Universidade Federal do Triângulo Mineiro

Programa de Pós-Graduação em Ciências da Saúde

Instituto de Pesquisa em Oncologia

Dinâmica da influência da imunoterapia de células dendríticas no infiltrado tumoral e em linfonodos de camundongos fêmeas de linhagem Balb/C tumor induzidos por linhagem de células 4T1

Saulo Fernando Moreira da Silva

Uberaba

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Orientação: Profa. Dra. Márcia Antoniazi Michelin

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Uberaba

2021

Catalogação na fonte: Biblioteca da Universidade Federal do Triângulo Mineiro

S583d	Silva, Saulo Fernando Moreira da Dinâmica da influência da imunoterapia de células dendríticas no infiltrado tumoral e em linfonodos de camundongos fêmeas de linhagem Balb/C tumor induzidos por linhagem de células 4T1 / Saulo Fernando Moreira da Silva. -- 2021. 85 f. : graf., tab. Tese (Doutorado em Ciências da Saúde) -- Universidade Federal do Triângulo Mineiro, Uberaba, MG, 2021 Orientadora: Profa. Dra. Márcia Antoniazi Michelin Coorientador: Prof. Dr. Eddie Fernando Cândido Murta 1. Neoplasias. 2. Imunoterapia. 3. Células dendríticas. 4. Neoplasias mamárias experimentais. 5. Moléculas de adesão celular. 6. Fatores de transcrição. I. Michelin, Márcia Antoniazi. II. Universidade Federal do Triângulo Mineiro. III. Título. CDU 616-006

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CRB-6/3461

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Uberaba, agosto de 2021.

Banca de defesa

Uberaba

2021

Dedicatória

Dedico este trabalho a todos com talento e inteligência para se tornarem ótimos pesquisadores e profissionais e que por motivos alheios a sua vontade não conseguiram.

Aos meus pais e minha família.

‘’Depois de escalar uma grande montanha se descobre que existem muitas outras montanhas para escalar’’

Nelson Mandela. Da autobiografia “O longo caminho para a liberdade”, 1994

Agradecimentos

A Deus por ter me dado saúde e determinação para prosseguir neste caminho que para alguns por algum momento pareceu loucura.

Aos meus orientadores Dra. Márcia Antoniazi Michelin e Dr. Eddie Fernando Cândido Murta pela paciência de sempre e por apresentarem para um recém formado que nada sabia sobre pesquisa este caminho lindo.

Aos meu pais João Francisco Moreira da Silva e Sonia Maria da Luz Silva por terem me dado a vida, me criado priorizando a educação, por exigirem boas notas e por todo amor.

Aos meus avos, os presentes e àqueles que não estão mais aqui. Obrigado por sonharem!

Ao meu irmão Lucas Fernando, e aos primos por tudo que vivenciamos juntos. Algumas daquelas experiências eu repeti no laboratório!

Aos amigos de jornada durante este tempo dentro da UFTM, Dr. Tharsus Dias Takeuti, Dr. André Adriano, Dr. Eduardo Arthur, Dra. Polyana Barbosa, Dra. Angela Moed, Dra. Alessandra Cunha, Kezia Aguiar, Eleni Solange, Ingrid Marques, Lenilson, Taissa. Aos amigos de jornada profissional no Hospital Nossa Senhora da Abadia em Ituiutaba – MG. Eduardo Carvalho, Annelise Amaral, Dr. Edigar Henrique, Bruna Lacerda e Cláudia Novais.

A todos o meu muito obrigado!

RESUMO

INTRODUÇÃO: As células dendríticas são uma das pontes entre as respostas imunológicas inata e adaptativa e ainda contribuírem no desenvolvimento de uma resposta especializada para cada tipo de tumor. No começo dos anos 2000 alguns pesquisadores levantaram a hipótese de as células dendríticas serem usadas no tratamento de alguns tipos de canceres como imunoterapia. Muitos resultados positivos foram alcançados desde então e a imunoterapia com células dendríticas contra o câncer foi estabelecida como uma imunoterapia tão eficiente quanto eficaz. **OBJETIVOS:** Para melhor entendermos a dinâmica que envolve os mecanismos pelos quais a imunoterapia com células dendríticas influência na resposta imunológica avaliamos linfonodos e tumores de camundongos induzidos a desenvolver tumor tratados com imunoterapia de células dendriticas. **METODOLOGIA:** foram utilizados 70 camundongos isogênicos da linhagem Balb/c e para indução tumoral $5x10^5$ de células de carcinoma mamário de camundongos Balb/c 4T1 foram injetado por animal. Estes animais foram utilizados para avaliarmos de maneira dinâmica a influência da imunoterapia no sistema imunológico dividimos estes animais em grupos 7 e 14 dias após a primeira dose da imunoterapia com células dendríticas. Ao final ficamos com 7 grupos experimentais: GI – grupo controle, GII – grupo controle CD 7 dias, GIII – grupo tumor 7 dias, GIV – grupo tumor CD 7 dias, GV – grupo controle CD 14 dias, GVI – grupo tumor 14 dias, GVII – grupo tumor CD 14 dias. **RESULTADOS:** Durante o período experimental o volume do tumor foi medido periodicamente e notamos que o desenvolvimento tumoral foi menor no grupo tumor CD (0,0003988mm^3 - 0,2670mm^3) quando comparado ao grupo tumor (0,03703mm^3 - 0,2670mm^3). Notamos ainda através do qPCR que a expressão do fatore de transcrição T-bet de perfil de células Th1, aquele que induz o combate ao tumor de maneira eficaz é maior no grupo tumor CD em 14 dias (1,83) quando comparado ai grupo tumor (1,08). Já o fator de transcrição de células Treg, FOXP3, é maior no grupo tumor tanto em 7 (3,85) quanto em 14 (20,73) dias quando comparado ao grupo tumor tratado em 7 (2,32) e 14 (2,16) dias; mostrando um cenário de difícil reversão. Através de imunofluorescência encontramos maior intensidade média de fluorescência de MHCI e moléculas coestimulatorias nos linfonodos do grupo tumor CD em 14 dias quando comparado ao grupo tumor. Observamos também que moléculas de adesão podem frente a estímulos diferentes durante a imunoterapia, notamos que ICAM1 tinha maior MFI nos linfonodos do grupo tumor tanto em 7 (79,32) quanto em 14 (147,3) dias quanto comparados ao grupo tratado 7 (39,59) e 14 (81,49) dias, já ICAM2 teve maior MFI nos linfonodos do grupo tumor CD em 14 (66,52) dias quando comparado ao grupo tumor 14 dias (56,4). **CONCLUSÃO:** Concluímos que a imunoterapia de células dendríticas no combate ao câncer, já comprovadamente eficaz, inicia o processo de modulação do sistema imunológico em 7 dias, porém com 14 dias a modulação é se torna mais eficaz. Para melhor entendimento deste mecanismo e melhoramento dos protocolos de vacinação mais estudos que analisem de forma dinâmica a resposta imunológica frente ao câncer deve ser realizados e outras moléculas devem ser analisadas.

Palavras-chave: Células dendríticas, câncer, imunoterapia, células 4T1, ICAM1, ICAM2, fator de transcrição

ABSTRACT

INTRODUCTION: Dendritic cells are one of the bridges between innate and adaptive immune responses and also contribute to the development of a specialized response for each type of tumor. In the early 2000s, some researchers hypothesized that dendritic cells could be used as immunotherapies in the treatment of some types of cancer. Promising results have been achieved since then and immunotherapy with dendritic cells against cancer has been established as an immunotherapy as efficient as it is effective. **OBJECTIVES:** To better understand the dynamics surrounding the mechanisms by which immunotherapy with dendritic cells influences the immune response, we evaluate lymph nodes and tumors of mice induced to develop tumors treated with dendritic cell immunotherapy. **METHODOLOGY:** 70 isogenic mice of the Balb/c lineage were used and for tumor induction $5x10^5$ of mammary carcinoma cells of Balb/c mice were (4T1) injected per animal. These animals were used to dynamically assess the influence of immunotherapy on the immune system. We divided these animals into groups 7 and 14 days after the first dose of immunotherapy with dendritic cells. Then we have 7 experimental groups: GI - control group, GII - control group CD 7 days, GIII - tumor group 7 days, GIV - tumor group CD 7 days, GV - control group CD 14 days, GVI - tumor group 14 days, GVII - CD tumor group 14 days. **RESULTS AND DISCUSSION:** During the experimental period, the tumor volume was measured periodically and we noticed that the tumor development was lower in the CD tumor group (0.0003988mm3 - 0.2670mm3) when compared to the tumor group (0.03703mm3 - 0.2670mm3). We also noticed through the qPCR that the expression of the T-bet transcription factor of the Th1 cell profile, the one that effectively induces the fight against the tumor is greater in the CD tumor group in 14 days (1.83) when compared to the tumor group (1.08). The Treg cell transcription factor, FOXP3, is higher in the tumor group both in 7 (3.85) and in 14 (20.73) days when compared to the tumor group treated in 7 (2.32) and 14 (2 ,16 days; showing a scenario of difficult reversal. Through immunofluorescence, we found a higher mean fluorescence intensity of MHCI and co-stimulatory molecules in the lymph nodes of the CD tumor group in 14 days when compared to the tumor group. We also observed that adhesion molecules can face different stimuli during immunotherapy, we noticed that ICAM1 had a higher MFI in the lymph nodes of the tumor group both in 7 (79.32) and in 14 (147.3) days as compared to the treated group 7 (39.59) and 14 (81.49) days, ICAM2 had higher MFI in the lymph nodes of the CD tumor group in 14 (66.52) days when compared to the tumor group 14 days (56.4). **CONCLUSION:** We conclude that immunotherapy with dendritic cells in the fight against cancer, already proven effective, starts the process of modulating the immune system in 7 days, however with 14 days the modulation becomes more effective. For a better understanding of this mechanism and improvement of vaccination protocols, further studies that dynamically analyze the immune response to cancer should be performed and other molecules should be analyzed.
Keywords: dendritic cells, cancer, immunotherapy, 4T1 cells, ICAM1, ICAM2, transcription factor

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Lista de abreviaturas ou siglas

INCA – instituto nacional do câncer

MHCI – Complexo Principal de Histocompatibilidade I

DNA – ácido desoxirribonucleico

APC – células apresentadoras de antígeno

CD4 – Grupamento de diferenciação 4

CD8 – Grupamento de diferenciação 8

mAbs – Anticorpos monoclonais

carT cell – receptor de antígeno quimérico de células T

Th2 – T helper 2

Th1 – T helper 1

IL-12 – Interleucina 12

CD40 – Grupamento de diferenciação 40

CD40L – Grupamento de diferenciação 40 ligante

MHCII – Complexo Principal de Histocompatibilidade II

TCR – receptor de células T

NK – natural killer

IFN-γ – Interferon γ

Stat1 – fator de transcrição

MHC – Complexo Principal de Histocompatibilidade

TGF-β – Fator de transformação do crescimento

Treg – T regulatório

M2 – Macrófagos M2

VEGF – Vascular endothelial growth fator

MMP2 – Metaloproteinase 2

IgSF – molécula de adesão

HLA I – Human Leucocyte Antigen I

HLA II – Human Leucocyte Antigen II

ICAM-1 – molécula de adesão intercelular-1

Anti-CD54 – anticorpo contra grupamento de diferenciação 54

MUC-1 – Mucina

TNF-α – Fatores de Necrose Tumoral Alfa

IL-1 – interleucina 1

ICAM-2 – Molécula de adesão intercelular 2

LFA-1 – Molécula de adesão

CD11a – grupamento de diferenciação 11a

4T1 – células tumorais de mama de camundongos Balb/c

qPCR – reação em cadeia da polimerase quantitativo

Balb/c – Camundongos isogênicos

IPON – Instituto de pesquisa em oncologia

GM-CSF – Fator de crescimento de colônias de macrófagos e granulócitos

RNA – (ácido ribonucleico

cDNA – DNA complementar

H_2O – água

RT-PCR – Transcrição reversa seguida de reação em cadeia da polimerase

Dntp – Desoxirribonucleotídeos Fosfatados

RNAm – ácido ribonucleico mensageiro

T-bet – fator de transcrição de linfócitos Th1

GATA3 – fator de transcrição de linfócitos Th2

RORγt – fator de transcrição de linfócitos Th17

FOXP3 – fator de transcrição de linfócitos Treg

CD49 – grupamento de diferenciação 49

CD102 – grupamento de diferenciação 102

CD54 – grupamento de diferenciação 54

IMDM – meio de cultura Iscove modificado do Dulbecco's Modified Eagle's Medium

CTLA4 – proteína CTLA-4

PBS – Tampão fosfato-salino

MFI – intensidade media de fluorescencia

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1. INTRODUÇÃO

INCA (Instituto Nacional do Câncer) define o câncer como o um conjunto de mais de 100 doenças que têm em comum a divisão desordenada de um grupo celular. Este crescimento desordenado se dá devido a uma ou mais mutações ocorridas em genes específicos(INSTITUTO NACIONAL DO CÂNCER, 2018). Os fatores de risco que podem causar mutações celulares, transformando-as em células tumorais são divididos em: químicos, físicos e biológicos (AZAR et al., 2004). Em conjunto com uma falha na imunovigilância e alterações genéticas, uma célula normal adquire características de células tumorais. A principal característica desta célula tumoral é a capacidade de divisão acelerada e baixa taxa de apoptose, formando então um conjunto de células transformadas (INSTITUTO NACIONAL DE CÂNCER; (INCA), 2008; MINISTÉRIO DA SAÚDE INSTITUTO NACIONAL DE CÂNCER (INCA), 2011). O INCA estima para o biênio 2018 – 2019 mais de 600 mil novos casos de câncer no Brasil (INSTITUTO NACIONAL DO CÂNCER, 2018). Entre as mulheres o câncer mais comum, segundo estimativa, é o câncer de mama; e entre os homens é o câncer de próstata, com 57.960 e 61.200 novos casos, respectivamente (INSTITUTO NACIONAL DO CÂNCER, 2018).

O conceito de imunovigilância postula que leucócitos patrulham tecidos a procura de algum patógeno ou célula transformada que possam vir causar alguma inflamação ou infecção maléfica para o organismo (ZITVOGEL et al., 2016). O conceito de imunovigilância nos leva ao conceito de imunoedição. A imunoedição complementa o conceito de imunovigilância e demonstra a dicotomia do sistema imunológico frente ao desenvolvimento ou supressão do tumor. A imunoedição é dividida nos chamados três *E's* da imunoedição: eliminação, equilíbrio e escape (TENG et al., 2015).

Na primeira fase da imunoedição, também chamada de eliminação, encontramos o conceito de imunovigilância. Nesta fase são utilizados mecanismos da imunidade inata e adaptativa. Durante a fase de eliminação células que estão constantemente patrulhando o organismo encontram células transformadas e reconhecem as mesmas como antígenos as eliminando ativando mecanismos de apoptose(DUNN; OLD; SCHREIBER, 2004). A segunda fase, também chamada de equilíbrio, apresenta uma dormência frente ao sistema imunológico as poucas células tumorais restantes. Nesta fase não ocorre resposta imunológica assim como também não ocorre desenvolvimento do tumor pelas células tumorais que não foram eliminadas durante a primeira fase (DEEPAK MITTAL, MATTHEW M GUBIN, ROBERT D SCHREIBER, 2014). Pouco se sabe sobre a fase

de equilíbrio por ser uma fase de difícil acesso em modelos experimentais (DEEPAK MITTAL, MATTHEW M GUBIN, ROBERT D SCHREIBER, 2014; TENG et al., 2015).

