias de 3 animais. As células medulares foram cultivadas em garrafas de cultura de 25cm^2, em meio IMDM suplementado com 0,1mM de vitaminas, 2mM de l-glutamina, 100µg/mL de gentamicina, 1mM de Piruvato de Sódio ($C_3H_3NaO_3$) e 5% Soro Bovino Fetal, incubadas em estufa de CO_2 a 5% de umidade e a 37°C. O protocolo de diferenciação celular tem a duração de 7 dias. No dia 1, as células foram estimuladas com 10ng/µL de GM-CSF e 10ng/µL de IL-4. No dia 5 foram estimuladas com 10ng/µL de TNF-α e antígeno tumoral da linhagem de células 4T1 (obtidos pelo congelamento e descongelamento das células 4T1). No

dia 07, as células dendríticas diferenciadas foram lavadas e ressuspensas em solução fisiológica 0,9%.

Foram administradas 5,0 x 10^6 células, em um volume de 50µL de solução fisiológica, em dose única nos animais do grupo Vacina DC. A avaliação das células dendríticas ocorreu de forma qualitativa, por controle visual através de microscópio óptico, pelo referido protocolo ser corriqueiro em pesquisas no laboratório e análises anteriores demonstrarem eficácia nesse processo de diferenciação (LOPES; MICHELIN; MURTA, 2017).

Análise de leucócitos esplênicos por citometria de fluxo

Amostras dos baços dos grupos experimentais foram analisadas por meio da técnica de citometria de fluxo, em citômetro FACS Calibur™ (BD Biosciences, San Diego, CA, EUA). A técnica foi realizada de acordo com o protocolo de citometria sugerido pelo fabricante, com utilização de anticorpos *BD Pharmigen™* para marcação extracelular de linfócitos T (CD3 FITC, CD4 PeCy 5.0 e CD8a APC), além dos respetivos marcadores para isotipo. Todos os anticorpos utilizados no protocolo de citometria de fluxo foram obtidos da BD Biosciences, San Diego, CA, EUA.

Estratégia de gate: linfócitos foram selecionados com base no tamanho e granularidade (FSC x SSC). Nos linfócitos, uma gate para CD3+ foi desenhada para marcar as células T. A partir desta gate foi traçado um gráfico CD4 vs. CD8 para separar as células T CD4 + e CD8 +.

Avaliação de metástases pulmonares (hematoxilina-eosina)

Para a avaliação da presença ou não de metástases foram confeccionadas lâminas de pulmão dos animais dos grupos Tumor e Vacina DC. Após a eutanásia, os órgãos foram excisados e fixados em solução de formalina 10%, seguido de inclusão em parafina. Os cortes foram feitos em micrótomo rotativo automatizado (Leica RM2255). Posteriormente seguiu-se com coloração histológica de hematoxilina-eosina (HE). As lâminas foram observadas em microscópio óptico (Olympus BX41). Para cada órgão, foram preparadas três lâminas para que todo o órgão fosse amostrado. Foram avaliados 45 campos às cegas por animal. A quantificação das metástases foi realizada no microscópio Nikon Eclipse Ti2 com o Software *Nikon Analyzes*.

Análise estatística

Gráficos e análise estatística foram realizados em *GraphPad Prism 8.4* (GraphPad Software). Os resultados foram testados quanto à distribuição por teste de normalidade *Shapiro-Wilk*. Foi utilizado teste de *t-Student* para 2 comparações e *One-way Anova* com pós-teste de *Tukey* para mais de duas comparações/grupos, com resultados expressos em média±SD. O teste

Chi-quadrado foi utilizado para estabelecer diferenças estatísticas na associação de vacinação e metástase.

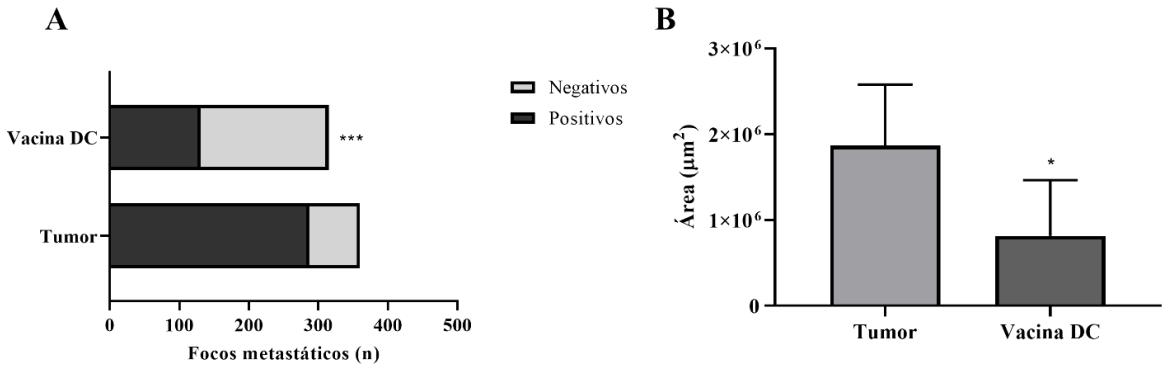
Resultados

A profilaxia com vacina de células dendríticas reduziu metástases pulmonares

O carcinoma mamário murino de células 4T1 possui capacidade de gerar metástases espontaneamente, podendo atingir linfonodo, fígado, pulmões, cérebro e ossos. A presença de metástases induzidas pela linhagem 4T1 foram avaliadas em lâminas de pulmão de animais submetidos ou não a imunoterapia profilática com células dendríticas.

Em todos os animais dos dois grupos avaliados foram observadas áreas metastáticas nos pulmões. Entretanto, os focos metastáticos encontravam em menor quantidade nos animais do grupo vacinado ($p<0,0001$). Além de menos focos, as áreas também eram menores; o grupo vacinado (812727±650484) apresentou redução significativa nas áreas metastáticas em relação ao grupo Tumor (1867885±711290) ($p=0.0106$). Logo, a vacina profilática foi uma poderosa ferramenta na indução de redução de áreas metastáticas nos pulmões (Figura 2).

Figura 2. Metástases pulmonares.



A imunoterapia profilática com células dendríticas reduziu a área metastática no pulmão de camundongos Balb/c induzidos a câncer de mama com 4T1. (A) Análise dos focos positivos. Teste estatístico: Chi-quadrado. ***$p<0,0001$. (B) Análise de áreas de metástases mensuradas em $\mu m^{2.}$ Teste estatístico: teste t não pareado. Os resultados estão expressos em média ± DP. * 0,0106.

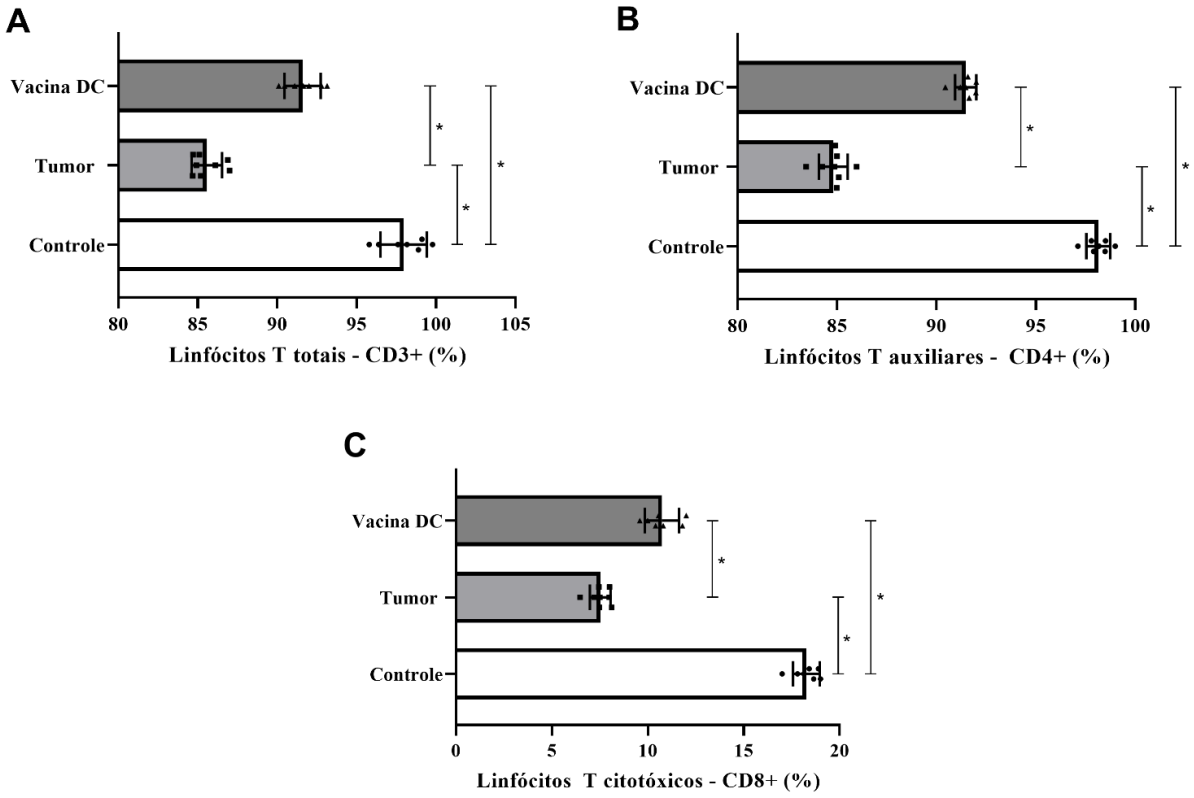
Profilaxia com células dendríticas aumenta porcentagem de linfócitos T esplênicos