Durante a última fase da imunoedição, também chamada de escape, por algum mecanismo ainda desconhecido, as células tumorais que se encontravam em estado de dormência na fase de equilíbrio começam a se dividir de maneira acelerada levando ao desenvolvimento do tumor (GAVIN P. DUNN LLOYD J. OLD, 2004). Durante a fase de escape as células tumorais remanescentes apresentam alguns mecanismos que burlam a vigilância imunologia, contribuindo para o desenvolvimento tumoral. Estas células apesar de mutadas e de se dividirem de maneira acelerada apresentam baixa imunogenicidade, ou seja, o sistema imunológico perde a capacidade de reconhecer estas células como células com potencial carcinogênico. De modo que podemos caracterizar este grupo celular como células que tem alta taxa de divisão, reduzida expressão de MHC I, e apresentam capacidade de produzir proteínas que visam reduzir a atividade leucocitária (DEEPAK MITTAL, MATTHEW M GUBIN, ROBERT D SCHREIBER, 2014; GAVIN P. DUNN LLOYD J. OLD, 2004; TENG et al., 2015).

Sabe-se que para o tratamento do câncer existem tratamentos seguramente eficazes.(WIRSDÖRFER; DE LEVE; JENDROSSEK, 2019). Ressecção cirurgica seguida de tratamento quimioterápico e/ou radioterápico tem eficácia comprovada e vem sendo usado ao longo do tempo como principal forma de tratamento para o combate ao câncer (BASKAR et al., 2012). Apesar de eficazes estes tratamentos desencadeiam reações adversas. A radiação utilizada na radioterapia é um agente físico e é chamada de radiação ionizante, pois quando atinge células tem a capacidade de formar íons e depositar energia nas células irradiadas. Estes íons causam danos no DNA de células atingidas impedindo que as mesmas se dividam de maneira acelerada ocasionando na apoptose das mesmas (FORMENTI; DEMARIA, 2013; SCHAUE; KACHIKWU; MCBRIDE, 2012).

A radiação ionizante da radioterapia atinge tanto células mutadas quanto células normais causando danos no tecido, algumas vezes irreversível (BASKAR et al., 2012). A quimioterapia tem como mecanismo de ação a administração de drogas citotóxicas que danificam células cancerígenas e células normais induzindo estas células a sofrer apoptose (ALAM, 2018). No início dos anos 2000, pesquisadores notaram potencial no uso de células dendríticas em imunoterapias contra o câncer (BANCHEREAU; PALUCKA, 2005; ZHANG et al., 2003), pelo fato de as mesmas serem células extremamente potentes na ação de ativar o sistema imunológico e por evitarem efeitos

colaterais como a necrose de tecidos submetidos a esta terapia, o que não acontece em radioterapia (DE LA PUENTE; AZAB, 2014) e quimioterapia.

Implícita no próprio nome, as imunoterapias podem ser definidas como terapias que visam ativar o sistema imunológico a combater um patógeno, de forma ativa ou passiva. As imunoterapias ativas são aquelas que visam ativar o sistema imunológico outrora inibido (MCCARTHY, 2006). Já as imunoterapias passivas visam o tratamento mais assertivo de uma determinada patologia (MCCARTHY, 2006). As células dendríticas são conhecidas como células apresentadoras de antígeno profissionais (APCs), pela sua grande capacidade e eficácia em realizar tal tarefa (MCDONNELL; ROBINSON; CURRIE, 2010). Exatamente por essa característica, as células dendríticas vêm sendo usadas em imunoterapias contra o câncer como vacinas (MCDONNELL; ROBINSON; CURRIE, 2010). Outra característica das células dendríticas que lhe dá o título de célula apresentadora de antígeno profissional é a capacidade de realizar apresentação cruzada, que consiste na apresentação de antígenos tanto para linfócitos T CD4+, gerando uma resposta imunológica de células T auxiliares, quanto para linfócitos T CD8+ citotóxicos, ativando estas, que são células especializadas na destruição de células infectadas (JOFFRE et al., 2012b).

Existem basicamente dois tipos de imunoterapias, as imunoterapias passivas e ativas(DAVIES, 2014; GAO et al., 2014). As imunoterapias passivas não visam a ativação do sistema imunológico, mas sim um combate direto ao tumor (KOKATE, 2017; OZVEREL; KARABOZ; NALBANTSOY, 2018). Podemos citar como exemplo as imunoterapias de imuno-check points. Estas imunoterapias são realizadas com anticorpos monoclonais (mAbs), estes anticorpos se ligam em regiões específicas das células tumorais impedindo que células ou citocinas imunossupressoras se liguem naquele determinado ponto (KOKATE, 2017).

De modo contrário as imunoterapias ativas visam ativar o sistema imunológico outrora inibido. Uma característica importante das imunoterapias ativa é que pelo fato de ativarem o sistema imunológico essas imunoterapias podem gerar memoria imunológica. Podemos citar dentre algumas as imunoterapias com células quiméricas (carT cell) (DANDAN XU1,*, GUOLIANG JIN1,*, DAFEI CHAI1, XIAOWAN ZHOU1, WEIYU GU1; CHONG1, 2018), imunoterapia com células T adotivas, imunoterapia com vacina de células dendríticas (DA CUNHA; MICHELIN; MURTA, 2014).

As células dendríticas, assim como outros leucócitos, são responsáveis pela secreção de citocinas que vão modular o sistema imunológico para uma resposta

dependendo do antígeno apresentado (KHAMAR, 2018; RODRIGUES et al., 2011). Por exemplo, parasitas e helmintos modulam uma resposta do perfil Th2, antígenos intracelulares e células infectadas modulam uma resposta do perfil Th1 (FAGUET, 2015; WAN, 2014). Dentre as citocinas produzidas e secretadas pelas células dendríticas, Bruno e colaboradores descreveram, em 2014, a IL-12 como a principal. A produção de IL-12 estimula um padrão de resposta imunológica de células T do perfil Th1, que seria o ideal para respostas antitumorais. Sabe-se que na ausência de produção de IL-12, o perfil de células T predominante é o perfil Th2 caracterizando o balanço Th1/Th2 (JANARDHAN; MARKS; GAJEWSKI, 2014; SABET-BAKTACH et al., 2013a) .

Após a captura do antígeno a célula dendrítica secreta algumas citocinas, que atuam na ativação e maturação de linfócitos T. Existem dois caminhos para indução da produção de IL-12 pelas células dendríticas: o primeiro caminho envolve a invasão de algum agente patogênico, essa invasão induz a rápida produção de IL-12 sem necessidade de interação com linfócitos T, um segundo caminho seria dependente da interação com linfócitos T, que requer a ligação do CD40-CD40L (presentes em linfócitos T e células dendríticas respectivamente), ou a interação do MHC II com o TCR (T cell receptor) (FUKAO et al., 2012).

IL-12 atua tanto em linfócitos T como em células NK induzindo a produção de INF-γ. No mesmo trabalho *Banchereau* relata que células T CD8+ produzidas por estimulo de IL-12 apresentam maior quantidade de CD40L aumentando desta forma a citotoxicidade dos linfócitos T CD8+. A polarização de células T em perfil Th1 estimula também a secreção de IFNs dentre os quais estão presentes IFN-γ (BANCHEREAU; PALUCKA, 2005; QIAN et al., 2011). O IFN-γ é uma citocina produzida principalmente por células como NK, e linfócitos do perfil Th1. Stat1 é um fator de transcrição responsável pela produção e secreção de IFN-γ (YLIKOSKI et al., 2005). Embora a ativação de Stat1 representa apenas uma fração de todos os genes indutores de IFN- γ os genes induzidos por Stat1 desempenham um papel importante na maioria das funções biológicas do IFN-γ(KRAUSE et al., 2006).

Atualmente muito se tem discutido a respeito da determinação do perfil de linfócitos T auxiliares de acordo com a produção de citocinas, pois muitas delas são comuns a vários perfis. Assim sendo, discute-se uma proposta mais precisa na determinação do perfil de células T, que seria através dos fatores de transcrição presentes nestas células. Tem sido proposto que A diferenciação de cada linfócito T $CD4^+$ em um perfil especifico de células T é determinado pela expressão de fatores de transcrição

específicos por parte destas células (WU et al., 2014) aonde: T-bet é essencial para polarização do perfil Th1 (POSSEMATO et al., 2003), GATA3 é essencial para polarização de um perfil Th2 (OKAMURA et al., 2014), RORγt é necessário para polarização do perfil Th17 (SABET-BAKTACH et al., 2013b) e finalmente o FOXP3 que é essencial para a polarização dos linfócitos T $CD4^+$ em linfócitos T regulatórios (treg) (CHEN; DU; HUANG, 2012).

T-bet descrito em 2000 por *SzaboS. J.et. al.* está presente no gene brachyury, neste gene está presente a família de fatores de transcrição T box (SZABO et al., 2000). Dentro dos genes desta família está presente o gene que codifica o INF-γ o IFNG. O T-bet induz a expressão do gene, ocorre então produção de IFN-γ que se liga a seu receptor e induz a ativação de STAT-1 que ativa genes responsáveis pela produção de IFN-γ e IL-12, os genes IFNG e IL12RB2 (OH; HWANG, 2014). Os linfócitos se diferenciam em linfócitos Th1 quando o fator de transcrição STAT 1 é ativado. Inicia-se a transcrição do fator de transcrição STAT 1 quando o IFN-γ se liga ao seu receptor. O STAT-1 ativa genes importantes na sobrevida e na função da célula. Como exemplo *Krause C. D. et. al.* citou em 2006 SOCS-1 que se liga no receptor do IFN-γ que permite a ativação de Jak-2. O perfil de célula Th1 é o ideal em uma resposta imunológica contra o câncer por produzir citocinas responsáveis pela ativação de células dendríticas por exemplo, citocinas capazes de ativar uma resposta contra um vírus ativando, consequentemente linfócitos T CD8+ conhecidos como citotóxicos (KRAUSE et al., 2006).

GATA3 é um fator de transcrição característico de linfócitos do perfil Th2. Altos níveis de GATA-3 foram observados pela primeira vez por (ZHENG; FLAVELL, 1997), quando comparada a expressão deste gene em linfócitos Th1. A ativação do GATA-3 estimula a secreção de IL-4, uma citocina responsável pela resposta imunológica a parasitas, a IL-4 que ativa a produção de IgE e aumenta a expressão de MHC de classe II. É produzida predominantemente por linfócitos Th2 e mastócitos (AKDIS et al., 2011).

RORγt é um fator de transcrição presente em linfócitos do perfil Th17, perfil este que produz citocinas pró-inflamatórias tais como: IL-6, IL-17, IL-21 e IL-23 (ZHENG, 2013). Basicamente estas citocinas tem a função de induzir a produção de citocinas, anticorpos atraindo e ativando outras células da resposta imune inata (AKDIS et al., 2011). Experimentos realizados em camundongos *knockout* para RORγt mostraram que estes animais não conseguiam induzir a polarização de linfócitos do perfil Th17 demosntrando que este fator de transcrição é essencial para a ativação do perfil Th17 (LIN et al., 2017a).

Descrito em 2002 o FOXP3 é o principal fator de transcrição das células T regulatórias e faz parte da grande família *forkhead winged-helix* e está ligado ao gene X. O FOXP3 é ativado quando o TGF-β se liga ao seu receptor (MANGAN et al., 2006). A expressão de FOXP3 em células T convencionais converte estas células em células do perfil Treg, com capacidade de induzir anergia e supressão na atividade destas células (FONTENOT; GAVIN; RUDENSKY, 2017).

FOXP3 não é obrigatoriamente necessário para o desenvolvimento de células Treg, porém a expressão de FOXP3 se mostra necessária para estabilizar as funções imunossupressoras destas células (FONTENOT; GAVIN; RUDENSKY, 2017). As células Treg têm função na homeostase do sistema imunológico, porém no câncer estas células têm papel imunossupressor, favorecendo o desenvolvimento do tumor. Ainda na homeostase há um desequilíbrio do balanço de células Treg quando FOXP3 é expresso por muito tempo (FONTENOT; GAVIN; RUDENSKY, 2017). As células Treg tem como principais marcadores a cadeia α do receptor de IL-2, o CD25, o CTLA-4 e o GITR (WARD-HARTSTONGE; KEMP, 2017). *Nakamura et. al.* relata em 2001 que estas células têm como citocinas características a IL-10 e TGF-β. Sabe-se que o microambiente tumoral produz dentre vários fatores, citocinas que induzem a polarização do perfil de células Treg como IL-10 e TGF-β (INFILTRATING et al., 2006).

Após fagocitar patógenos, as células dendríticas têm a capacidade de apresentá-los como peptídeos via MHC (COMBER; PHILIP, 2014). Formado este complexo, a célula dendrítica expõe o mesmo em sua superfície e nos linfonodos ocorre a apresentação deste antígeno aos linfócitos T CD4, sendo que para ativação dos linfócitos T CD4 a interação com o receptor do linfócito (TCR) é feita exclusivamente com o MHC II e para a ativação de linfócitos citotóxicos T CD8 a interação é feita exclusivamente com o MHC I (COMBER; PHILIP, 2014; ZHU; YAMANE; PAUL, 2010).

Os linfócitos pertencentes a família dos linfócitos T CD8 apresentam características diferentes dos linfócitos T CD4. Os linfócitos T CD8 são ativados através da interação do seu TCR com o MHC I da célula alvo e moléculas coestimulatórias (KOKATE, 2017). A função do linfócito T CD8 é de destruir as células alvo, o linfócito T CD8 apresenta em seu interior, assim como as células NK, grânulos de perforinas, granzimas e outros com a função de perfurar a membrana citoplasmática e induzir a apoptose das células alvo(RODRIGUES et al., 2011). Embora a resposta do sistema imunológica ao câncer se mostre eficaz, o tumor dispõe de mecanismos chamados mecanismos de escape tumoral, que fazem com que o tumor burle a resposta imunológica

e se desenvolva cada vez mais (HEGDE; CHEN, 2020; TRINCHIERI, 2015). Entre os principais mecanismos de escape tumoral podemos citar: a produção de citocinas imunossupressoras por parte do tumor, como o TGF-β, o que polariza o perfil das células no microambiente tumoral para um perfil de células imunossupressoras, como os linfócitos T regulatórios (Treg) (LIAO et al., 2011), macrófagos M2 (FARHOOD; NAJAFI; MORTEZAEE, 2019), entre outros tipos celulares.

Outro mecanismo de escape tumoral é a perda da expressão do MHCI por parte das células tumorais (LUCKHEERAM et al., 2012). O microambiente tumoral tem também a capacidade de produzir fatores que estimulam a vascularização do tumor, como o VEGF e de MMP2 (HUMAR et al., 2014; LEE et al., 2018; PALUCKA; BANCHEREAU, 2012), que têm a função de destruir a matriz extracelular, facilitando, deste modo, a invasão de células tumorais para outros tecidos, dando início a metástase.