A avaliação de células da resposta imune funciona como importante sinalizador de uma boa resposta imune. Nós avaliamos por citometria de fluxo a porcentagem de linfócitos totais

(CD3+), T auxiliares (CD4+) e T citotóxico (CD8+) no baço dos camundongos, como uma ferramenta indicativa das respostas imunes sistêmicas.

Foi observado aumento na porcentagem de linfócitos T totais, T auxiliares e T citotóxico no grupo Vacina DC em relação ao grupo Tumor: linfócitos T totais Vacina DC (91,61±1,137) versus Grupo Tumor (85,58±0.9578); T auxiliar Vacina DC (91.68±0.5321) versus Tumor (84.82±0.7265); T citotóxico Vacina DC (10,73±0,8918) versus Tumor (7,525±0,5377). Os dados (Figura 3) mostraram que a vacina profilática foi capaz de melhorar os padrões de resposta imune de forma sistemática.

Figura 3. Influência da vacinação profilática de células dendríticas na porcentagem de linfócitos T esplênicos.



(A) Linfócitos T totais. (B) Linfócitos T auxiliares. (C) Linfócitos T citotóxicos. Os resultados foram analisados por One-way ANOVA com *Tukey´s multiple comparisons test* e foram representados por média±SD. As diferenças foram consideradas significantes quando $p<0.05$; *$p<0.0001$.

Discussão

A maioria das vacinas com células dendríticas, no contexto do carcinoma, têm sido estudadas na forma de tratamento, seja pré ou pós intervenção cirúrgica. Elas têm sido desenvolvidas com base no papel chave das células dendríticas na ativação de resposta imunes

específicas e na coordenação dos mecanismos imunes antitumorais (BAUER et al., 2011; PALUCKA; BANCHEREAU, 2013).

Nesse estudo, buscamos avaliar o papel da vacina de células dendríticas, aplicada em dose única, como forma profilática na ativação de linfócitos T esplênicos e metástases pulmonares em um modelo de carcinoma mamário murino. Foi observado que a imunoterapia profilática com células dendríticas reduziu os focos e áreas metastáticas pulmonares.

A vacina de células dendríticas tem sido utilizada como tratamento em alguns subtipos de câncer. Inclusive uma imunoterapia personalizada já foi aprovada pela *U.S.Food and Drug Administration* (FDA), a *Sipuleucel-T*, para câncer de próstata resistente à castração (ANASSI; NDEFO, 2011). Porém, o uso da vacina de células dendríticas na terapia profilática não tem sido muito investigada. Desta forma, o desenvolvimento de mecanismos que ativem a imunidade antitumoral específica, robusta e com longa duração é essencial para promover a gestão de abordagens combinatórias na clínica de pacientes com câncer. E os estudos experimentais proporcionam a introdução dessas discussões.

Assim como demonstramos nesse trabalho, um estudo em modelo metastático de melanoma, em um contexto um pouco diferente do apresentado aqui, demonstrou eficiência da imunoterapia na inibição de metástases (MARKOV et al., 2015). Em um outra trabalho, a profilaxia com células dendríticas controlou crescimento do câncer em um modelo experimental de câncer pancreático (SHANGGUAN et al., 2020). Porém, a maioria das intervenções com vacina de células dendríticas demonstram o uso da vacina como forma de tratamento, atuando no processo de recidiva da doença.

Na colonização metastática, acredita-se que exista um padrão órgão-específico. Devido à localização anatômica pulmonar, ele tende a ser local de colonização por células de carcinoma mamário, o que impacta diretamente na sobrevida global das pacientes, representando um mal prognóstico (SLEEMAN, 2012; TANG; AHMAD; SARKAR, 2012) . Os estudos acerca do processo metastático ainda são limitados, mas há um consenso de que diversos fatores associados ao microambiente tumoral e a resposta imune antitumoral sistêmica possam subsidiar esse processo (RIIHIMÄKI et al., 2013; SLEEMAN, 2012; SLEEMAN; STEEG, 2010). Logo, os mecanismos do sistema imunológico atuam de forma intrínseca e direta nesse processo, o limitando ou o favorecendo.

Como é bem estabelecido pela literatura, as células dendríticas são apresentadoras antigênicas profissionais, atuando no desenvolvimento e modulação de respostas imunes antitumorais e/ou antimetastáticas. Esse papel envolve a ativação de linfócitos T (PALUCKA et al., 2011). Portanto, a capacidade de ativação de linfócitos T tem sido considerada um dos

mecanismos de avaliação da eficiência das vacinas de células dendríticas, e utilizados na discussão do potencial dessa ferramenta terapêutica.

Desta forma, observamos nesse trabalho que a imunoterapia profilática com células dendríticas aumentou a porcentagem de linfócitos T esplênicos em relação ao grupo com desenvolvimento tumoral e sem a vacina. No nosso trabalho, utilizamos a análise de células esplênicas como parâmetro de análise sistêmica, por ser o baço, um órgão linfóide secundário clássico e estar conectado com a circulação sanguínea.

Um aumento de linfócitos T está associado, em alguns casos, a melhora das respostas antitumorais. Entretanto, os linfócitos T auxiliares podem se diferenciar em diferentes subtipos, que podem tanto atuar na progressão quanto na regressão da atividade metastática, modulando a eficácia da resposta imune antitumoral (SELEDTSOV; GONCHAROV; SELEDTSOVA, 2015). Assim, linfócitos T auxiliares do tipo 1 (Tbet+) são o principal subtipo de linfócito T auxiliares no processo de eliminação das células tumorais pelo sistema imune (LAZAREVIC; GLIMCHER; LORD, 2013).

Os linfócitos T citotóxicos são os principais subtipos celulares da resposta imune antitumoral. Em muitos tipos de cânceres são utilizados como marcadores da eficiência dos mecanismos imunes antitumorais, sendo considerado um bom marcador prognóstico (MAHMOUD et al., 2011). Portanto, para a eliminação de células tumorais e células em metástase pelo sistema imune é necessária atuação de células T auxiliares e T citotóxicas. As citocinas produzidas por essas células, IFN-γ e TNF-α, promovem a morte das células tumorais e são pontuais para a atividade imune antitumoral (LIUDAHL; COUSSENS, 2017; WANG et al., 2018).

Além do mais, algumas células T geradas da resposta antígeno-específica previamente induzida pela vacina com células dendríticas, podem se manter por um período de tempo, como células de memória. As células de memória podem promover uma proteção rápida em casos de reincidência do processo tumoral ou tentativa de colonização de outros nichos (SALLUSTO; GEGINAT; LANZAVECCHIA, 2004), sendo esse o ponto crucial que norteia a atividade profilática da vacina de células dendríticas. Entretanto, nesse trabalho, não avaliamos marcadores de memória celular.

Logo, outras análises se fazem necessárias. Em trabalhos futuros, análises do papel desempenhado por células T auxiliares e T citotóxicas, compreensão dos mecanismos que são mediados pela profilaxia com a vacina de células dendríticas, inclusão de marcadores de memória, assim como avaliação de outros subtipos celulares da resposta antitumoral serão

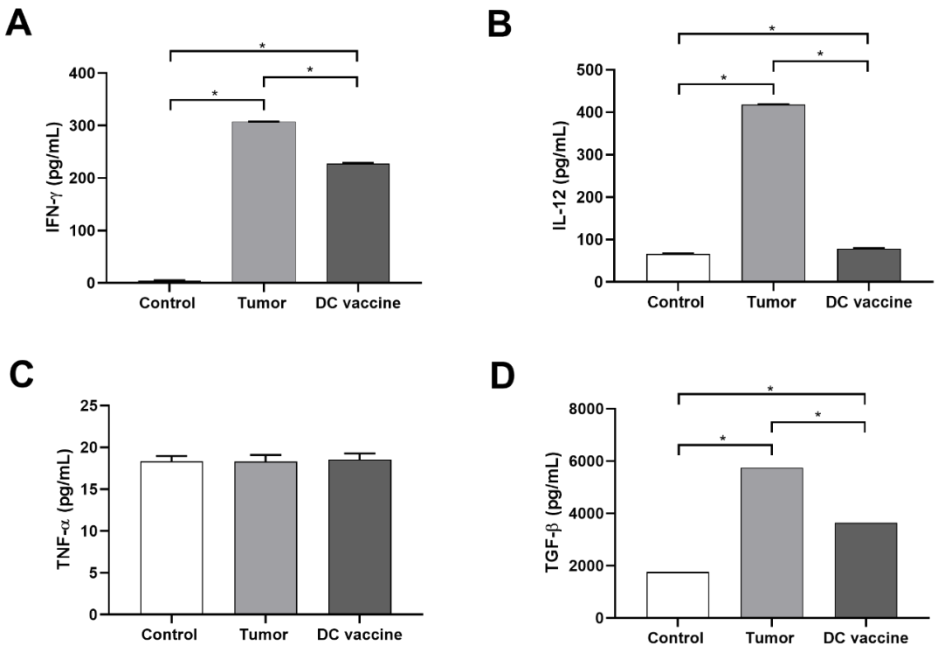
incluídos. Mas de início, abrimos as discussões do papel da imunoterapia profilática na redução de metástases pulmonares e aumento na porcentagem de linfócitos T no baço.

Conclusão

A profilaxia com vacina de células dendríticas promoveu redução dos focos e áreas metastáticas pulmonares. Promoveu ainda aumento na ativação de linfócitos T (totais, auxiliares e citotóxicas), células estas indicativas, em alguns contextos, da promoção e eficiência da resposta imune antitumoral. Isso pode fornecer uma discussão oportuna quanto à ativação de vigilância imunológica antitumoral na ausência de carga tumoral e sua manutenção.

5.4 Síntese de citocinas por células leucocitárias do baço (ELISA)

Foi avaliado por ELISA, IFN-γ, IL-12, TNF-α, TGF-β, IL-4, IL-10 e IL-2 no sobrenadante de culturas de células leucocitárias do baço dos animais dos grupos estudados após 24h de incubação (Figura 8). O grupo DC vaccine exibiu redução significativa dos níveis de IFN-γ, IL-12, TGF-β e IL-4 em relação ao grupo Tumor, porém aumentados em relação ao Controle ($p<0.0001$). Os níveis de TNF-α não exibiram alterações significativas; e os níveis de IL-10 e IL-2 exibiram aumento significativo em DC vaccine versus Tumor ($p<0.0001$).



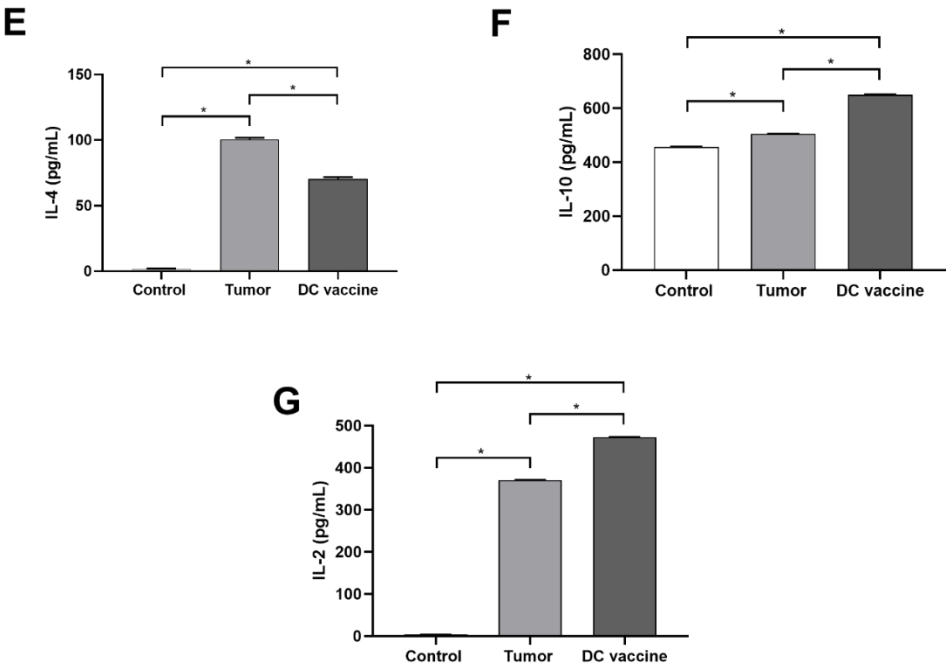


Figura 7. Representação da síntese de citocinas no sobrenadante (perfil de citocinas) de cultura de células leucocitárias do baço. Os níveis de (A) IFN-γ, (B) IL-12, (C) TNF-α, (D) TGF-β, (E) IL-4, (F) IL-10 e (G) IL-2 no sobrenadante de leucócitos esplênicos dos diferentes grupos determinado por ELISA. Os resultados foram analisados por One-way ANOVA com Tukey´s multiple comparisons *test* (representados por média±SD. Concentrações das citocinas em pg/mL. As diferenças foram consideradas significantes quando $p<0.05$; *$p<0.0001$.

Porém, as citocinas apresentam diferenças cinéticas, em resposta a alguns estímulos, que podem ser causadas por funções cruzadas de mecanismos. Considerando isso, avaliamos ainda a cinética da produção de citocinas em 12h, 24h e 36h no grupo DC vaccine em resposta ao LPS. O IFN-γ e a IL-10 exibiram síntese máxima em 12h, TNF-α, IL-4 e TGF-β exibiram síntese máxima em 24h. A IL-12 e IL-2 exibiram síntese máxima em 36h (Figura 9). Isso pode estar relacionado com a baixa em algumas citocinas vistas no gráfico anterior.

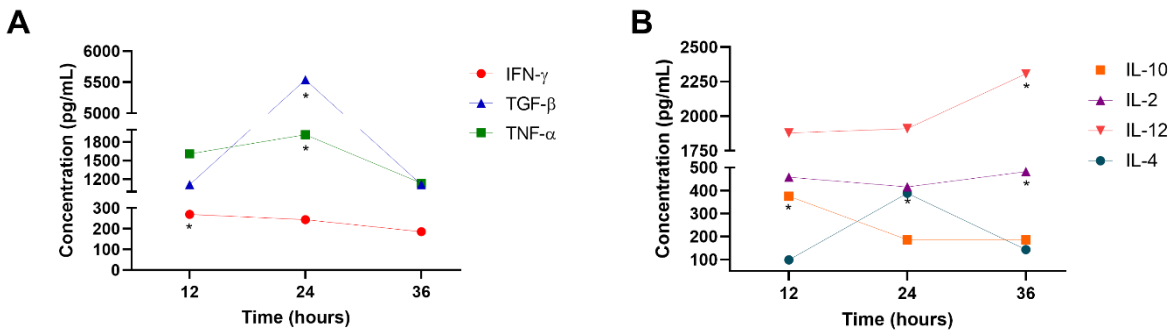


Figura 8. Concentração de citocinas no sobrenadante de leucócitos totais esplênicos, dentro de 12h, 24h e 36h a partir da estimulação com LPS. Os níveis de (A) IFN-γ, TGF-β e TNF-α, (B) IL-12, IL-4, IL-10 e IL-2 no sobrenadante de leucócitos esplênicos do grupo DC

vaccine determinado por ELISA. Os resultados foram analisados por One-way ANOVA com Tukey´s multiple comparisons test e foram representados por média±SD. Concentrações das citocinas em pg/mL. As diferenças foram consideradas significantes quando $p<0.05$; $*p<0.0001$.