Voglestein e *Kinzelr* descreveram que para um tumor se tornar maligno existem três etapas. Na primeira etapa um gene modificado leva uma célula a ter potencial de se proliferar de maneira desordenada, a segunda etapa é caracterizada como a fase de expansão clonal desta célula modificada, e por fim, a terceira etapa está relacionada com a capacidade que as células tumorais ganham de invadir tecidos adjacentes caracterizando assim seu potencial de malignidade (SÖKELAND; SCHUMACHER, 2019). A capacidade que as células tumorais têm de sair do seu tecido de origem e invadirem tecidos adjacentes está fortemente relacionada às moléculas de adesão.

Durante o desenvolvimento do tumor todas as células do sistema imunológico interagem com a células tumorais de várias formas. O tráfico de células para o sítio da infecção, a ativação de células nos linfonodos, o recrutamento de novas células para o local da infecção, assim como a adesão de uma célula tumoral a outra ou a não adesão de uma célula tumoral a outra o que pode gerar as metástases. Todos estes processos necessitam das chamadas moléculas de adesão. E através da presença ou não delas podemos justificar a presença ou não de metástases o que poderia nos dar também um prognostico com relação ao desenvolvimento da doença(HARJUNPÄÄ et al., 2019).

O processo pelo qual uma célula tumoral passa para invasão de outro tecido começa quando ela se desprende do sítio inicial e através da destruição da matriz extracelular dos vasos sanguíneos atinge a corrente sanguínea (SÖKELAND; SCHUMACHER, 2019). A partir daí a célula tumoral segue o fluxo sanguíneo, acontece então a adesão inicial desta célula tumoral através das selectinas na porção luminal dos vasos sanguíneos. A célula tumoral ligada as selectinas passa a rolar no sentido do fluxo

sanguíneo até que ocorra uma adesão mais forte com as integrinas, também chamado de ancoragem (SÖKELAND; SCHUMACHER, 2019). Após ancoragem desta célula tumoral a diapedese ocorre com ajuda das caderinas, permitindo então que esta célula tumoral invada tecidos diferentes do seu tecido inicial(SÖKELAND; SCHUMACHER, 2019).

As moléculas de adesão são divididas em cinco classes: integrinas, selectinas, caderinas, IgSF membro da superfamília das imunoglobulinas e mucinas (HARJUNPÄÄ et al., 2019). As selectinas são divididas em selectinas P, E e L-selectinas, baseado em qual tipo celular elas são encontradas(KAPPELMAYER; NAGY, 2017). Há uma diferença básica nas selectinas com relação ao momento da expressão das mesmas, as P-selectinas são expressas em minutos, as E-selectinas são expressas horas. As selectinas são importantes para a migração leucocitária, migração de leucócitos para os linfonodos e para pele(KAPPELMAYER; NAGY, 2017). As integrinas são grandes heterodímeros com uma cadeia α com tamanho de 120-170 kDa e outra cadeia β com tamanho de 90-100 kDa (HARJUNPÄÄ et al., 2019). Cada subunidade das integrinas tem uma função diferente, as subunidades α têm função de determinar a especificidade do ligante da integrina, já a subunidade β está conectada ao citoesqueleto e influencia várias vias de sinalização (BARCZYK; CARRACEDO; GULLBERG, 2010).

A IgSF é uma das mais diversas e maiores famílias de proteínas. Todos os membros desta família contem pelo menos um domínio de imunoglobulina ou semelhante a imunoglobulina, e a maioria dos membros são proteínas transmembrana tipo I com domínio extracelular e uma cauda citoplasmática (HARJUNPÄÄ et al., 2019). Os membros mais conhecidos desta família incluem HLA I e II e as proteínas do TCR (KAPPELMAYER; NAGY, 2017).

O ICAM-1 é expresso nas células endoteliais tem sua função juntamente ao desenvolvimento de novos tumores bem esclarecida. Sabe-se que o ICAM-1 faz a adesão célula-célula, célula-matriz extra celular (ROLAND et al., 2007). Um estudo clássico publicado em 2006, mostrou que o bloqueio de ICAM-1 com anti-CD54 inibiu o crescimento em um modelo de melanoma em camundongos (WANG et al., 2006). Quando MUC-1 se liga à ICAM-1 presente nas células endoteliais é induzido intracelularmente um sinal de cálcio que resulta em dois efeitos importantes: a liberação de quimiocinas, e a superexpressão de ICAM-1 por parte das células tumorais (ROLAND et al., 2007). É bem definido também que a secreção de TNF-α, IFN-γ e IL-1 aumenta a

expressão de ICAM-1 nas células endoteliais (FIGENSCHAU et al., 2018; ROLAND et al., 2007).

O ICAM-2 é expresso em tecidos normais predominantemente por células endoteliais neovasculares e por alguns leucócitos (YOON; MILLER; KREITZBURG, 2015). Quando expresso por células endoteliais vasculares, o domínio extracelular do ICAM-2 se liga a β_2-integrinas nas superfície dos leucócitos como um passo inicial para a resposta imune (DE FOUGEROLLES et al., 1991). Nas células tumorais foi identificado que o ICAM-2 conferiu um fenótipo não metastático em células de neuroblastoma (YOON et al., 2008). Assim como o ICAM-1 o ICAM-2 tem como principal ligante o LFA-1 ou CD11a (HARJUNPÄÄ et al., 2019; YOON et al., 2008).

A expressão de LFA-1 em células hematopoiéticas o torna uma alvo potencial em leucemias e linfomas(REINA; ESPEL, 2017), tumores de origem hematopoiéticas expressam LFA-1 e podem ser alvos com anti-LFA-1(COHEN; HAIMOVICH; HOLLANDER, 2003). A expressão de LFA-1 em tumores não hematopoiéticos também tem sido relatada, em um modelo experimental de tumor cerebral em ratos a presença de LFA-1 em células metastáticas contribuiu com o desenvolvimento tumoral (SOTO et al., 2016). Os estudos com tumores não hematopoiéticos não são suficientes para tornar o LFA-1 um alvo direto em tumores não hematopoiéticos (REINA; ESPEL, 2017). Porém as células Treg presentes no infiltrado tumoral e a importância do LFA-1 para a função dessas células sugere que o bloqueio desta proteína limitaria a função das células Treg e melhoraria a ação do sistema imunológico contra o tumor (REINA; ESPEL, 2017).

2. JUSTIFICATIVA

O uso de animais na pesquisa se justifica pelo fato de o modelo animal ser isogênico. Este modelo isogênico diminui o as variações dando abertura para que estudos experimentais se transformem em estudos clínicos (KNIGHT, 2007). Resultados prévios em grupos experimentais e em grupos de estudo clínico demonstraram que a vacina de células dendríticas é eficaz, reduzindo o volume tumoral, aumentando a produção de citocinas que polarizam as células T auxiliares para um perfil ideal na resposta imune contra o câncer e aumenta-se também, a expressão do principal fator de transcrição deste perfil de células T, o perfil de células Th1.

Porém, para que haja uma melhor compreensão da resposta imunológica, são necessários aperfeiçoamentos nos modelos experimentais, analisando o desenvolvimento tumoral e perfil das células em diferentes estágios de tratamento, verificando os mecanismos envolvidos em cada etapa e as células da resposta imune e seus mediadores produzidos durante esta resposta antitumoral.

Para que possamos descrever de maneira clara o que acontece com o sistema imunológico durante o tratamento com vacinas de células dendríticas em animais induzidos a desenvolver câncer de mama com células 4T1, faz-se necessário a análise dos parâmetros anteriormente analisados somente em 30 dias após o início do tratamento com vacina de células dendríticas, e agora em 7 e 14 dias após o início do tratamento, para que possamos entender a cinética da resposta antitumoral, analisando os dados obtidos em datas diferentes, com o intuito de analisar pequenas mudanças no sistema imunológico.

3. OBJETIVOS

3.1 Objetivos gerais

Analisar a dinâmica que envolve as células da resposta imune frente aos mecanismos que são desencadeados pela imunoterapia com células dendríticas. Utilizando técnicas como imunofluorescência analisamos quando ocorre maior ou menor expressão de moléculas de adesão, analisamos ainda a presença de moléculas que fazem parte da ativação do sistema imunológico adaptativo nos linfonodos e nos tumores dos animais dos grupos experimentais. Ainda por imunofluorescência analisamos o infiltrado de células nos tumores dos grupos experimentais. Com qPCR analisamos a expressão dos fatores de transcrição de linfócitos T auxiliares.

3.2 Objetivos específicos

- Verificar a expressão dos fatores de transcrição de linfócitos T auxiliares $CD4^+$ T-bet, GATA3, RORγt no baço de camundongos induzidos e não induzidos a desenvolver tumor com células 4T1, tratados e não tratados com vacina de células dendríticas por qPCR.

- Verificar a interação das células dendríticas com linfócitos nos linfonodos através de imunofluorescência com os marcadores CD3, CD4, MHCI, TCR, CD80, CD86, CD49d, CD11a, CD152, CD106, CD54 e CD102.
- Avaliar, por imunofluorescência, o perfil das células do infiltrado tumoral no microambiente tumoral obtidos de camundongos com câncer de mama induzido por células 4T1, assim como moléculas de adesão envolvidas no processo de migração celular. Os marcadores analisados serão: CD4, CD8, CD11a, CD49d, CD54, CD80, CD86, CD102, CD106, CD152 E LFA-1.

4. METODOLOGIA

4.1 Delineamento Experimental

O experimento utilizou 88 camundongos fêmeas da linhagem Balb/c, com idade entre 6 a 8 semanas, provenientes do biotério setorial do IPON – Instituto de Pesquisa em Oncologia. Os animais ficaram alojados em gaiolas (dimensões - 305x198x133mm), agrupados em não mais que 5 animais por gaiola, separados nos diferentes grupos experimentais, sendo submetidos a ambientes com ciclo claro/escuro (12h), temperatura controlada em torno de 22°C, +/-2°C, com alimentação e água *ad libitum.* Destes 88 camundongos, 70 foram divididos nos grupos experimentais.

Os 18 animais restantes foram utilizados para a confecção das vacinas de células dendríticas. Para cada 1 animal eutanasiado foram confeccionadas 4 doses de vacina de células dendríticas. Desta forma os 18 animais produziram doses suficientes de vacina de células dendríticas para vacinar os 40 animais dos grupos GII, GIV, GV e GVII. Para a produção da vacina aplicada no GII, GV (Grupo Vacina de Células Dendríticas) e no GIV e GVII (Grupo Tumor tratado com Vacina de Células Dendríticas) foram retiradas as células das medulas ósseas dos fêmures e das tíbias.

Então, as células foram colocadas em garrafas de $25cm^2$ (volume 7,5 ml) a uma quantidade de $32{,}5x10^6$, onde sob estímulos de GM-CSF (13µL; 10ng/µL), IL-4 (13µL; 10ng/µL), TNF-α (23 µL; 10ng/µL) e antígeno tumoral de células 4T1 (23µL), as mesmas sofreram diferenciação em células dendríticas.

Para compreender a cinética da resposta imunológica, os protocolos de vacinação contarão com 1 dose aplicada nos animais submetidos a 7 dias de tratamento e 2 doses de

vacina aplicadas nos grupos submetidos a 14 dias de tratamento. Desta forma a divisão dos animais em sete grupos experimentais:

- GI (Grupo controle) = 10 animais, sendo 5 agrupados em GI, que foram eutanasiados no sétimo dia do período experimental. Lembrando que que este grupo não foi induzido ao desenvolvimento tumoral pela linhagem de células de tumor de mama 4T1 e também não foi tratado com vacina de células dendríticas;

- GII (Grupo Vacina de Células Dendríticas 7 dias) = formado por 10 animais que receberam 1 dose de vacina de células dendríticas e não receberam células de linhagem tumoral mamário de camundongos 4T1. Estes animais foram eutanasiados no sétimo dia do período experimental.
- GIII (Grupo Tumor 7 dias) = sendo formado por 10 animais que receberam células de linhagem tumoral mamário de camundongos 4T1, não foram tratados com a imunoterapia de células dendríticas e foram eutanasiados no sétimo dia do período experimental.
- GIV (Grupo Tumor tratado com imunoterapia de células dendríticas 7 dias) = constituído por 10 animais que receberam células de linhagem tumoral mamário de camundongos 4T1, foram tratados com imunoterapia de células dendríticas e foram eutanasiados no sétimo dia do período experimental.
- GV (Grupo Vacina de Células Dendríticas 14 dias) = formado por 10 animais que receberam 2 doses de vacina de células dendríticas e não receberam células de linhagem tumoral mamário de camundongos 4T1. Estes animais foram eutanasiados no decimo quarto dia do período experimental.
- GVI (Grupo Tumor 14 dias) = sendo formado por 10 animais que receberam células de linhagem tumoral mamário de camundongos 4T1, não foram tratados com a imunoterapia de células dendríticas e foram eutanasiados no decimo quarto dia do período experimental.
- GVII (Grupo Tumor tratado com imunoterapia de células dendríticas 14 dias) = constituído por 10 animais que receberam células de linhagem tumoral mamário de camundongos 4T1, foram tratados com 2 doses da imunoterapia de células dendríticas e foram eutanasiados no decimo quarto dia do período experimental.

Sendo assim no total temos 7 grupos, GI (grupo controle), GII (grupo controle células dendríticas 7 dias), GII (grupo tumor 7 dias), GIV (grupo tumor células dendríticas

7 dias), GV (grupo células dendríticas 14 dias), GVI (grupo tumor 14 dias) e GVII (grupo tumor células dendríticas 14 dias).

Logo, no dia 7 do período experimental foram eutanasiados animais relativos aos grupos GI, GII, GIII E GIV, os demais animais relativos aos grupos GV, GVI E GVII foram eutanasiados no dia 14 do período experimental. Os resultados obtidos foram verificados e analisados pelo software *GraphPad Prism 5.0*, utilizando os testes estatísticos *Mann-Whitney*, *T Student* a depender da distribuição dos dados. As correlações entre as variáveis contínuas foram realizadas pelo teste de *Pearson* para as distribuições simétricas, e para as assimétricas pelo teste de *Spearman*.

4.2 Extração de material genético

Após a eutanásia uma parte do baço dos animais foi submetida a divulsão mecânica e as amostras foram completadas com TRIzol® até chegar a 1 mL. Em seguida, estas amostras foram incubadas e homogeneizadas por 5 min. a 15-30°C. Foi adicionado 200 µL de clorofórmio, homogeneizado vigorosamente por 15s e incubado por 30°C por 3 min. com centrifugação não mais que 12000g, 15min., 2-8 °C. Neste momento, houve a separação de uma fase inferior fenol-clorofórmio, uma interfase e uma fase superior aquosa, na qual foi removida cuidadosamente e adicionou-se 300 µL de etanol 100% para precipitação do DNA, homogeneização e incubação a 30°C por 3 minutos, seguida por remoção do sobrenadante fenol-etanol para isolamento da proteína.