6. CONSIDERAÇÕES FINAIS

Nos últimos anos, o desenvolvimento de vacinas antitumorais tem representado significativos pontos na história da imunologia antitumoral. Os avanços na compreensão da imunobiologia de células dendríticas, e de outros componentes do sistema imune, engajaram o desenvolvimento de novas estratégias para melhorar a manipulação desse processo fisiopatológico (câncer).

As células dendríticas são um ponto chave na conexão das atividades do sistema imune, o que a torna uma ferramenta de potencialização das respostas imunes antitumorais, como a melhoria dos processos de imunomodulação. Os recursos fornecidos por essas células, a capacidade de apresentação de antígenos tumorais, ativação de linfócitos Th1, T CTL e NK foram primordiais para seu uso como estratégia imunoterapêutica contra tumores. Nesse contexto, as células dendríticas são diferenciadas e carregadas com antígenos tumorais autólogos, o que permite e favorece uma resposta imune direcionada e efetiva. Assim, as imunoterapias com células dendríticas emergem como um processo terapêutico promissor. Porém, avanços nessa área do conhecimento, como os mecanismos moleculares e todo o cerne que envolve a sua imunobiologia, se faz necessário.

Vários estudos clínicos têm sido desenvolvidos nesses âmbitos, considerando as células dendríticas como uma boa estratégia terapêutica (ANGUILLE et al., 2014; SALGALLER et al., 1997). Os resultados têm sido promissores, demonstrando segurança, viabilidade na indução das respostas imunes. Inclusive já foi aprovada pela FDA, a *Sipuleucel-T*, uma imunoterapia personalizada para câncer de próstata (ANASSI; NDEFO, 2011). Assim como a forma profilática apresentada nesse trabalho, *Sipuleucel-T* é uma vacina personalizada que engloba as células dendríticas que expressam o antígeno chave (fosfatase ácida prostática), maturadas *ex vivo*.

Mesmo com os resultados promissores na clínica, a eficácia na vacina de células dendríticas ainda permanece limitada devido a perspectivas que envolvem a carga antigênica que as células dendríticas apresentam e aos ensaios, que habitualmente são realizados em pacientes com câncer em fase terminal, situação em que o sistema imune encontra-se debilitado ao extremo e considerável imunossupressão. Além do mais, outras características que incidem sobre a eficácia podem ser levantadas, como o que tange a momentos de administração, vias de administração, tipos de tumores, se usado como adjuvante, neoadjuvante e até mesmo em uma perspectiva profilática, o que tem apresentado resultados poucos conclusivos ainda. Portanto, o

desenvolvimento de uma vacina profilática como forma preventiva de tratamento seria de grande importância.

Este trabalho apresenta algumas limitações. O número de animais foi pequeno por grupo, apesar de ser suficiente para as análises estatísticas e estar pautado no princípio dos 3Rs (Declaração da Basileia). Em estudos futuros, grupos experimentais com maior número de animais deverá ser considerado. Análises de citotoxidade *in vitro* podem ser incluídas para rastrear a atividade de citocinas secretadas pelos linfócitos T CTL e averiguar a capacidade de indução de morte celular.

Futuramente, a inclusão de outros marcadores das respostas imunes; outras citocinas, como a IL-12, deverão ser consideradas; marcadores de memória para as células T e outras células da resposta imune antitumoral, como NK e linfócitos B também. Além do mais, deve-se levar em consideração a inclusão de novos protocolos de vacinação de DCs, assim como, diferentes vias de vacinação, analisando se isso pode alterar o padrão de resposta imune.

Assim, o contexto de uma vacina profilática, conforme foi apresentado nesse estudo, é pensado na condição de pessoas com alguma predisposição genética ao câncer (com algum risco alto de desenvolvimento de neoplasia maligna) ou pessoas sem uma situação de tumor existente, com alta capacidade de desenvolvimento metastático. Ou ainda, na recorrência e prevenção de recidiva, considerando uma profilaxia pré e pós exposição, que é uma perspectiva ainda diferente do tratamento em si.

É necessária e essencial uma avaliação mais ampla, para melhorar as estratégias, pensando até mesmo na combinação de imunoterapias com radioterapia, quimioterapia e até outras formas de imunoterapia e terapias angiogênicas. Afinal, os avanços da imunoterapia de tumores são promissores e tem um vasto campo a ser analisado.

7. CONCLUSÕES

Este trabalho demonstrou eficácia da imunoterapia profilática com células dendríticas (situação de pré-exposição) sobre o crescimento tumoral no modelo estudado, levando a uma diminuição dos seus volumes. Demonstrou ainda melhora da resposta imune antitumoral ao promover um aumento na ativação de células da resposta imune antitumoral (Th1 e T CTL), o que pode fornecer uma ativação oportuna de vigilância imunológica antitumoral na ausência de carga tumoral.

No fígado, a imunoterapia profilática com células dendríticas reduziu os focos de metástases, o mesmo sendo observado no pulmão. Alterou o fenótipo da resposta imune no órgão, demonstrando que o tecido hepático funciona como um importante regulador da imunidade sistêmica, seja apenas na presença de um processo patológico ou na intervenção preventiva a esse processo. Foi observado aumento na porcentagem de linfócitos T totais, T CTL, porém sem alterações significativas em Th. As células Treg aumentaram no grupo vacinado, assim como a produção de IL-10 e IL-17 simultaneamente, demonstrando que a vacina pode induzir a um estado de controle das respostas pró-inflamatórias, o que pode favorecer um ambiente menos favorável para instalação metastática. Os demais subtipos de Th (Th1, Th2 e Th17) estavam reduzidos, assim como de a expressão de IL-12, IFN-γ e TNF-α em Th.

Em conjunto esses dados revelam que o fígado subsidia processos funcionais distintos em relação a resposta preventiva mediada pela vacina, atuando como sentinela, o que pode levar a uma interferência dos processos metastáticos, os evitando. Pesquisas adicionais são necessárias para abordar os fenótipos e as suas relações.

Evidenciamos ainda que no baço, a imunoterapia profilática com células dendríticas aumentou a porcentagem de linfócitos T totais, Th e T CTL, e que nos ensaios de citocinas, IL-10 e IL-2 foram as únicas citocinas com picos aumentados, após 24h. Isso está relacionado com os diferentes tempos de pico de secreção dessas citocinas, como demonstrado no ensaio *in vitro* com LPS.

Assim, foi observado que a resposta imune após imunoterapia profilática com células dendríticas ocorreu de maneiras distintas a depender dos nichos. Isso nos dá indicativos de que cada um dos nichos possui mecanismos peculiares na indução e manutenção das respostas imunes antitumorais, e demonstra que a vacina altera as respostas presentes nesses meios, favorecendo a resposta imune antitumoral. Investigações futuras são necessárias para a maior compreensão desses processos.

REFERÊNCIAS

ADAM, R. et al. Is liver resection justified for patients with hepatic metastases from breast cancer? **Annals of Surgery**, 2006.

AHRENDS, T. et al. CD4+ T cell help creates memory CD8+ T cells with innate and help-independent recall capacities. **Nature Communications**, v. 10, n. 1, 1 dez. 2019.

ALEIXO, A.; MICHELIN, M.; MURTA, E. Dendritic Cell Vaccine and Cancer Treatment: New Patents. **Recent Patents on Endocrine, Metabolic & Immune Drug Discovery**, 2014.

ALMAND, B. et al. Clinical significance of defective dendritic cell differentiation in cancer. **Clinical Cancer Research**, 2000.

ALTSHULER, D. L. et al. A map of human genome variation from population-scale sequencing. **Nature**, 2010.

ALY, H. A. A. Cancer therapy and vaccination. **Journal of Immunological Methods**, v. 382, n. 1–2, p. 1–23, ago. 2012.

ANASSI, E.; NDEFO, U. A. Sipuleucel-T (provenge) injection: the first immunotherapy agent (vaccine) for hormone-refractory prostate cancer. **P & T : a peer-reviewed journal for formulary management**, v. 36, n. 4, p. 197–202, abr. 2011.

ANGUILLE, S. et al. Clinical use of dendritic cells for cancer therapy. **The Lancet Oncology**, v. 15, n. 7, p. e257–e267, jun. 2014.

ASLAKSON, C. J.; MILLER, F. R. Selective Events in the Metastatic Process Defined by Analysis of the Sequential Dissemination of Subpopulations of a Mouse Mammary Tumor. **Cancer Research**, 1992.

BALKWILL, F. The Inflammatory Tissue Microenvironment and the Early Stages of Malignancy. In: **Pre-Invasive Disease: Pathogenesis and Clinical Management**. New York, NY: Springer New York, 2011. p. 21–29.

BALKWILL, F. R. The chemokine system and cancer. **The Journal of Pathology**, v. 226, n. 2, p. 148–157, jan. 2012.

BALKWILL, F. R.; MANTOVANI, A. Cancer-related inflammation: Common themes and therapeutic opportunities. **Seminars in Cancer Biology**, v. 22, n. 1, p. 33–40, fev. 2012.

BANCHEREAU, J.; STEINMAN, R. M. Dendritic cells and the control of immunity. **Nature**, v. 392, n. 6673, p. 245–252, mar. 1998.

BAUER, C. et al. Dendritic cell-based vaccination of patients with advanced pancreatic carcinoma: results of a pilot study. **Cancer Immunology, Immunotherapy**, v. 60, n. 8, p. 1097–1107, 6 ago. 2011.

BERG, M. et al. Cross-presentation of antigens from apoptotic tumor cells by liver sinusoidal endothelial cells leads to tumor-specific CD8+ T cell tolerance. **European Journal of Immunology**, 2006.

BERTOLINO, P.; MCCAUGHAN, G. W.; BOWEN, D. G. Role of primary intrahepatic T-cell activation in the “liver tolerance effect”. **Immunology and cell biology**, v. 80, n. 1, p. 84–92, fev. 2002.

BOGDANOS, D. P.; GAO, B.; GERSHWIN, M. E. Liver immunology. **Comprehensive Physiology**, 2013.

BOUDREAU, J. E. et al. **Engineering dendritic cells to enhance cancer immunotherapyMolecular Therapy**, 2011.

BOYLE, P. Triple-negative breast cancer: Epidemiological considerations and recommendations. **Annals of Oncology**, 2012.

BURKE, J. D.; YOUNG, H. A. **IFN-Γ: A cytokine at the right time, is in the right placeSeminars in Immunology**Academic Press, , 1 jun. 2019. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/31221552/>. Acesso em: 21 jun. 2021

BURNET, F. M. **The concept of immunological surveillance.Progress in experimental tumor research. Fortschritte der experimentellen Tumorforschung. Progres de la recherche experimentale des tumeurs**, 1970.

CALMEIRO, J. et al. **Dendritic cell vaccines for cancer immunotherapy: The role of human conventional type 1 dendritic cellsPharmaceutics**MDPI AG, , 1 fev. 2020. Disponível em: </pmc/articles/PMC7076373/>. Acesso em: 30 abr. 2021

CHANG, M. R. et al. Synthetic RORγt Agonists Enhance Protective Immunity. **ACS Chemical Biology**, v. 11, n. 4, p. 1012–1018, 15 abr. 2016.

CHAUDHRY, S.; EMOND, J.; GRIESEMER, A. **Immune cell trafficking to the liverTransplantation**, 2019.

CHEMIN, K.; GERSTNER, C.; MALMSTRÖM, V. Effector Functions of CD4+ T Cells at the Site of Local Autoimmune Inflammation—Lessons From Rheumatoid Arthritis. **Frontiers in Immunology**, v. 10, 12 mar. 2019.

CHEUNG, K. J.; EWALD, A. J. **A collective route to metastasis: Seeding by tumor cell clustersScience**, 2016.

CLARK, A. M. et al. Liver metastases: Microenvironments and ex-vivo models. **Experimental Biology and Medicine**, 2016.

CORTHAY, A. et al. Primary Antitumor Immune Response Mediated by CD4+ T Cells. **Immunity**, v. 22, n. 3, p. 371–383, mar. 2005.

CRISPE, I. N. **Immune tolerance in liver diseaseHepatology**, 2014.

CRUVINEL, W. DE M. et al. Immune system - part I. Fundamentals of innate immunity with emphasis on molecular and cellular mechanisms of inflammatory response. **Revista brasileira de reumatologia**, v. 50, n. 4, p. 434–61, 2012.

CUNHA, L. L. et al. RORγt may Influence the Microenvironment of Thyroid Cancer

Predicting Favorable Prognosis. **Scientific Reports**, v. 10, n. 1, p. 4142, 5 dez. 2020.

DA CUNHA, A.; ANTONIAZI MICHELIN, M.; CÂNDIDO MURTA, E. F. Phenotypic profile of dendritic and T cells in the lymph node of Balb/C mice with breast cancer submitted to dendritic cells immunotherapy. **Immunology Letters**, 2016.

DHABEKAR, G.; DANDEKAR, R.; KINGAONKAR, A. Role of macrophages in malignancy. **Annals of Maxillofacial Surgery**, 2011.

DIEHL, L. et al. Tolerogenic maturation of liver sinusoidal endothelial cells promotes B7-homolog 1-dependent CD8+ T cell tolerance. **Hepatology**, 2008.

DOUGAN, M.; DRANOFF, G. **Immune therapy for cancerAnnual Review of Immunology**, 2009.

DRAKE, C. G. Update on Prostate Cancer Vaccines. **The Cancer Journal**, v. 17, n. 5, p. 294–299, set. 2011.

DUPRÉ, S. A.; REDELMAN, D.; HUNTER, K. W. The mouse mammary carcinoma 4T1: Characterization of the cellular landscape of primary tumours and metastatic tumour foci. **International Journal of Experimental Pathology**, 2007.

DURAND, F.; FRANCOZ, C. The future of liver transplantation for viral hepatitis. **Liver international : official journal of the International Association for the Study of the Liver**, v. 37 Suppl 1, p. 130–135, 1 jan. 2017.

DURGEAU, A. et al. **Recent advances in targeting CD8 T-cell immunity for more effective cancer immunotherapyFrontiers in Immunology**Frontiers Media S.A., , 22 jan. 2018. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/29403496/>. Acesso em: 21 jun. 2021

EGBERTS, J. H. et al. Anti-tumor necrosis factor therapy inhibits pancreatic tumor growth and metastasis. **Cancer Research**, 2008.

FARES, J. et al. **Molecular principles of metastasis: a hallmark of cancer revisitedSignal Transduction and Targeted Therapy**, 2020.

FARHOOD, B.; NAJAFI, M.; MORTEZAEE, K. **CD8+ cytotoxic T lymphocytes in cancer immunotherapy: A reviewJournal of Cellular Physiology**Wiley-Liss Inc., , 1 jun. 2019. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/30520029/>. Acesso em: 21 jun. 2021

FONG, L. et al. Dendritic Cells Injected Via Different Routes Induce Immunity in Cancer Patients. **The Journal of Immunology**, v. 166, n. 6, p. 4254–4259, 15 mar. 2001.

FONSECA, C.; DRANOFF, G. Capitalizing on the Immunogenicity of Dying Tumor Cells. **Clinical Cancer Research**, v. 14, n. 6, p. 1603–1608, 15 mar. 2008.

FU, C.; JIANG, A. **Dendritic Cells and CD8 T Cell Immunity in Tumor MicroenvironmentFrontiers in immunology**NLM (Medline), , 2018. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/30619378/>. Acesso em: 21 jun. 2021

GARCÍA PAZ, F. et al. The relationship between the antitumor effect of the IL-12 gene therapy and the expression of th1 cytokines in an HPV16-positive murine tumor model. **Mediators of Inflammation**, 2014.

GARDNER, A.; RUFFELL, B. Dendritic Cells and Cancer Immunity. **Trends in Immunology**, v. 37, n. 12, p. 855–865, dez. 2016.

GEIGER, T. R.; PEEPER, D. S. **Metastasis mechanismsBiochimica et Biophysica Acta - Reviews on Cancer**, 2009.

GRAHAM, T. A.; SOTTORIVA, A. **Measuring cancer evolution from the genomeJournal of Pathology**, 2017.

GROSS, K. et al. **Cell fate decisions during breast cancer developmentJournal of Developmental Biology**, 2016.

GUN, S. Y. et al. **Targeting immune cells for cancer therapyRedox Biology**, 2019.

HANAHAN, D.; WEINBERG, R. A. **Hallmarks of cancer: The next generationCell**, 2011.

HE, Z. Y. et al. Up-regulation of RFC3 promotes triple negative breast cancer metastasis and is associated with poor prognosis via EMT. **Translational Oncology**, 2017.