O DNA, precipitado, foi lavado 2 vezes com solução 0,1M de citrato de sódio a 10% de etanol: 1 mL da solução, e incubação por 30 minutos de 15 a 30°C com homogeneização periódica com centrifugação de 2000g/5 min./4°C. Cuidadosamente, foi removida a solução de lavagem deixando apenas o pellet de DNA. Em seguida, suspendeu-se este pellet de DNA em 2 ml de etanol a 75% e incubou-se a 30°C por 20 minutos com homogeneização periódica outra vez. Após isto, centrifugou-se a 2000G por 5 minutos a temperatura de 2 a 8 °C e em seguida, removeu-se de forma cuidadosa o etanol 75% deixando o pellet do DNA, guardando em tubo aberto.

As amostram foram incubadas e homogeneizadas por 5 minutos de 15 a 30°C, adicionado então 200 µL de clorofórmio, com homogeneização vigorosa por 15s e incubação a 30°C por 3 minutos. Novamente, ocorreu uma homogeneização vigorosa por 15s e incubação de 30°C por 3 minutos e centrifugando a não mais que 12000G, por 15 minutos de 2 a 8 °C.

Após esta etapa foi adicionado 500 µL de álcool isopropílico e foram incubadas as amostras em 15 a 30°C por 10 minutos. Então centrifugadas, não mais que 12000G, por 10 minutos, 2 a 8 °C. O RNA precipitado forma um gel no fundo do tubo. O sobrenadante foi removido, adicionando então 1 mL de etanol 75%, e, em seguida homogeneização e centrifugação a não mais que 7500G, por 5 minutos de 2 a 8 °C. Por fim, retirou-se então o sobrenadante e se deixando o tubo semiaberto.

4.3 qPCR

Após realizar a eutanásia dos camundongos Balb/c por deslocamento cervical os baço dos animais dos 7 grupos foi divulsionado mecanicamente com auxílio de pinças e posteriormente submetidos a extração de RNA. O cDNA (DNA complementar) foi obtido por meio de RNA utilizando o kit GoScript™ Reverse Transcription System. O pellet de RNA foi homogeneizado em 15 µL H_2O tratada DEPEC (dietilpirocarbonato). Em outro frasco montou-se uma solução mix inicial para transcrição reserva com volume final de 24,5 µL. A solução submetida ao termociclador *Veriti®* da life *Technologies* com ciclo de 5 minutos e desnaturação a 65°C e incubação de 1 minuto. Em seguida foi adicionado 6,0 µL em tubo de solução enzimática (*Superscript IIIrt First Standard System Synhtheis for RT-PCR*) responsável pela transformação de RNA em cDNA, para essas soluções utilizou-se:

Tabela 1 – Composição de solução mix inicial para transcrição reserva e solução enzimática *Volume sugerido pelo Kit do fabricante. Concentrações já estabelecidas no Kit.

Reação cDNA	
Reagentes	Volume*
Oligo dt	0,5 µL
RNA	10 µl
Dntp	1,0 µL
H2O	13,0 µL
Tampão Kit 5x	4,0 µL
DTT 0,1 mol	1,0 µL
Enzima Superscript IIIrt	1,0 µL

Posteriormente, as amostras foram incubadas por 5 minutos a 25°C, seguida de incubação de 60 minutos a 50°C e inativação da reação a 70°C por 15 minutos. Por fim o

cDNA foi quantificado no *Qubit® 2.0 fluotomerter* (Life Technologies®) para posterior submissão a reação de qPCR com o kit GoTaq® qPCR MasterMix (Promega®) para verificar a expressão de RNAm dos fatores de transcrição T-bet, GATA3, RORγt e FOXP3 com controle endógeno de β-actina.

Tabela 2 – Representação de reação qPCR com o kit GoTaq® qPCR MasterMix da Promega. *Reagentes estabelecidos em Kit único com [ ] de fábrica.

Reação qPCR		
Reagentes	Volume	[ ] Aprox. *
Go Taq PCR master mix	12,5 µL	1.5 x
CXR reference DYE	0,2 µl	300 nM
Primer R	0,45 µL	1µM
Primer F	0,45 µL	1µM
Água nuclease free	11,3 µL	-
Amostra	0,1 µL	-

O qPCR foi realizado no aparelho 7900HT Fast Real Time System® da *life Technologies* e contiveram reações com volume de 25µL com seguinte ciclo, 95°C por 2 minutos para desnaturação do cDNA, 40 ciclos de 95°C por 15 segundos e temperatura determinada pelo fabricante do primer por 1 minuto para anelamento e extensão e uma curva de dissociação de 95°C por 2:00 minutos, 95°C por 15 segundos e 57° por 1 minuto.

Tabela 3 - demonstrando o primers *forward e reverse* dos fatores de trancrição de células T auxiliates dos perfis Th1 – T-bet, Th2 – GATA3, Th17 -RORγt e Treg- FOXP3, bem como a temperatura de anelamento dos mesmos em °C (graus Celsius)

GENE	PRIMERS	TEMPERATURA DE ANELAMENTO (°C)
T-BET FORWARD	5’-TCAACCAGCACCAGACAGAG-3’	65
T-BET REVERSE	5'-AAACATCCTGTAATGGCTTGTG-3’	
GATA3 FORWARD	5’-TTATCAAGCCCAAGCGAAG-3’	65
GATA3 REVERSE	5’-TGGTGGTGGTCTGACAGTTC-3’	
RORγt FORWARD	5’-ATGGACAGGGCCCCACACAGAGA-3’	58
RORγt REVERSE	5’CAAGTTCAGGACGCCTGGTTTCCTC-3’	

Foxp3 FORWARD	5’-ACTGCTGGCAAATGGAGTCT-3’	61
Foxp3 REVERSE	5’-AAGTAGGCGAACATGCGAGT-3'	
β-ACTINA FORWAR	5´TGTGATGGTGGGAATGGGTCAG 3´	65
β-ACTINA REVERSE	5´-TTTGATGTCACGCACGATTTCC- 3´	

4.4 Imunofluorescência

Para realização das imunofluorescência dos tumores e linfonodos segue protocolo para marcação. Leva-se a amostra congelada previamente em *tissue-tek* para o setor de imunofluorescência aonde a mesma é descongelada e colocada em um molde preenche-se o mesmo com tissue-tek e coloca-se a amostra a ser congelada. Este molde com tissue-tek e o fragmento a ser analisado é colocado no nitrogênio líquido por 4 minutos. Uma vez congelado a amostra não deve ser descongelada.

O bloco de tissue-tek congelado com a amostra segue para o criostato. O bloco foi colado no suporte usando tissue-tek. O criostato estava a temperatura de -24°C, se chegasse a -20°C o bloco começaria a descongelar prejudicando o manuseio e o corte do mesmo.

Por diferença de temperatura o corte (5µm) cola na lâmina que está à temperatura ambiente. Depois de cortada usamos solução de metanol fixar o corte na lâmina por 10 minutos. Lavamos a lamina 10 vezes com PBS previamente preparado com fosfato de potássio monobásico, fosfato de sódio bi básico, cloreto de sódio. Seguimos então a coloração que foi feita em cuba escura a fim de preservar os anticorpos da exposição direta a luz, que pode prejudicar a leitura da lâmina. Colocamos o anticorpo (volume variável) e a mesma teve incubação de 120 minutos. Lavamos 10 vezes com o PBS. Para fixação das lamínulas foi utilizado glicerol 3%.

5. RESULTADOS

5.1 Artigo 1 – Submissão para *Immunotheraphy – Future Medicine*

ICAM2 could be a marker of good prognosis in mice submitted to breast cancer development with 4T1 cells treated with dendritic cell immunotherapy

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ABSTRACT

INTRODUCTION: Dc Immunotherapy has taken a promising path. **OBJECTIVE:** evaluate behavior of adhesion molecules in dc immunotherapy. **MATERIALS AND METHODS:** 88 female Balb/c were divided into experimental groups, evaluated 7 and 14 days after dc immunotherapy. **RESULTS:** higher MFI of ICAM-1 in the lymphnodes and tumors in tumor group 14 days. Higher MFI of ICAM2 in tumor dc 14 days. Positive correlation in the lymphnodes with the ICAM-1 and the tumor volume in tumor group. Negative correlation between ICAM-2 and tumoral volume in lymph nodes of tumor group. **CONCLUSION:** increase in ICAM-2 in tumor dc and decrease in ICAM-1 leads us to conclude that dc vaccine positively influences immune system and that ICAM-2 could be a marker of good prognosis.

Key Words: Dendritic cell immunotherapy, Breast cancer, ICAM1, ICAM2, Active immunotherapy, CD49d, LFA1

LAY ABSTRACT

Previous results in experimental groups and in clinical study groups have shown that the dendritic cell vaccine is effective, reducing tumor volume, increasing the production of cytokines that polarize helper T cells for an ideal profile in the immune response against cancer and increase if also, the expression of the main transcription factor of this T cell profile, the Th1 cell profile.

However, for a better understanding of the immune response, improvements are needed in experimental models, analyzing tumor development and cell profile at different stages of treatment, checking the mechanisms involved in each step and the immune response cells and their mediators produced during this antitumor response.

In order to clearly describe what happens to the immune system during treatment with dendritic cell vaccines in animals induced to develop breast cancer with 4T1 cells, it is necessary to analyze the parameters previously analyzed only 30 days after the beginning

of treatment with dendritic cell vaccine, and now in 7 and 14 days after the start of treatment, so that we can understand the dynamics of the antitumor response, analyzing the data obtained at different dates, in order to analyze small changes in the immune system.

Introduction

Even though radiotherapy and chemotherapy are the most chosen anti-tumor therapies [1,2], some factors may limit their effectiveness. The imbalance between aggressive side effects of these therapies and a lack of response often lead patients to give up on treatments and stop attending the sections[2,3].

In recent years, immunotherapies have been gaining visibility in research mostly because they can be as effective as conventional therapies, but presenting minimal side effects. Among a myriad of immunotherapies, there are passive immunotherapies like immunological checkpoint block [4] and active immunotherapies, from which we can highlight the use of adaptive T cells [5] and dendritic cells vaccination [6].

Immunotherapies aim to overcome the immunological inhibition often caused by several tumor escape mechanisms, thus activating the immunologic system [7,8]. The final target of immunotherapies is always the destruction of the target cell by immune system cells, and it can be achieved by different manners. In this way, there is activation of cytotoxic T lymphocytes in a context of specific response while there could also be NK cells activation in a nonspecific response. However, the presence of molecules known as adhesion molecules is vital to lead these cells into maximum effective function [9].

Every cell of the immune system interacts with tumor cells in different ways during tumor development. Adhesion molecules are required in processes like cell trafficking to the site of infection, cell activation in lymph nodes, recruitment of new cells to the site of infection, tumor cell adhesion to another cell or even non-adhesion of tumor cells leading to metastasis. Moreover, it is possible to justify the presence or absence of metastases by the presence or not of adhesion molecules, which could also point to a prognosis regarding the development of the disease [9].

Adhesion molecules are divided into five classes: integrins, selectins, cadherins, IgSF immunoglobulin superfamily members and mucin [9]. Selectins are divided into P, E and L-selectins, based on which type of cell they are found [10]. Selectins are basically different in terms of time of expression, in which P-selectins are expressed in minutes while E-selectins are expressed in hours. Selectins are important for leukocyte migration to lymph nodes and skin [10]. Integrins are large heterodimers with one α and β chain [9]. There are different functions for each integrin subunit, where α subunits are responsible for determining the specificity of integrin ligands whilst β subunit is connected to the cytoskeleton and influences several signaling pathways [11].

IgSF is one of the largest and most diverse protein families. All members of this family contain at least one immunoglobulin or immunoglobulin-like domain and most members

are type I transmembrane proteins with extracellular domain and a cytoplasmic tail [9]. The best known members of this family include HLA I and II and TCR proteins [10].

ICAM-1 is expressed in endothelial cells and its role in the development of new tumors is well understood. ICAM-1 is known for participating in cell-to-cell, cell-to-cell matrix adhesion [12]. A classic study published in 2006 showed that blocking ICAM-1 with anti-CD54 inhibited growth in a mouse model of melanoma [13]. When MUC-1 binds to ICAM-1 present in endothelial cells, a calcium signal is induced intracellularly which results in two important effects: the release of chemokines, and the overexpression of ICAM-1 by tumor cells [12]. It is also well defined that the secretion of TNF-α, IFN-γ and IL-1 increases the expression of ICAM-1 in endothelial cells [12,14].

The expression of LFA-1 in hematopoietic cells makes it a potential target in leukemias and lymphomas [15], tumors of hematopoietic origin express LFA-1 and can be targeted with anti-LFA-1 [16]. The expression of LFA-1 in non-hematopoietic tumors has also been reported: in an experimental model of brain tumor in rats the presence of LFA-1 in metastatic cells contributed to tumor development [17].

Studies with non-hematopoietic tumors are not sufficient to make LFA-1 a direct target in non-hematopoietic tumors [15]. However, Treg cells present in the tumor infiltrate and the importance of LFA-1 for their function suggest that blocking this protein could limit the function of the Treg cells and improve the immune system's action against the tumor [15]

This study aims to evaluate the kinetics of the role of adhesion molecules ICAM-1, ICAM-2, LFA-1 and CD49d in tumor regression induced by immunotherapy with dendritic cells in mice bearing breast tumors. It looks forward to a possible prognosis marker for neoplasms. In order to accomplish this, analysis were performed through immunofluorescence techniques in tumors and lymph nodes from Balb/c lineage mice, where the molecules CD11a PE, CD49d PE, CD102 PE and CD54 PE were stained in lymph nodes and tumors from breast cancer bearing mice submitted or not submitted to dendritic cells immunotherapy.

MATERIAL AND METHODS

Experimental design

The experiment was submitted to the ethics committee on the use of animals at the Federal University of Triangulo Mineiro under protocol number 402. Experiments were performed with 6 to 8-weeks-old female Balb/c mice (n=88) housed in an animal facility in IPON - Oncology Research Institute. Animals were housed in cages (dimensions - 305x198x133mm) grouped in no more than 5 animals per cage and separated into different experimental groups. They were in controlled conditions under light/dark cycle (12h), controlled temperature around 22 °C (+/- 2 ° C), and allowed food and water *ad libitum*. Seventy mice were divided experimental groups like described below:

The 4T1 cells were cultured in RPMI complete medium in a 37°C humidified incubator with 5% CO_2. For transplants, cells were removed from incubator, washed twice with saline and then centrifuged at 290g for 10 min at 4 °C. After this, a solution of 50 µL with 2.0×10^5 4T1 cells were injected in the left breast of each animal through a 13x4.5 syringe. Beginning on day 7, tumor of the animals from groups that received breast tumor induction was measured at least every three days. Tumor volume was determined using the following formula:

$$\frac{[largest\ diameter\ x\ (smallest\ diameter)^{2}]}{2}$$

18 animals were used to produce dendritic cell vaccines. Four doses of dendritic cell vaccine were prepared for each euthanized animal. Therefore, 18 animals produced sufficient doses of dendritic cell vaccine to vaccinate 40 animals in groups GII, GV (Dendritic Cell Vaccine Group 7 and 14 days respectively), GIV and GVII (Treated Tumor group with Dendritic Cells Vaccine 7 and 14 days respectively). In order to produce vaccines, cells were removed from the bone marrow of femurs and tibias. For the production of the dendritic cell vaccine applied in the experimental groups, cells were taken from the bone marrow of the femurs and tibiae of the mice. These cells were placed in bottles of 25cm^2 (volume 7.5 ml) at an amount of 32.5×10^6, where under stimuli of GM-CSF (13µL; 10ng/µL), IL-4 (13µL; 10ng/µL), TNF -α (23 µL; 10ng/µL) and tumor antigen being a lysate of 4T1 cells (23 µL), they were differentiated into dendritic cells.

Groups were divided into groups where animals were euthanized seven days after the first dose of dendritic cell vaccine and 14 days after the first dose of dendritic cell vaccine. So there are 7 groups, GI (Control group), GII (Control group dendritic cells-7 days), GIII (Tumor group-7 days), GIV (Tumor group dendritic cells-7 days), GV (Group dendritic cells-14 days) , GVI (Tumor group-14 days) and GVII (Tumor group dendritic cells-14 days).

Therefore, animals related to groups GI, GII, GIII and GIV were euthanized on day 7 while other animals related to groups GV, GVI and GVII were euthanized on day 14 of the experimental period.

Immunofluorescence

The immunofluorescence of tumors and lymph nodes was performed in accordance with the following protocol. The sample previously frozen in tissue-tek was thawed and placed in a mold. It was filled with TISSUE TEK®. Both mold with TISSUE TEK® and the fragment to be analyzed were placed in liquid nitrogen for 4 minutes. The sample frozen with TISSUE TEK® went to the cryostat. The block was glued to the support using TISSUE TEK®.

Due to the difference of temperature, the cut (5µm) glued on the blade at room temperature. After cutting, we used a 30% methanol solution for 10 minutes in order to fix the sample on the blade. The slide was washed 10 times with PBS previously prepared

with monobasic potassium phosphate, bi-basic sodium phosphate and sodium chloride. We then followed the staining performed in a dark vat in order to preserve the antibodies from direct exposure to light, which could impair the reading of the slide. We placed the antibody (variable volume) and incubated for 120 minutes. We washed 10 times with PBS. It was used 3% of glycerol to cover the coverslips. The following markings were performed to this article: CD11a PE, CD49d PE, CD102 PE and CD54 PE.

The slides were analyzed using the inverted microscope system Eclipse TI-E Nikon. Subsequently, the images were quantified and analyzed using the software NISElements Advance Research, developed by Nikon. The slides were evaluated by 2 independent analyzers. The slides were scanned in zig zag until the end of the tissue. a total of 25 images were captured for each slide. Images were analyzed using the NIS-Elements Advance Research Analyzer software, where the medium fluorescence intensity of each marker point was determined in pixels. These values were transferred and analyzed with graphpad prism 5.0 software.

Statistical Analysis

The data obtained was analyzed using the GraphPad Prism 5 software. Kolmogorov-smirnov and Shapiro-wilk normality tests were performed. If the results passed the normality tests, ANOVA and T test parametric tests were performed; if they did not pass the normality tests, non-parametric Kruskal-Walis and Mann-Whitney tests were performed. Tests with a result of $p < 0.0001$ were considered statistically significant. For the correlations, normality tests and Pearson's correlation were performed.

RESULTS

Tumoral volume during experiment

Analyzing the development of the tumor volume, we noticed that the Treated group (GIV) 0 - 0.0018 (5.625e-005) mm3 presented a smaller tumor volume when compared to the group without treatment (GII) 0.0018 - 0.0640 (0.0426) mm^3 at the day 7th day. A Kruskal-Wallis nonparametric test was performed to obtain a $p < 0.0001$. At the 14th day, the tumor volume was even greater in the Tumor group without treatment (GVI) 0.045 - 1.944 (0.562) mm3 when compared to the Tumor group treated with dendritic cell immunotherapy (GVII) 0.0150 - 0.785 (0.143) mm3. Kruskal- nonparametric test Wallis also obtained a $p < 0.0001$ comparing the two groups, as shown in the figure 1.

Higher amount of ICAM2 in tumors from the group treated with dendritic cell immunotherapy in 14 days (GVII)

Analyzing ICAM1 in tumors from experimental groups, it is possible to see that the most MFI was in the Tumor group without treatment (GVI) at the 14th day 420.4 - 1529 (772.2) when compared to the others, as shown in the figure 2A. There is no significant difference in ICAM1 MFI when comparing the Tumor groups without treatment (GIII) 435.9 - 688.2 (551) and Treated tumor with dendritic cell immunotherapy (GIV) 416.4 - 860.1 (533) at the 7th day. In 14 days, it is possible to notice a significant difference when between both groups in question, where Tumor group (GVI) 420.4 - 1529 (772.2) and Treated tumor with dendritic cell immunotherapy (GVII) 340.9 - 914.6 (622, 1) at the 14th day. The untreated tumor group has a higher MFI when compared to the Treated tumor group with dendritic cell immunotherapy.

Analyzing ICAM2 in tumors from experimental groups, it is possible to notice the same pattern in both 7 and 14 days. In Figure 2C, it is possible to see that the Tumor group without treatment (GIII) 532.7 - 1073 (825.6) presents a higher MFI when compared to the Treated tumor group with dendritic cell immunotherapy (GIV) 409.1 - 801 (592.4) at the 7th day. The same pattern is repeated at the 14th day. However, in a comparison of groups that received immunotherapy with dendritic cells, the group GVII (at the 14th day) 584.9 - 888.4 (717.9) has a higher MFI compared to the group GIV (at the 7th day) 409.1 - 801 (592.4).

The presence of ICAM1 in the lymph nodes analyzed may be a sign of poor prognosis

Analyzing the CD54 (ICAM-1), we noticed that the Tumor group-14 days (GVI) obtained a higher mean fluorescence intensity when compared to the other groups 139.2 - 159.2 (147.3) and consequently also higher when compared to the Tumor group-7 days (GIII) 73, 06 - 88.10 (79.32). When comparing groups, we noticed that the Tumor group treated with dendritic cell vaccine-14 days (GVII) 59.45 - 87.81 (81.48) has a higher mean fluorescence intensity when compared to the tumor group treated with a 7-day dendritic cell vaccine (GIV) 36.27 - 64.28 (38.59). After performing the analysis of variance, we obtained a $p < 0.0001$ when comparing GI vs GIV, GII vs GIV, GIII vs GVI, GIV vs GVI and GVI vs GVII.

After analyzing the CD102 (ICAM-2), we noticed that the highest mean fluorescence intensity was found in the Control group (GI) 67.12 - 85.02 (83.42). When the Tumor groups 7 and 14 days were compared, we noticed that the highest mean fluorescence intensity was found in the Tumor group 7 days (GIII) 63.55 - 73.05 (66.9). When comparing the Tumor groups treated with the dendritic cell vaccine, we noticed that the Tumor group treated with the dendritic cell vaccine 14 days (GVII) 61.46 - 68.25 (66.52) obtained a higher mean fluorescence intensity as shown in the figure 3B. All groups passed the normality test, then the analysis of variance was performed and $p < 0.0001$ was obtained when comparing GIII vs GVI and GIV vs GVII.

As for CD49d, we noticed that the highest mean fluorescence intensity was found in the Tumor group at 14 days (GVI) 93.22 - 114.7 (98.65) followed by the Tumor group at

7 days (GIII) 91.38 - 101 (91.38). Regarding the Tumor group treated with the dendritic cell vaccine, we note that there is a slight increase in the group of 14 days (GVII) when compared to the group treated with 7 days (GIV) 60.12 - 66.07 (63.66), but both are smaller than the Tumor groups at days 7 and 14 respectively (GIII and GVI, respectively) (5A). The group treated only with the dendritic cell vaccine had a higher mean fluorescence intensity in 14 days (GVII) 77.59 - 89.57 (80.41) when compared to the group of 7 days (GIV) 65.13 - 69.68 (66.65). When Turkey's multiple comparison test was performed, we did not obtain a statistically significant “p”.

The LFA-1 showed higher mean fluorescence intensity in Tumor group at the 7th day (GIII) 96.19 - 143.5 (119.7) followed by tumor group at the 14th day (GVI) 88.47 - 102.7 (94.47). The highest mean fluorescence intensity among both groups is from the tumor group at the 7th day (GIII). The Tumor groups treated with dendritic cell vaccine had an average similar fluorescence intensity at the 7th day (GIV) 62.16 - 83.12 (71.05) when compared to group at the 14th day (GVII) 58.16 - 76.42 (65.67) as shown in the figure 5B. In GIV and GVII there was a higher MFI in 7 days (GIV) 68.65 - 86.52 (75.22) when compared to the group of 14-days (GVII) 5.96 - 49.49 (38.13). Variance analysis showed a $p < 0.0001$ when comparing GIII vs GVI.

Reverse behavior of ICAM1 and ICAM2 in analyzed lymph nodes and tumors

For tumors, there was a strong correlation when compared tumor development to ICAM1 ($r = 0.9150$) in Tumor group (6A). In Tumor group treated with dendritic cell immunotherapy, this correlation was weaker ($r = -0.1410$) (6C). Still in tumor, when analyzing the correlation between tumor development and ICAM2, negative correlation ($r = -0.5976$) (6B) in Tumor group and a positive correlation in Tumor group treated with dendritic cell immunotherapy ($r = 0.5544$) were noticed as shown in the figure 6D.

In lymph nodes, an opposite behavior was found when compared to tumors. A strong correlation ($r = 0.9297$) was found when analyzing ICAM1 to tumor development in the Tumor group as shown in the figure 7A. In the Tumor group treated with dendritic cell immunotherapy, this correlation became negative ($r = -0.06481$) (7C). Still in lymph nodes, a negative correlation was found when analyzing tumor development to ICAM3 in the Tumor group ($r = -0.7310$) (7B) while in Tumor group treated with cell immunotherapy there was a negative correlation ($r = -0.6482$) as shown in the figure 7D.

DISCUSSION

Described in 1974 by Steinman, dendritic cells are known as professional antigen presenting cells[18,19]. In the last two decades, immunotherapies have been gaining a prominent place in the fight against cancer [6]. Check point immunotherapies are already used as treatment for some types of cancer [20].

The use of dendritic cell immunotherapy is promising because of its ability to modulate both the innate and adaptive immune system for an effective response against tumor development [21–24].

In addition, dendritic cells have MHCI and MCHII molecules on their surface, which allows them to do what is known as cross-presentation, activating CD4 and CD8 lymphocytes [25]. The modulation caused by dendritic cells is capable of altering even the presence of some adhesion molecules involved in the immune response, such as ICAM1 and ICAM2 [9,26,27]. The role of some of these molecules will be discussed.

As previously demonstrated, the presence of LFA-1 is important to the immune response of cytotoxic lymphocytes and NK cells against the tumor, once its binding directs the released granules in a more favorable way to the destruction of tumoral cells [15]. However, if these cells have an immunosuppressive profile (i.e. as an infiltration of Treg lymphocytes), the immunosuppressive function of this cell will be exerted if this infiltrate is not inflammatory [28]. Our results demonstrate that, at all times, the Tumor group had a greater amount of LFA-1 in the lymph nodes when compared to the other groups. It has been shown that the infiltration of Treg lymphocytes in tumors is consistent with a poor prognosis [29].

Another important integrin in the process of combating the tumor is CD49d. As well as LFA-1, the presence of CD49d in tumor infiltrates of Treg profile cells is consistent with a poor prognosis [30]. Regarding this integrin, our results demonstrate the same profile as LFA-1, where the Untreated groups at 7 and 14 days (GIII and GVI) have a higher mean fluorescence intensity when compared to the Tumor Treated groups at 7 and 14 days (GIV and GVII).

Regarding the LFA-1 ligand, ICAM-1, our results demonstrate that the highest mean fluorescence intensity was found in the Tumor group without treatment at 7 and 14 days timepoints (GIII and GVI, respectively) when compared to the Treated Tumor group. From 7th to the 14th day, there is a significant increase in ICAM-1 in the untreated Tumor group. ICAM-1 is a marker of triple negative and more aggressive breast tumors [31]. Our results demonstrate that immunotherapy with dendritic cells may be attenuating this pro-tumor immune response. In the case of a prolonged tumor immune response, the tendency is a predominant T cell balance of Th17/Treg [32,33], which is favorable to the development of tumor and a poor prognosis. As we know, the production of inflammatory cytokines induces the highest expression of ICAM-1 [14]. It is possible to infer with our results that the treatment with dendritic cell vaccine makes it difficult to activate ICAM-1 due to the fact that the vaccine activates a Th1 cell profile, which would be necessary to generate an efficient antitumor response [34].

ICAM1 has been reported to be associated with more aggressive breast tumors [14] in addition to poor prognosis during an antitumor immune response [35]. The expression of ICAM1 is deeply associated with the proinflammatory cytokine TNF-α [36]. TNF-α is a cytokine present in the Th17 cell profile, a cell profile that induces an exacerbated inflammatory response [37] and that during an antitumor response has a dichotomous role [38–41]. These roles might be against tumor development by inducing an inflammatory response or favoring the tumor development by facilitating the formation of metastatic sites [40].

It is known that ICAM-2 expression is beneficial. Studies show that the role of ICAM-2, differently from ICAM-1, decreases tumor development and its metastatic potential [42,43]. Given that, it can be inferred that ICAM2 would be a marker of good prognosis. Our results showed that the Treated Tumor group-14 days (GVII) showed a higher mean fluorescence intensity when compared to the Tumor group-14 days (GVI). The Treated Tumor group (GIV) had a lower mean fluorescence intensity at the 7th day when compared to the Tumor group (GIII) at the same time point. This increase in ICAM-2 in the Treated Tumor group leads us to the conclusion that the dendritic cell vaccine positively influences the immune system in fighting the tumor.

From what has been described in the literature regarding cancer-related ICAM2, it is known that the protein is associated with an antitumor immune response in the early stages of cancer [44,45]. It has also been shown that once induced by p53, a protein that provides protection to the genome, ICAM2 plays a role in inhibiting the migration and invasion; it has also been shown that p53 activation is related to the higher expression of ICAM2 [46]. Another study published in 2011 demonstrated that ICAM2 plays a role in the anti-tumor immune response by inducing the infiltration of immature myeloid dendritic cells in patients with pancreatic cancer [44].

CONCLUSION

With our results, we demonstrated that in dendritic cell immunotherapy, ICAM2 effectively acts as a marker of good prognosis. We can infer this according to results found as an increase in ICAM-2 in the Treated Tumor group and a decrease in adhesion molecules that could facilitate tumor development and a possible metastasis such as LFA-1, ICAM-1 and CD49d. Corroborating these results we noticed that the tumor volume in the Treated Tumor group in general is smaller when compared to the Tumor group. The role of adhesion molecules in immunotherapies in the context of cancer is yet not very clear, more studies should be carried out in this way so that we can define the role of these molecules in the response of immunotherapies.