HINES, I. N.; SON, G.; KREMER, M. **Contribution of gut bacteria to liver pathobiologyGastroenterology Research and Practice**, 2010.

HONG, X. et al. Synergistical toll-like receptors activated dendritic cells induce antitumor effects against carcinoembryonic antigen-expressing colon cancer. **International Journal of Colorectal Disease**, v. 28, n. 1, p. 25–33, 10 jan. 2013.

HUDIS, C. A.; GIANNI, L. Triple-Negative Breast Cancer: An Unmet Medical Need. **The Oncologist**, 2011.

HUNTER, K. W.; CRAWFORD, N. P. S.; ALSARRAJ, J. **Mechanisms of metastasisBreast Cancer Research**, 2008.

INCA. Estimativa 2020 : incidência de câncer no Brasil / Instituto Nacional de Câncer José Alencar Gomes da Silva. **Instituto Nacional de Câncer José Alencar Gomes da Silva.**, 2019.

INSTITUTO NACIONAL DO CÂNCER. **Estimativa Incidência de Câncer no Brasil - Biênio 2018-2019**. [s.l: s.n.].

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER (IARC). GLOBOCAN 2018: Latest global cancer data. **CA: A Cancer Journal for Clinicians**, 2018.

IVANOVA, E. A.; OREKHOV, A. N. **T Helper lymphocyte subsets and plasticity in autoimmunity and cancer: An overviewBioMed Research International**, 2015.

JAESCHKE, H. et al. Mechanisms of hepatotoxicity. **Toxicological Sciences**, 2002.

JOMANTAITE, I. et al. Hepatic dendritic cell subsets in the mouse. **European Journal of**

Immunology, 2004.

JORGOVANOVIC, D. et al. **Roles of IFN-γin tumor progression and regression: A reviewBiomarker Research**BioMed Central Ltd, , 29 set. 2020. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/33005420/>. Acesso em: 21 jun. 2021

KACHLER, K. et al. The role of Foxp3 and Tbet co-expressing Treg cells in lung carcinoma. **OncoImmunology**, 2018.

KANDALAFT, L. E. et al. Autologous lysate-pulsed dendritic cell vaccination followed by adoptive transfer of vaccine-primed ex vivo co-stimulated T cells in recurrent ovarian cancer. **OncoImmunology**, v. 2, n. 1, p. e22664, 27 jan. 2013.

KANTOFF, P. W. et al. Sipuleucel-T Immunotherapy for Castration-Resistant Prostate Cancer. **New England Journal of Medicine**, v. 363, n. 5, p. 411–422, 29 jul. 2010.

KAPLAN, R. N.; PSAILA, B.; LYDEN, D. **Bone marrow cells in the "pre-metastatic niche": Within bone and beyondCancer and Metastasis Reviews**, 2006.

KELLY, A. M. et al. Changes in hepatic immunoregulatory cytokines in patients with metastatic colorectal carcinoma: Implications for hepatic anti-tumour immunity. **Cytokine**, 2006.

KIDD, P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. **Alternative medicine review : a journal of clinical therapeutic**, v. 8, n. 3, p. 223–46, ago. 2003.

KIM, E. J. et al. Dietary fat increases solid tumor growth and metastasis of 4T1 murine mammary carcinoma cells and mortality in obesity-resistant BALB/c mice. **Breast Cancer Research**, 2011.

KOIDO, S. et al. Induction of Antitumor Immunity by Vaccination of Dendritic Cells Transfected with MUC1 RNA. **The Journal of Immunology**, v. 165, n. 10, p. 5713–5719, 15 nov. 2000.

KORKAYA, H.; LIU, S.; WICHA, M. S. Breast cancer stem cells, cytokine networks, and the tumor microenvironment. **The Journal of clinical investigation**, v. 121, n. 10, p. 3804–9, 3 out. 2011.

KRETSCHMANN, K. L.; WELM, A. L. Mouse models of breast cancer metastasis to bone. **Cancer and Metastasis Reviews**, 2012.

KUBES, P.; JENNE, C. Immune Responses in the Liver. **Annual Review of Immunology**, 2018.

KUMAR, V.; ASTER, J.; ABBAS, A. Robbins & Cotran Patologia - Bases Patológicas das Doenças. In: **Elsevier Brasil**. [s.l: s.n.].

KUSHWAH, R.; HU, J. **Complexity of dendritic cell subsets and their function in the host immune systemImmunology**, 2011.

LANZAVECCHIA, A.; SALLUSTO, F. **Understanding the generation and function of memory T cell subsetsCurrent Opinion in Immunology**, 2005.

LAZAREVIC, V.; GLIMCHER, L. H.; LORD, G. M. T-bet: a bridge between innate and adaptive immunity. **Nature Reviews Immunology**, v. 13, n. 11, p. 777–789, 11 nov. 2013.

LEE, H. L. et al. Inflammatory cytokines and change of Th1/Th2 balance as prognostic indicators for hepatocellular carcinoma in patients treated with transarterial chemoembolization. **Scientific Reports**, v. 9, n. 1, p. 3260, 1 dez. 2019.

LELEKAKIS, M. et al. A novel orthotopic model of breast cancer metastasis to bone. **Clinical and Experimental Metastasis**, 1999.

LEÓN, B.; LÓPEZ-BRAVO, M.; ARDAVÍN, C. Monocyte-derived dendritic cells. **Seminars in Immunology**, 2005.

LEWIS, C. E.; POLLARD, J. W. **Distinct role of macrophages in different tumor microenvironmentsCancer Research**, 2006.

LIANG, Y. et al. **Metastatic heterogeneity of breast cancer: Molecular mechanism and potential therapeutic targetsSeminars in Cancer Biology**, 2020.

LIN, J. H. et al. Type 1 conventional dendritic cells are systemically dysregulated early in pancreatic carcinogenesis. **Journal of Experimental Medicine**, v. 217, n. 8, 3 ago. 2020.

LIN, Z.-W. et al. The Expression Levels of Transcription Factors T-bet, GATA-3, RORγt and FOXP3 in Peripheral Blood Lymphocyte (PBL) of Patients with Liver Cancer and their Significance. **International Journal of Medical Sciences**, v. 12, n. 1, p. 7–16, 2015.

LIU, M. et al. **A new perspective: Exploring future therapeutic strategies for cancer by understanding the dual role of B lymphocytes in tumor immunityInternational Journal of Cancer**, 2019.

LIUDAHL, S. M.; COUSSENS, L. M. To Help or To Harm: Dynamic Roles of CD4+ T Helper Cells in Solid Tumor Microenvironments. In: **Immunology: Immunotoxicology, Immunopathology, and Immunotherapy**. [s.l: s.n.].

LIZOTTE, P. H. et al. In situ vaccination with cowpea mosaic virus nanoparticles suppresses metastatic cancer. **Nature Nanotechnology**, 2016.

LOKHOV, P. G.; BALASHOVA, E. E. Cellular Cancer Vaccines: an Update on the Development of Vaccines Generated from Cell Surface Antigens. **Journal of Cancer**, p. 230–241, 2010.

LOOSE, D.; VAN DE WIELE, C. **The immune system and cancerCancer Biotherapy and Radiopharmaceuticals**, 2009.

LOPES, A. M. M.; MICHELIN, M. A.; MURTA, E. F. C. Monocyte-derived dendritic cells from patients with cervical intraepithelial lesions. **Oncology Letters**, 2017.

LURJE, I.; HAMMERICH, L.; TACKE, F. **Dendritic cell and T cell crosstalk in liver fibrogenesis and hepatocarcinogenesis: Implications for prevention and therapy of liver cancerInternational Journal of Molecular Sciences**MDPI AG, , 1 out. 2020. Disponível

em: <https://pubmed.ncbi.nlm.nih.gov/33036244/>. Acesso em: 21 jun. 2021

MACCALLI, C.; PARMIANI, G.; FERRONE, S. Immunomodulating and Immunoresistance Properties of Cancer-Initiating Cells: Implications for the Clinical Success of Immunotherapy. **Immunological Investigations**, v. 46, n. 3, p. 221–238, 3 abr. 2017.

MAHMOUD, S. et al. Cd8+ T lymphocytes infiltrating breast cancer a promising new prognostic marker? **OncoImmunology**, 2012.

MAHMOUD, S. M. A. et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. **Journal of Clinical Oncology**, 2011.