FUTURE PERSPECTIVES

We hope that with this work there will be a greater interest in understanding the dynamics of the immune response in active immunotherapies and that in the coming years the mechanisms that involve the immune response will be better described. This could bring the possibility of better vaccination protocols, being able to combat tumor development more effectively and that with that dendritic cell vaccination protocols can be improved.

Summary points

- Kinetic evaluation of the immune system in dendritic cell immunotherapy against breast cancer.

- Difference of tumoral volume in tumor treated and untreated groups.

- Different behavior of ICAM2 compared to ICAM1 in experimental groups.

- Can ICAM2 act as a marker of good prognosis?

Figure and tables Legends

Table 1. Table showing the experimental design. Among the 7 experimental groups, the table shows which group was challenged with 4T1 cells, the day during the experimental period in which the groups were euthanized and which group were treated with dendritic cell immunotherapy.

Figure 1. Increase in tumor volume in the untreated tumor group compared to the tumor group treated with dendritic cell immunotherapy. Graphical representation of the tumor volume from the Tumor and Treated Tumor groups throughout the experimental period. The tumors from the Tumor and Treated Tumor groups were measured on days 7 and 14 of the experimental period and are represented in this graph in mm3. The Tumor group received tumor induction of 4T1 cells and was not treated with dendritic cell immunotherapy, while the Treated group received tumor induction of 4T1 cells and was treated with up to 3 doses of dendritic cell immunotherapy. **** represents the value of $p < 0.0001$ in the kruskal-wallis analysis of variance.

Figure 2. Graphical representation of the increase in ICAM1 in the tumor and tumor treated groups (A, B) and differentiated behavior of ICAM2 in the tumor and treated tumor groups in 7 and 14 days. Graphic representation showing the mean fluorescence intensity (MFI) of ICAM1(A, B) and ICAM2 (C, D) in the tumors of the experimental groups. The groups that presented tumors were analyzed so there is a Tumor group-7 day (GIII), a Treated tumor group with dendritic cell immunotherapy-7 days (GIV), a Tumor group-14 days (GVI) and a Treated tumor group with dendritic cell immunotherapy-14 days (GVII). Mann-Whitney test was performed between groups Tumor 7 and Tumor 14 days, obtaining $p=0.002$.

Figure 3. Graphical representation of the increase in ICAM1 in the lymph nodes of the control dendritic cell group, tumor untreated and tumor group treated with immunotherapy of dendritic cells in 7 and 14 days. (A) Average fluorescence intensity of ICAM1 in the Control group **(GI)**, Control group 7 days dendritic cells **(GII)**, Tumor group 7 days **(GIII)** and Tumor group treated with dendritic cell immunotherapy 7 days **(GIV)** . **(B)** Mean fluorescence intensity of ICAM1 in the Control groups **(GI)**, Control group dendritic cells 14 days **(GV)**, Tumor group 14 days **(GVI)** and Tumor group treated with dendritic cell immunotherapy 14 days **(GVII)**. **(C)** Mean fluorescence intensity of ICAM1 in the **GII, GIII, GIV, GV, GVI and GVII** groups. Mann-Whitney test was performed between groups Tumor 7 and Tumor 14 days, obtaining $p=0.0016$.

Figure 4. Graphical representation of the increase in ICAM2 in the lymph nodes of the dendritic cell control group, untreated tumor group and tumor group treated with dendritic cell immunotherapy in 7 and 14 days. (A) Average fluorescence intensity of ICAM2 in the Control group **(GI)**, Control group 7 days dendritic cells **(GII)**, Tumor group 7 days **(GIII)** and Tumor group treated with dendritic cell immunotherapy 7 days **(GIV) . (B)** Mean fluorescence intensity of ICAM2 in the Control groups **(GI)**, Control group dendritic cells 14 days **(GV)**, Tumor group 14 days **(GVI)** and Tumor group treated with dendritic cell immunotherapy 14 days **(GVII). (C)** Mean fluorescence intensity of ICAM2 in groups **GII, GIII, GIV, GV, GVI and GVII**. * represents $p < 0.0001$ after performing the Kruskal-Wallis non-parametric test.

Figure 5. Graphical representation of CD49d (A) and LFA-1 (B) in the lymph nodes of the dendritic cell control groups, tumor and tumor treated with dendritic cell immunotherapy in 7 and 14 days. Graph of MFI by the immunofluorescence technique in the lymph nodes of the various experimental groups of the CD49d (A) and LFA-1 (B) molecules. GI represents Control group, GII Control group CD 7 days, GIII Tumor group 7 days, GIV Treated Tumor Group 7 days, GV Control group CD 14 days, GVI Tumor Group 14 days and GVII Treated Tumor treated 14 days.

Figure 6. Pearson's correlation coefficient showing different behavior of ICAM1 vs tumor volume and ICAM2 vs tumor volume in tumors of the tumor and tumor group treated with dendritic cell immunotherapy. It is showing a correlation between tumor development and the adhesion molecules ICAM1 and ICAM2 in tumors from experimental groups induced to develop 4T1 cells breast tumor treated and untreated with dendritic cell immunotherapies. (A) ICAM1 vs tumor volume in Tumor group, (B) ICAM1 vs tumor volume in Treated tumor group, (C) ICAM2 vs tumor volume in Tumor group, (D) ICAM2 vs tumor volume in Treated tumor group.

Figure 7. Pearson's correlation coefficient showing different behavior of ICAM1 vs tumor volume and ICAM2 vs tumor volume in the lymph nodes of the tumor and tumor groups treated with dendritic cell immunotherapy. It is showing correlation between tumor development and the adhesion molecules ICAM1 and ICAM2 in lymph nodes from the experimental groups induced to develop 4T1 cells breast tumor treated and untreated with dendritic cell immunotherapies. (A) ICAM1 vs tumor volume in Tumor group, (B) ICAM1 vs tumor volume in Treated tumor group, (C) ICAM2 vs tumor volume in Tumor group, (D) ICAM2 vs tumor volume in Treated tumor group.

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Figures and tables

	GI	GII	GIII	GIV	GV	GVI	GVII
EUTHANASIA 7 DAYS AFTER FIRST IMMUNOTHERAPY	X	X	X	X			
TUMOR INDUCTION WITH 4T1 CELLS			X	X		X	X
SUBMITTED TO DENDRTIC CELL IMMUNOTHERAPY		X		X	X		X
EUTHANASIA 14 DAYS AFTER FIRST IMMUNOTHERAPY					X	X	X

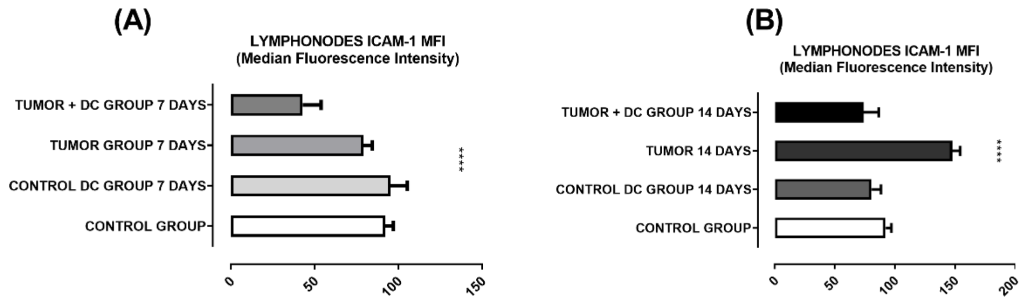
****
****
****
TUMORAL VOLUME mm^3
2.5
2.0
1.5
1.0
0.5
0.0
TUMOR 7 DAYS
TUMOR 14 DAYS
TUMOR DC 7 DAYS
TUMOR DC 14 DAYS

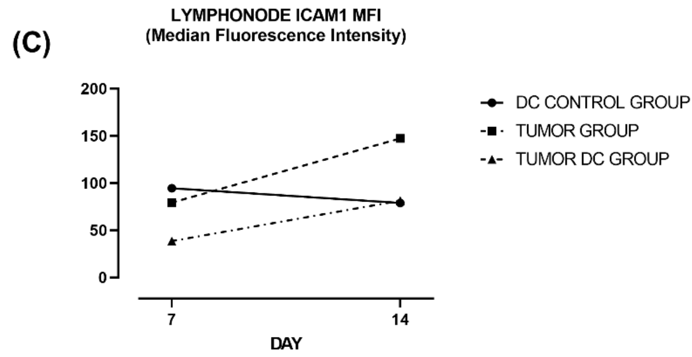
(A)
ICAM-1 MFI (Median Fluorescence Intensity)
TUMOR + DC GROUP 14 DAYS
TUMOR GROUP 14 DAYS
TUMOR + DC GROUP 7 DAYS
TUMOR GROUP 7 DAYS
****
0 500 1000 1500

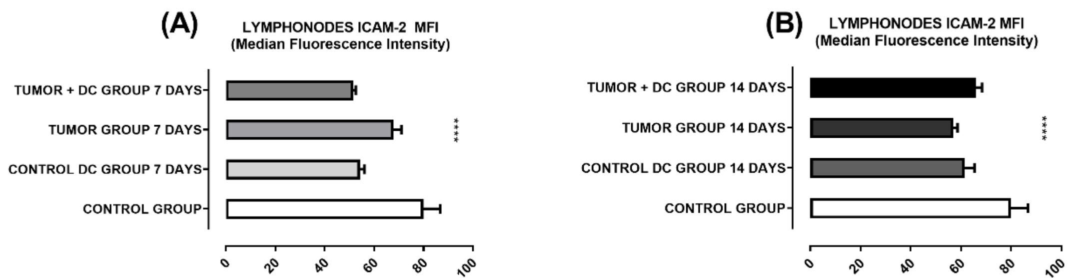
(B)
TUMOR ICAM1
MFI (Median Fluorescence Intensity)
700 650 600 550 500 450
7 14
DAY
TUMOR GROUP
TUMOR DC GROUP

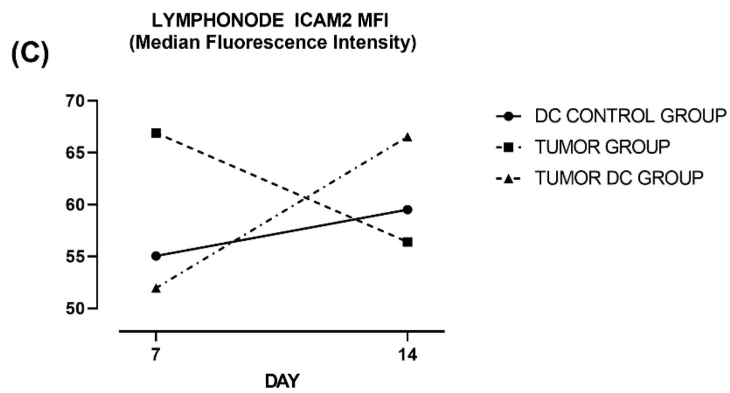
(C)
ICAM-2 MFI (Median Fluorescence Intensity)
TUMOR + DC GROUP 14 DAYS
TUMOR GROUP 14 DAYS
TUMOR + DC GROUP 7 DAYS
TUMOR GROUP 7 DAYS
****
0 500 1000 1500

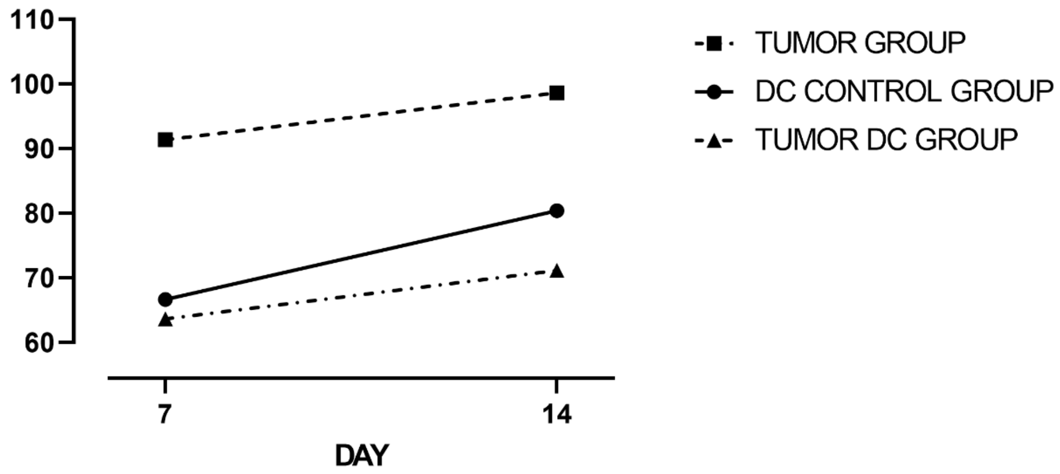
(D)
TUMOR ICAM2
MFI (Median Fluorescence Intensity)
800 750 700 650 600
7 14
DAY
TUMOR GROUP
TUMOR DC GROUP

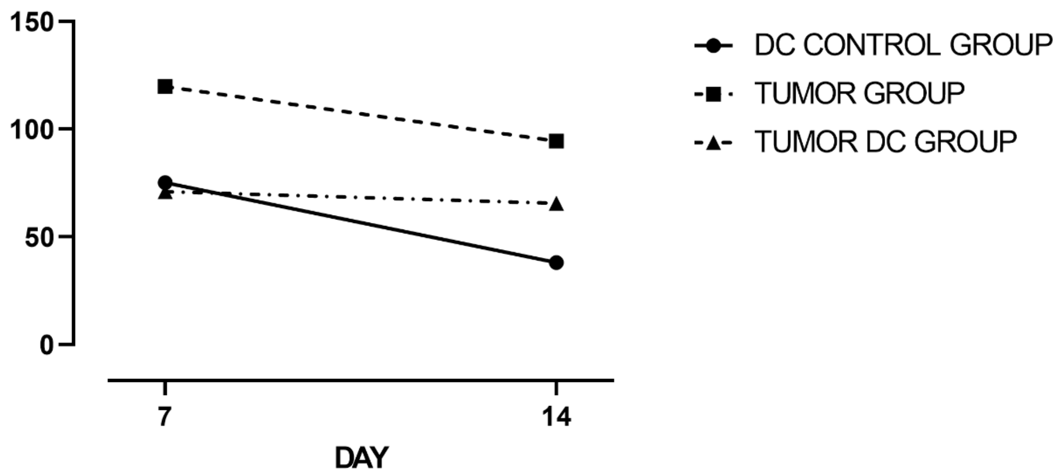

(A)
LYMPHONODES ICAM-1 MFI
(Median Fluorescence Intensity)
TUMOR + DC GROUP 7 DAYS
TUMOR GROUP 7 DAYS
CONTROL DC GROUP 7 DAYS
CONTROL GROUP
****
0
50
100
150
(B)
LYMPHONODES ICAM-1 MFI
(Median Fluorescence Intensity)
TUMOR + DC GROUP 14 DAYS
TUMOR 14 DAYS
CONTROL DC GROUP 14 DAYS
CONTROL GROUP
****
0
50
100
150
200