MARKOV, O. V. et al. Prophylactic Dendritic Cell-Based Vaccines Efficiently Inhibit Metastases in Murine Metastatic Melanoma. **PLOS ONE**, v. 10, n. 9, p. e0136911, 1 set. 2015.

MARTÍNEZ-LOSTAO, L.; ANEL, A.; PARDO, J. How Do Cytotoxic Lymphocytes Kill Cancer Cells? **Clinical Cancer Research**, 2015.

MATHEOUD, D. et al. Cross-presentation by dendritic cells from live cells induces protective immune responses in vivo. **Blood**, v. 115, n. 22, p. 4412–4420, 3 jun. 2010.

MATHIS, D.; BENOIST, C. Back to Central Tolerance. **Immunity**, v. 20, n. 5, p. 509–516, maio 2004.

MATIAS, B. F. et al. Influence of Immunotherapy with Autologous Dendritic Cells on Innate and Adaptive Immune Response in Cancer. **Clinical Medicine Insights: Oncology**, v. 7, p. CMO.S12268, 28 jan. 2013.

MERCOGLIANO, M. F. et al. **Tumor Necrosis Factor α Blockade: An Opportunity to Tackle Breast CancerFrontiers in Oncology**, 2020.

MILDNER, A.; JUNG, S. Development and Function of Dendritic Cell Subsets. **Immunity**, v. 40, n. 5, p. 642–656, maio 2014.

MILLER, F. R.; MILLER, B. E.; HEPPNER, G. H. Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: Heterogeneity in phenotypic stability. **Invasion and Metastasis**, v. 3, n. 1, p. 22–31, 1 jan. 1983.

MIRZA, A. H. et al. **Importance of the immune system in head and neck cancerHead and Neck**, 2019.

MITCHELL, D.; CHINTALA, S.; DEY, M. **Plasmacytoid dendritic cell in immunity and cancerJournal of Neuroimmunology**, 2018.

NIELSEN, S. R.; SCHMID, M. C. **Macrophages as Key Drivers of Cancer Progression and MetastasisMediators of Inflammation**, 2017.

NOACK, M.; MIOSSEC, P. **Th17 and regulatory T cell balance in autoimmune and inflammatory diseasesAutoimmunity Reviews**, 2014.

O'NEILL, L. A. J.; PEARCE, E. J. Immunometabolism governs dendritic cell and macrophage function. **Journal of Experimental Medicine**, v. 213, n. 1, p. 15–23, 11 jan. 2016.

ODA, Y. et al. Prophylactic immunization with Bubble liposomes and ultrasound-treated dendritic cells provided a four-fold decrease in the frequency of melanoma lung metastasis. **Journal of Controlled Release**, v. 160, n. 2, p. 362–366, jun. 2012.

OSTUNI, R. et al. **Macrophages and cancer: From mechanisms to therapeutic implicationsTrends in Immunology**, 2015.

OTT, E. et al. The density of Tbet+ tumor-infiltrating T lymphocytes reflects an effective and druggable preexisting adaptive antitumor immune response in colorectal cancer, irrespective of the microsatellite status. **OncoImmunology**, 2019.

PALUCKA, K. et al. Dendritic cells and immunity against cancer. **Journal of Internal Medicine**, v. 269, n. 1, p. 64–73, jan. 2011.

PALUCKA, K.; BANCHEREAU, J. Cancer immunotherapy via dendritic cells. **Nature Reviews Cancer**, v. 12, n. 4, p. 265–277, 22 abr. 2012.

PALUCKA, K.; BANCHEREAU, J. Dendritic-Cell-Based Therapeutic Cancer Vaccines. **Immunity**, v. 39, n. 1, p. 38–48, jul. 2013.

PARDOLL, D. M.; TOPALIAN, S. L. The role of CD4+ T cell responses in antitumor immunity. **Current Opinion in Immunology**, v. 10, n. 5, p. 588–594, out. 1998.

PENTHEROUDAKIS, G. et al. Metastatic breast cancer with liver metastases: A registry analysis of clinicopathologic, management and outcome characteristics of 500 women. **Breast Cancer Research and Treatment**, 2006.

PEREZ, C. R.; DE PALMA, M. **Engineering dendritic cell vaccines to improve cancer immunotherapyNature Communications**, 2019.

PHUPHANICH, S. et al. Phase I trial of a multi-epitope-pulsed dendritic cell vaccine for patients with newly diagnosed glioblastoma. **Cancer Immunology, Immunotherapy**, v. 62, n. 1, p. 125–135, 31 jan. 2013.

PILLARISETTY, V. G. et al. Liver Dendritic Cells Are Less Immunogenic Than Spleen Dendritic Cells because of Differences in Subtype Composition. **The Journal of Immunology**, 2004.

PROTZER, U.; MAINI, M. K.; KNOLLE, P. A. Living in the liver: hepatic infections. **Nature reviews. Immunology**, v. 12, n. 3, p. 201–13, 24 fev. 2012.

PULASKI, B. A.; OSTRAND-ROSENBERG, S. Mouse 4T1 Breast Tumor Model. **Current Protocols in Immunology**, 2000.

RAPHAEL, I. et al. **T cell subsets and their signature cytokines in autoimmune and inflammatory diseasesCytokine**, 2015.

REIS, E. S. et al. **Complement in cancer: Untangling an intricate relationshipNature**

Reviews Immunology, 2018.

RIIHIMÄKI, M. et al. Comparison of survival of patients with metastases from known versus unknown primaries: Survival in metastatic cancer. **BMC Cancer**, v. 13, p. 36, 28 jan. 2013.

ROBINSON, M. W.; HARMON, C.; O'FARRELLY, C. **Liver immunology and its role in inflammation and homeostasisCellular and Molecular Immunology**, 2016.

RODRIGUES, C. M. et al. The Role of T Lymphocytes in Cancer Patients Undergoing Immunotherapy with Autologous Dendritic Cells. **Clinical Medicine Insights: Oncology**, v. 5, p. CMO.S6927, 25 jan. 2011.

ROLAND, C. L. et al. Inhibition of vascular endothelial growth factor reduces angiogenesis and modulates immune cell infiltration of orthotopic breast cancer xenografts. **Molecular Cancer Therapeutics**, v. 8, n. 7, p. 1761–1771, 1 jul. 2009.

SABADO, R. L.; BALAN, S.; BHARDWAJ, N. **Dendritic cell-based immunotherapyCell Research**, 2017.

SAITO, T. et al. Two FOXP3 + CD4 + T cell subpopulations distinctly control the prognosis of colorectal cancers. **Nature Medicine**, 2016.

SAKAGUCHI, S. et al. **FOXP3 + regulatory T cells in the human immune systemNature Reviews Immunology**, 2010.

SALGALLER, M. L. et al. **Dendritic cell-based immunotherapy of prostate cancer**. Critical Reviews in Immunology. **Anais**...1997

SALLUSTO, F.; GEGINAT, J.; LANZAVECCHIA, A. C entral M emory and E ffector M emory T C ell S ubsets : Function, Generation, and Maintenance . **Annual Review of Immunology**, 2004.

SALLUSTO, F.; LANZAVECCHI, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor

plus iuterleukin 4 and downregulated by tumor necrosis factor α. **Journal of Experimental Medicine**, 1994.

SANA, G. et al. Adult human hepatocytes promote CD4+ T-cell hyporesponsiveness via interleukin-10-producing allogeneic dendritic cells. **Cell Transplantation**, 2014.

SANTANA-DAVILA, R.; PEREZ, E. A. **Treatment options for patients with triple-negative breast cancerJournal of Hematology and Oncology**, 2010.

SELEDTSOV, V.; GONCHAROV, A.; SELEDTSOVA, G. Clinically feasible approaches to potentiating cancer cell-based immunotherapies. **Human Vaccines & Immunotherapeutics**, v. 11, n. 4, p. 851–869, 3 abr. 2015.

SENGUPTA, N. et al. **Cancer immunoediting and "spontaneous" tumor regressionPathology Research and Practice**, 2010.

SHANGGUAN, A. et al. Prophylactic dendritic cell vaccination controls pancreatic cancer growth in a mouse model. **Cytotherapy**, v. 22, n. 1, p. 6–15, jan. 2020.

SHEVCHENKO, J. A. et al. Autologous dendritic cells and activated cytotoxic T-cells as combination therapy for breast cancer. **Oncology Reports**, 2020.

SIMON, T.; FONTENEAU, J.-F.; GRÉGOIRE, M. Dendritic cell preparation for immunotherapeutic interventions. **Immunotherapy**, v. 1, n. 2, p. 289–302, mar. 2009.

SLEEMAN, J. P. The metastatic niche and stromal progression. **Cancer and Metastasis Reviews**, 2012.

SLEEMAN, J.; STEEG, P. S. Cancer metastasis as a therapeutic target. **European Journal of Cancer**, 2010.

STANTON, S. E.; DISIS, M. L. Clinical significance of tumor-infiltrating lymphocytes in breast cancer. 2016.

TANG, J.; AHMAD, A.; SARKAR, F. H. **The role of microRNAs in breast cancer migration, invasion and metastasisInternational Journal of Molecular Sciences**MDPI AG, , 2012. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/23202960/>. Acesso em: 19 abr. 2021

TAO, K. et al. Imagable 4T1 model for the study of late stage breast cancer. **BMC Cancer**, v. 8, n. 1, p. 228, 9 dez. 2008.

TELLI, M. L. Triple-negative breast cancer. In: **Molecular Pathology of Breast Cancer**. [s.l: s.n.].

THOMPSON-SNIPES, L. et al. Interleukin 10: A novel stimulatory factor for mast cells and their progenitors. **Journal of Experimental Medicine**, 1991.

TINDEMANS, I. et al. **GATA-3 function in innate and adaptive immunityImmunity**, 2014.

TOMASICCHIO, M. et al. An autologous dendritic cell vaccine polarizes a Th-1 response which is tumoricidal to patient-derived breast cancer cells. **Cancer Immunology, Immunotherapy**, 2019.

TÖPFER, K. et al. Tumor Evasion from T Cell Surveillance. **Journal of Biomedicine and Biotechnology**, v. 2011, p. 1–19, 2011.

TORREZINI, T.; ATHANAZIO, D. A. Imunovigilância e Imunoedição de Neoplasias: Implicações Clínicas e Potencial Terapêutico. **Revista Brasileira de Cancerologia**, 2008.

TOSOLINI, M. et al. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, Th2, Treg, Th17) in patients with colorectal cancer. **Cancer Research**, 2011.

TSUJI, T.; IBARAGI, S.; HU, G. F. **Epithelial-mesenchymal transition and cell cooperativity in metastasisCancer Research**, 2009.

TSUJI, W.; PLOCK, J. A. Breast Cancer Metastasis. In: **Introduction to Cancer Metastasis**.

[s.l: s.n.].

TU, Z. et al. The activation state of human intrahepatic lymphocytes. **Clinical and Experimental Immunology**, 2007.

VAHIDIAN, F. et al. **Interactions between cancer stem cells, immune system and some environmental components: Friends or foes?Immunology Letters**, 2019.

VIK-MO, E. O. et al. Therapeutic vaccination against autologous cancer stem cells with mRNA-transfected dendritic cells in patients with glioblastoma. **Cancer Immunology, Immunotherapy**, v. 62, n. 9, p. 1499–1509, 2 set. 2013.

WANG, Q.-S. et al. Interferon-gamma induces autophagy-associated apoptosis through induction of cPLA2-dependent mitochondrial ROS generation in colorectal cancer cells. **Biochemical and Biophysical Research Communications**, v. 498, n. 4, p. 1058–1065, abr. 2018.

WANG, X. et al. Th17/Treg imbalance in triptolide-induced liver injury. **Fitoterapia**, 2014.

WCULEK, S. K. et al. **Dendritic cells in cancer immunology and immunotherapyNature Reviews Immunology**Nature Research, , 1 jan. 2020. Disponível em: <https://www.nature.com/articles/s41577-019-0210-z>. Acesso em: 19 jun. 2021

WINDISCH, R. et al. **Oncogenic deregulation of cell adhesion molecules in LeukemiaCancers**, 2019.

WORLD HEALTH ORGANIZATION. WORLD HEALTH STATISTICS - MONITORING HEALTH FOR THE SDGs. **World Health Organization**, 2016.

WU, Q. et al. Breast cancer subtypes predict the preferential site of distant metastases: A SEER based study. **Oncotarget**, 2017.

YAN, H. et al. **Targeting C-type lectin receptors for cancer immunityFrontiers in Immunology**, 2015.

YAN, J. et al. Prevalence and clinical relevance of T-helper cells, Th17 and Th1, in hepatitis B virus-related hepatocellular carcinoma. **PLoS ONE**, 2014.

YONEDA, T. et al. **Actions of bisphosphonate on bone metastasis in animal models of breast carcinoma**. Cancer. **Anais**...2000

ZENG, R. et al. Positive effect of RORγt on the prognosis of thyroid papillary carcinoma patients combined with Hashimoto's thyroiditis. **American journal of translational research**, v. 10, n. 10, p. 3011–3024, 2018.

ZHANG, H.; JIANG, Z.; ZHANG, L. **Dual effect of T helper cell 17 (Th17) and regulatory T cell (Treg) in liver pathological process: From occurrence to end stage of diseaseInternational Immunopharmacology**, 2019.

ZHANG, J. P. et al. Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients. **Journal of Hepatology**, 2009.

ZONG, J. et al. Tumor-derived factors modulating dendritic cell function. **Cancer Immunology, Immunotherapy**, v. 65, n. 7, p. 821–833, 16 jul. 2016.

ANEXO 1 – CEUA (parecer técnico)

Universidade Federal do Triângulo Mineiro
Comissão de Ética no Uso de Animais
R. Conde Prados, nº 191 - Bairro Abadia Uberaba/MG CEP 38025-350
(34) 3700-6802 E-mail: ceua@uftm.edu.br

Uberaba, 13 de novembro de 2018

PARECER Nº **37/2018/CEUA/PROPPG**
PROCESSO Nº 23085.010283/2018-48
INTERESSADO: MARCIA ANTONIAZI MICHELIN

ASSUNTO: **Parecer da CEUA sobre pedido de alteração do protocolo 379**

Senhora Prof.ª Drª Márcia Antoniazi Michelin,

1. Confirmamos o recebimento do Memorando 58/2018/DMIP/ICBN (0121314) que solicita alteração no protocolo 379 - "Avaliação da resposta imunológica em camundongos com câncer de mama submetidos a imunoterapia preventiva com vacina de células dendríticas."

2. O pedido de alteração foi apreciado em reunião da CEUA realizada no dia 9/11/2018 e considerado **aprovado**. Desta forma, fica aprovada a alteração do biotério de fornecimento dos 30 camundongos fêmeas Balb/c, que passa a ser o Biotério Central da UFTM.

À consideração superior.

Aldo Rogelis Aquiles Rodrigues
Coordenador da CEUA

Documento assinado eletronicamente por **ALDO ROGELIS AQUILES RODRIGUES**, **Coordenador(a) da Comissão de Ética no Uso de Animais**, em 13/11/2018, às 15:08, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015 e no art. 14 da Resolução nº 34, de 28 de dezembro de 2017.

A autenticidade deste documento pode ser conferida no site http://sei.uftm.edu.br/sei/controlador_externo.php?acao=documento_conferir&id_orgao_acesso_externo=0, informando o código verificador **0122051** e o código CRC **A0D1451E**.

ANEXO 2 – CEUA (certificado protocolo 379)

Ministério da Educação
Universidade Federal do Triângulo Mineiro
CEUA - Comissão de Ética no Uso de Animais
Rua Madre Maria José, nº122 – Unidade Administrativa Temporária II - Bairro Abadia
CEP: 38025-100 – Uberaba - MG - Telefone: (034) 37006764 - E-mail: ceua@pesqpg.uftm.edu.br

CERTIFICADO

Certificamos que a proposta intitulada "Avaliação da resposta imunológica em camundongos com câncer de mama submetidos à imunoterapia preventiva com vacina de células dendríticas", registrada com o nº **379**, sob a responsabilidade de Márcia Antoniazi Michelin – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794 de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle e Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal do Triângulo Mineiro, em 25/07/2016.

Finalidade	( ) Ensino (x) Pesquisa Científica
Vigência da autorização	01/08/2016 à 01/08/2020
Espécie/Linhagem/Raça	Camundongos Isogênicos Balb/c
Nº de animais	55
Peso/idade	20 à 30g/ 6 à 8 semanas
Gênero	Fêmeas
Origem	Biotério Setorial do Instituto de Pesquisa em Oncologia - UFTM

Prof. Dr. Carlo José Freire de Oliveira
Coordenador da CEUA