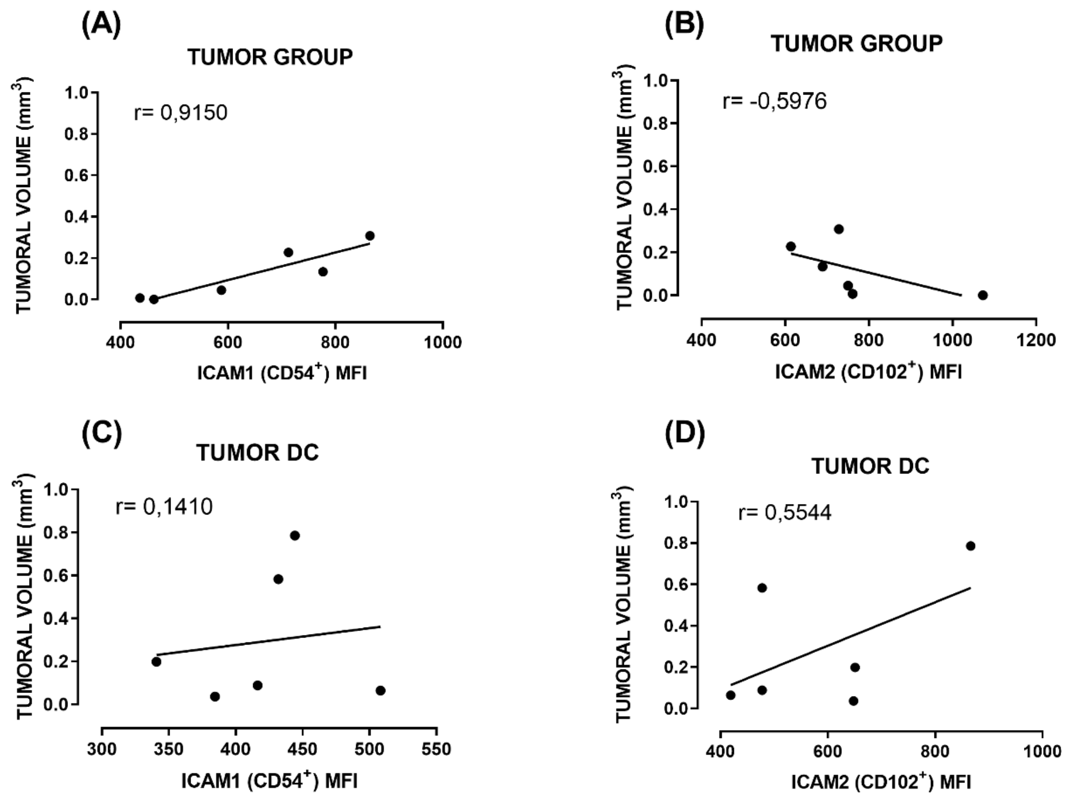

(C)
LYMPHONODE ICAM1 MFI
(Median Fluorescence Intensity)
200
150
100
50
0
7
14
DAY
DC CONTROL GROUP
TUMOR GROUP
TUMOR DC GROUP

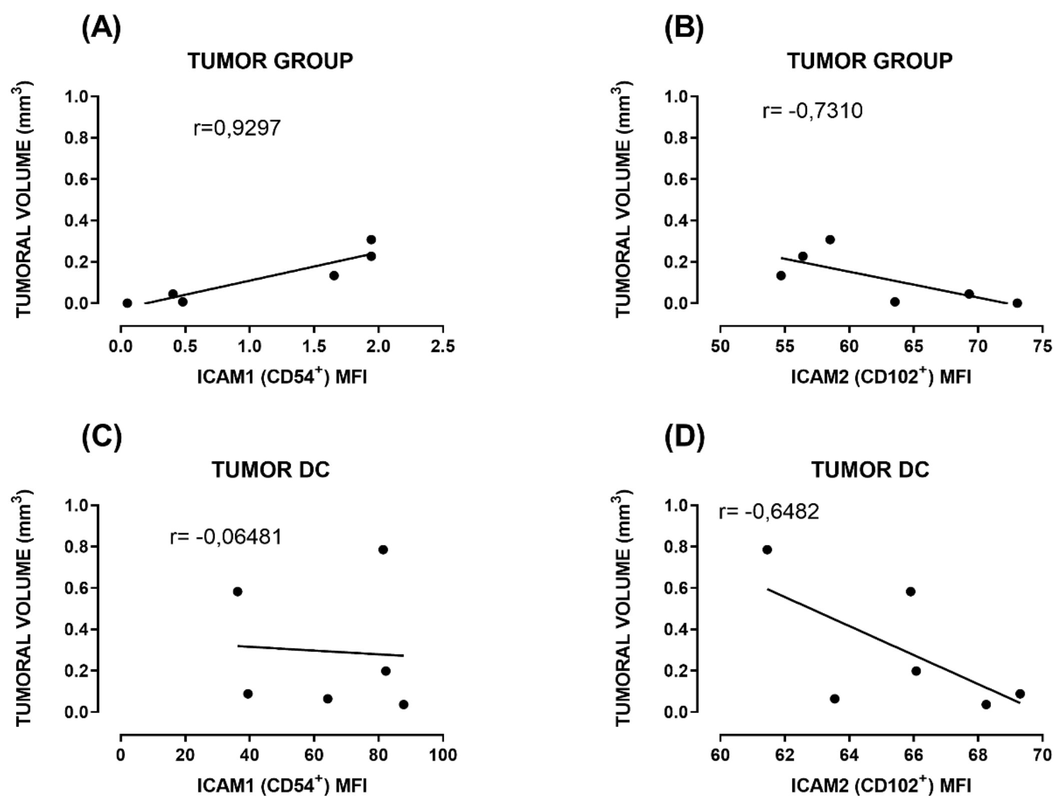

(A)
LYMPHONODES ICAM-2 MFI
(Median Fluorescence Intensity)
TUMOR + DC GROUP 7 DAYS
TUMOR GROUP 7 DAYS
CONTROL DC GROUP 7 DAYS
CONTROL GROUP
****
0
20
40
60
80
100
(B)
LYMPHONODES ICAM-2 MFI
(Median Fluorescence Intensity)
TUMOR + DC GROUP 14 DAYS
TUMOR GROUP 14 DAYS
CONTROL DC GROUP 14 DAYS
CONTROL GROUP
****
0
20
40
60
80
100


(C)
LYMPHONODE ICAM2 MFI
(Median Fluorescence Intensity)
70
65
60
55
50
7
14
DAY
DC CONTROL GROUP
TUMOR GROUP
TUMOR DC GROUP


(A)
LYMPHONODE CD49 MFI
(Median Fluorescence Intensity)
110
100
90
80
70
60
7
14
DAY
TUMOR GROUP
DC CONTROL GROUP
TUMOR DC GROUP


(B)
LYMPHONODE LFA1 MFI
(Median Fluorescence Intensity)
150
100
50
0
7
14
DAY
DC CONTROL GROUP
TUMOR GROUP
TUMOR DC GROUP


TUMOR
(A)
TUMOR GROUP
r= 0,9150
TUMORAL VOLUME (mm3)
ICAM1 (CD54+) MFI
(B)
TUMOR GROUP
r= -0,5976
TUMORAL VOLUME (mm3)
ICAM2 (CD102+) MFI
(C)
TUMOR DC
r= 0,1410
TUMORAL VOLUME (mm3)
ICAM1 (CD54+) MFI
(D)
TUMOR DC
r= 0,5544
TUMORAL VOLUME (mm3)
ICAM2 (CD102+) MFI


LYMPHONODE
(A)
TUMOR GROUP
r=0,9297
TUMORAL VOLUME (mm3)
ICAM1 (CD54+) MFI
(B)
TUMOR GROUP
r= -0,7310
TUMORAL VOLUME (mm3)
ICAM2 (CD102+) MFI
(C)
TUMOR DC
r= -0,06481
TUMORAL VOLUME (mm3)
ICAM1 (CD54+) MFI
(D)
TUMOR DC
r= -0,6482
TUMORAL VOLUME (mm3)
ICAM2 (CD102+) MFI

5.2 Artigo 2 – Submetido e aceito na revista Brazilian Journal od development

DYNAMIC ANALYSIS OF THE IMMUNOLOGICAL RESPONSE OF BALB/C MICE WITH EXPERIMENTAL BREAST CANCER SUBMITTED TO IMMUNOTHERAPY TREATMENT OF DENDRITIC CELL

ANÁLISE DINÂMICA DA RESPOSTA IMUNOLÓGICA DE CAMUNDONGOS BALB/c COM CÂNCER DE MAMA EXPERIMENTAL SUBMETIDO A IMUNOTERAPIA DE CÉLULAS DENDRÍTICAS

INTRODUCTION: Cancer is a complex disease because it is capable of inhibiting the immune response through tumor escape mechanisms. **OBJECTIVE:** analyze the dynamics of the immune system during cancer treatment with dendritic cell immunotherapy. We evaluated the presence of tumor infiltrate of CD8 T lymphocytes, transcription factors of CD4 T lymphocytes in the spleen of these animals, the development of tumor volume and the behavior of coestimulatory molecules. **METHODS:** 70 female Balb/c mice were divided into experimental and control groups, they were evaluated on the 7th e 14th days after tumor challenge and dendritic cell immunotherapy. **RESULTS:** Molecules such as T-bet, showed an increased expression in treated tumor group. Our results also demonstrated the higher MFI of CD8 + T lymphocytes infiltrate in the treated groups. Thus, there is a greater MFI of protumoral co-stimulatory molecules such as CTLA4 in the untreated groups. **CONCLUSION:** the immune system is able to modulate an immune response against tumor within 14 days if the organism is being treated with dendritic cell immunotherapy.
Keywords: *dendritic cell immunotherapy, breast cancer, active immunotherapy.*
INTRODUÇÃO: O câncer é uma doença complexa, pois é capaz de inibir a resposta imune por meio de mecanismos de escape tumoral. **OBJETIVO:** analisar a dinâmica do sistema imunológico durante o tratamento do câncer com imunoterapia de células dendríticas. Avaliamos a presença de infiltrado tumoral de linfócitos T CD8, fatores de transcrição de linfócitos T CD4 no baço desses animais, o desenvolvimento do volume tumoral e o comportamento de moléculas coestimulatórias. **MÉTODOS:** 70 camundongos Balb/c fêmeas foram divididos em grupos experimental e controle, avaliados no 7º e 14º dias após o desafio tumoral e imunoterapia com células dendríticas. **RESULTADOS:** Moléculas como a T-bet, mostraram uma expressão aumentada no grupo de tumor tratado. Nossos resultados também demonstraram a maior IMF de infiltrado de linfócitos T CD8 + nos grupos tratados. Assim, há uma maior MFI de moléculas coestimulatórias protumorais, como CTLA4, nos grupos não tratados. **CONCLUSÃO:** o sistema imune é capaz de modular uma resposta imune contra o tumor em 14 dias se o organismo estiver sendo tratado com imunoterapia com células dendríticas.
Palavras-chave: imunoterapia com células dendríticas, câncer de mama, imunoterapia ativa.

INTRODUCTION

Immunotherapies are therapies that have the function of activating the immune system against an infection, and in a case of cancer, immunotherapy aims to activate the

immune system previously inhibited by tumor escape mechanisms [1]. Immunotherapies are being highlighted in research in recent years precisely because they are as effective as conventional therapies and have minimal side effects when compared to conventional therapies [1–3] . Among a plenty of immunotherapies, there are passive immunotherapies such as the immune checkpoint blockade [4] and active immunotherapies such as the use of adoptive T cell therapy [5], dendritic cell vaccination [6], and others.

The immune surveillance theory postulates that the body's own cells verify the body looking for infections that can be combated [7]. Once the tumor formation is an inflammatory process, the concept of Immunoediting divides immune response to tumors into three phases: recognition, where the cells of the immune system recognize tumor development and destroy tumor cells [7]. The equilibrium phase, where a group of cells with potential to become cancer are in anergy state, so they do not induce immune response and do not present cancer characteristics [8,9]. Finally the escape phase, where the anergic state cells begin an unregulated division, thus presenting high tumorigenic potential, also called tumor escape mechanisms. These mechanisms are different ways that the tumor can circumvent the immune response in order to keep growing [7–9].

In an ideal immune response against cancer, tumor antigens are processed in small peptides into antigen presenting cells and presented via MHC molecule to T lymphocytes [10]. T lymphocytes are divided in two classes, CD8+ T lymphocytes and CD4+ T lymphocytes. CD8+ T lymphocytes, also known as cytotoxic T lymphocytes, have the function of first recognizing the antigen presented via MHCI and then destroying the cell that presented that antigen [11]. CD4+ T lymphocytes recognize the antigen presented via MHCII and, when activated, the cell modulates the immune response by producing cytokines that will lead the immune response to fight against the presented antigen [12].

Cancer is seen as a complex disease because it inhibits the immune response through tumor escape mechanisms. Among these mechanisms, it is possible to cite the tumor ability of the producing soluble mediators that contribute tumor development such as immunosuppressive cytokines, metalloproteinases, and vascular growth factors [13]. Another tumor escape mechanism is the suppression of the immune response through the suppression of the MHCI, thus preventing tumor cells from being destroyed by CD8+ T lymphocytes [14]. Dendritic cells are used in active immunotherapies because they are also antigen-presenting cells [15,16]. They are able to process and present antigens both via

MHCI and MHCII without being destroyed by CD8 + T lymphocytes, which is a mechanism called cross-presentation [17].

It is extremely important to understand the mechanisms of action of dendritic cells in immunotherapies and the mechanisms triggered by it, so researchers will be able to develop new and better protocols for differentiation and maturation of dendritic cells *in vitro* in order to improve dendritic cell immunotherapy quality [18].

OBJECTIVE

Our aim is to evaluate the immune response against breast cancer in mice submitted to dendritic cells immunotherapy and also understand how long it takes to a modulation of the immune response towards tumor control and reduction of tumor volume by performing techniques such as immunofluorescence and qPCR .

In order to do this, immunofluorescence will evaluate the presence of tumor infiltrate and also the presence of co-stimulatory molecules in lymphoid organs. In addition, CD8+ will be stained in tumors while CD3+, CD152 (CTLA4), and IA (MHCI) will be stained in lymph nodes. Furthermore, it is going to be evaluated if dendritic cells immunotherapy plays a role on the differentiation of auxiliary T lymphocytes to Th1, Th2, Th17 and Treg profiles through qPCR expression assessment of transcription factors T-bet, GATA3, RORγt, and FOXP3, respectively.

METHODOLOGY

EXPERIMENTAL DESIGN

The experiment used 88 female Balb/c mice, aged 6 to 8 weeks, from the sectoral vivarium of IPON - Oncology Research Institute. The animals were housed in cages (dimensions - 305x198x133mm), grouped in no more than 5 animals per cage, separated into different experimental groups, being subjected to environments with light/dark cycle (12h), controlled temperature around 22 ° C, +/- 2 ° C, with food and water *ad libitum*. Of these 88 mice, 70 were divided into the experimental groups described below:

GI- Control group consisting of 10 animals. This group was not induced to tumor development by the 4T1 breast tumor cell line and was also not treated with a dendritic cell vaccine;

GII - Dendritic Cell Vaccine Group, consisting of 20 animals that were not induced to tumor development by the 4T1 cell line, but were submitted to the dendritic cell vaccination protocol;

GIII - Tumor Group without treatment with dendritic cell vaccine, this group consists of 20 animals that were induced to tumor development by the 4T1 cell line, but were not submitted to the dendritic cell vaccination protocol.

GIV - Tumor Group Treated with Dendritic Cell Vaccine, consisting of 20 animals that were induced to tumor development by the 4T1 cell line and also submitted to a dendritic cell vaccination protocol.

The 4T1 cells were maintained in complete RPMI media in a CO2 incubator with 5% humidity at 37°C. For transplants, cells were removed from the incubator, washed twice with saline and centrifuged for 10 min. at 290g at 4°C. Then they were injected with a 13x4.5 syringe in the left breast of each animal with a transplant of 2.0 x 10^5 4T1 cells in 50 µL of solution. From day 7 the tumor of animals in groups submitted to developing a breast tumor was measured at least every three days. The tumor volume was determined using the following formula (Carlsson G et. al; 1983):

$$\frac{[\text{largest diameter } \times (\text{smallest diameter})^2]}{2}$$

The remaining 18 animals were used to make dendritic cell vaccines. For each 1 euthanized animal, 4 doses of dendritic cell vaccine were made. In this way the 18 animals produced sufficient doses of dendritic cell vaccine to vaccinate the 40 animals in groups GII and GIV. For the production of vaccines applied in GII (Dendritic Cell Vaccine Group) and GIV (Tumor Group treated with Dendritic Cell Vaccine) cells were removed from the bone marrow of femurs and tibiae.

The removed cells were then placed in 25cm^2 flasks (volume 7.5 ml) in an amount of 32.5x10^6 per flask, with supplemented IMDM culture medium. Under stimuli of GM-CSF (13µL; 10ng / µL), IL-4 (13µL; 10ng / µL), TNF-α (23 µL; 10ng / µL) and tumor antigen of 4T1 cells (23µL), the cells were differentiated into dendritic cells.

Subsequently, the groups were subdivided into other groups in which animals were euthanized seven days after the first dose of dendritic cell vaccine and other groups where the animals were euthanized 14 days after the first dose of dendritic cell vaccine. In this way, the total amount of groups increased from 4 to 7 groups: GI (control group), GII (control group dendritic cells 7 days), GII (tumor group 7 days), GIV (tumor group dendritic cells 7 days), GV (control group dendritic cells 14 days) , GVI (tumor group 14 days) and GVII (tumor group dendritic cells 14 days).

Therefore, on the 7th day of the experimental period, animals related to the groups GI, GII, GIII and GIV were euthanized, the other animals related to the groups GV, GVI and GVII were euthanized on the 14th day of the experimental period. The results obtained were verified and analyzed by the GraphPad Prism 5.0 software.

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The following protocol was used for immunofluorescence staining of tumors and lymph nodes. The sample previously frozen in tissue-tek is taken to the immunofluorescence sector where it is thawed and placed in a mold, it is filled with TISSUE TEK and the sample to be frozen is placed. This mold with TISSUE TEK and the fragment to be analyzed are placed in liquid nitrogen for 4 minutes. Once frozen, the sample must not be defrosted.

The TISSUE TEK block frozen with the sample was taken to the cryostat. The block was placed in the holder using TISSUE TEK. The cryostat was at a temperature of -24°C, if it reached -20°C the block would start to thaw, impairing its handling and cutting.

Due to the temperature difference, the cut (5µm) adheres to the blade at room temperature. After cutting, we used a 30% methanol solution to fix the cut on the blade for 10 minutes. We washed the slide 10 times with PBS previously prepared with monobasic potassium phosphate, bi-basic sodium phosphate, sodium chloride. We then followed the staining that was done in a dark vat in order to preserve the antibodies from direct light exposure, which can impair the reading of the slide. We placed the antibody (variable volume) and incubated for 120 minutes. We washed 10 times with PBS. To

cover the coverslips, 3% glycerol was used. The markings performed were as follows: CD11a PE, CD49d PE, CD102 PE and CD54 PE.

qPCR

Part of the spleen was stored in trizol and RNA was subsequently extracted. The cDNA obtained from the RNA using the GoScript ™ Reverse Transcription System kit, according to the manufacturer's protocol, was subjected to a qPCR reaction to check the gene expression of the following transcription factors: T-bet, GATA-3, FOXP3 and RORγt. For the same samples, the endogenous control used was β-actin. The pre-assembled primers are:

GENE	PRIMERS	ANNEALING TEMPERATURE (CELSIUS)
T-BET FORWARD	5’-TCAACCAGCACCAGACAGAG-3’	
T-BET REVERSE	5'-AAACATCCTGTAATGGCTTGTG-3’	65
GATA3 FORWARD	5’-TTATCAAGCCCAAGCGAAG-3’	
GATA3 REVERSE	5’-TGGTGGTGGTCTGACAGTTC-3’	65
RORγt FORWARD	5’-ATGGACAGGGCCCCACACAGAGA-3’	
RORγt REVERSE	5’CAAGTTCAGGACGCCTGGTTTCCTC-3’	58
Foxp3 FORWARD	5’-ACTGCTGGCAAATGGAGTCT-3’	
Foxp3 REVERSE	5’-AAGTAGGCGAACATGCGAGT-3'	61
β-ACTIN FORWARD	5´TGTGATGGTGGGAATGGGTCAG 3´	
β-ACTIN REVERSE	5´-TTTGATGTCACGCACGATTTCC- 3´	65

TABLE 1 – Genes used for qPCR assay. Nucleotide sequence and annealing temperature for each primer.

STATISTICAL ANALYSIS

Data obtained were analyzed using the GraphPad Prism 5 software. Kolmogorov-smirnov and Shapiro-wilk normality tests were carried out, if the results passed the normality tests, ANOVA and T test parametric tests were performed, if they did not pass the normality tests, non-parametric Kruskal-Wallis and Mann-Whitney tests were chosen. Tests with a result of $p < 0.0001$ were considered statistically significant. For the correlations, normality tests and Pearson or Spearman correlation were performed.

RESULTS

Dendritic cell vaccine in the activation of T lymphocytes

Our results demonstrated that the mean fluorescence intensity of cells with CD3 in the lymph nodes among all experimental groups is the highest in the control group DC 7 days (76.49 - 116) 92.8, followed by the tumor group 7 days (55.63 - 98.73) 74.45 with a statistical difference of $p < 0.05$ between the two groups. When comparing the groups Tumor 14 days (68.25 - 78.92) 71.99 and Tumor Treated 14 days (67.66 - 78.36) 71.27, we did not notice any statistically significant difference. When comparing CD3 + in the Treated Tumor and Tumor groups, we noticed that in 7 days the MFI is higher in the Tumor group (55.63 - 98.73) 74.45 when compared to the Treated Tumor group (47.20 - 53.20) 49 , 33. In 14 days, we noticed that MFI levels are close when we compare the Tumor group (68.25 - 78.92) 71.99 to the Treated Tumor group (67.66 - 78.36) 71.27.

In terms of the CD8+ cells in the tumor, we noticed the highest MFI in the Tumor Treated 14 days (740.6 - 1347) 1123 when compared to the other groups. When comparing the group Tumor 14 days (544.9 - 1873) 544.9, there is a statistically significant difference with a value of $p < 0.0001$. When comparing the CD8+ MFI kinetics in the tumors from treated and untreated groups, we noticed first that the Treated Tumor group had a higher MFI when compared to the Tumor group in 7 days and second, the Treated Tumor group had a higher MFI when compared to the Tumor group in 14 days.

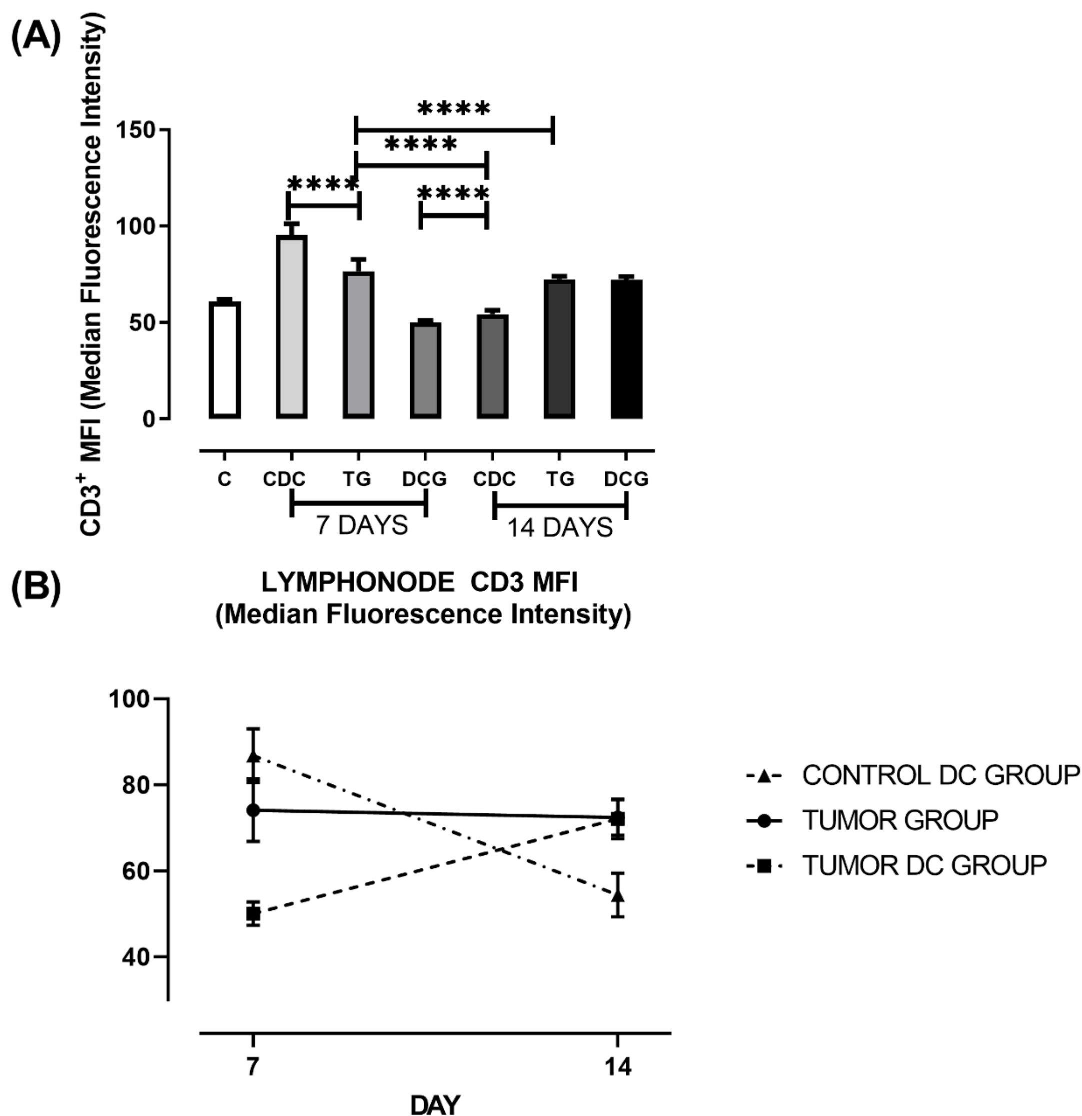

FIGURE 1 – Mean Fluorescence Intensity (MFI) of CD3+ cells in lymph nodes with immunofluorescence technique. (A) represents MFI of CD3+ cells in experimental groups. C= control group, CDC= control dendritic cell group, DCG= dendritic cell group. (B) representation of the kinetics involving MFI of CD3 + cells in the tumor and tumor DC groups from 7 to 14 days after the first dose of dendritic cell immunotherapy. **** represents the value of p <0.0001.

Activation of co-stimulatory molecules

CTLA-4 or C152 is known as a co-stimulatory molecule holding an inhibitory role. It was performed a correlation between the tumor volume and the presence of this molecule in the lymph nodes. Our results demonstrate that the treated groups show a negative correlation, wherein the Tumor group DC 7 days (r = -0.7826) and the Tumor

group DC 14 days (r = -0.4472). This means that the greater the presence of CTLA4, the lower the tumor volume in both groups. In groups without any treatment, for both 7 and 14 days, there is a positive correlation for the Tumor group 7 days(r = 0.2236) and the Tumor group 14 days (r = 0.2236), which means that the greater the tumor volume, the greater the presence of co-stimulatory molecules. The presence of dendritic cell immunotherapy can unbalance this correlation.

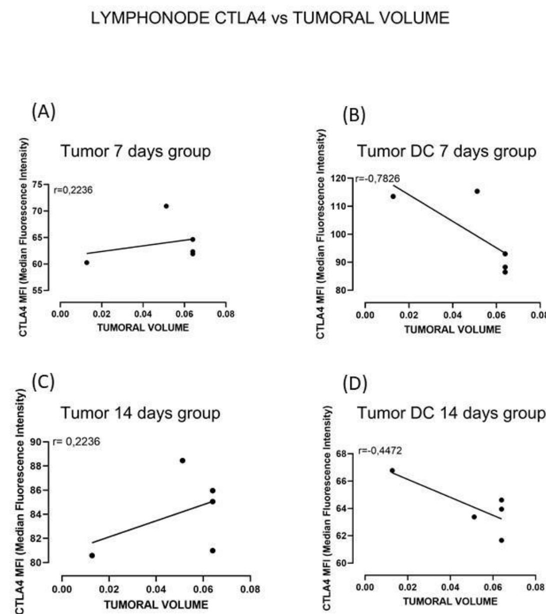

FIGURE 2 - Spearman's correlation showing the correlation between tumor development and the presence of CTLA4 in the lymph nodes of the experimental groups. (A) represents the correlation between the tumor volume and the presence of CTLA4 in the lymph nodes of the tumor group 7 days. (B) represents the correlation between the tumor volume and the presence of CTLA4 in the lymph nodes of the DC tumor group 7 days. (C) represents the correlation between the tumor volume and the presence of CTLA4 in the lymph nodes of the tumor group 14 days. (D) represents the correlation between the tumor volume and the presence of CTLA4 in the lymph nodes of the DC tumor group 14 days.

In order to evaluate an essential molecule in the activation of cells such as CD8+ T lymphocytes, we performed a correlation between the MFI of MHCI in lymph nodes and the tumor infiltrate of CD8+ T lymphocytes. The treated tumor groups, for both 7 and 14 days, present an r=1,000 and therefore a strong positive correlation. It indicates that the greater the presence of tumor infiltrate of CD8 + T lymphocytes, the greater the presence of MHC molecules in the lymph nodes. In the untreated tumor groups, we noticed an r = 1,000 and a strong positive correlation in 7 days , which indicates that the greater the presence of CD8+ T lymphocyte infiltrate in the tumor, the greater the presence of MHCI in the lymph nodes. This strong positive correlation turns into a

negative correlation with a r = -0.02000 in 14 days, thus suggesting that the immune system needs this time to modulate its immune response against many pro-tumor stimuli.

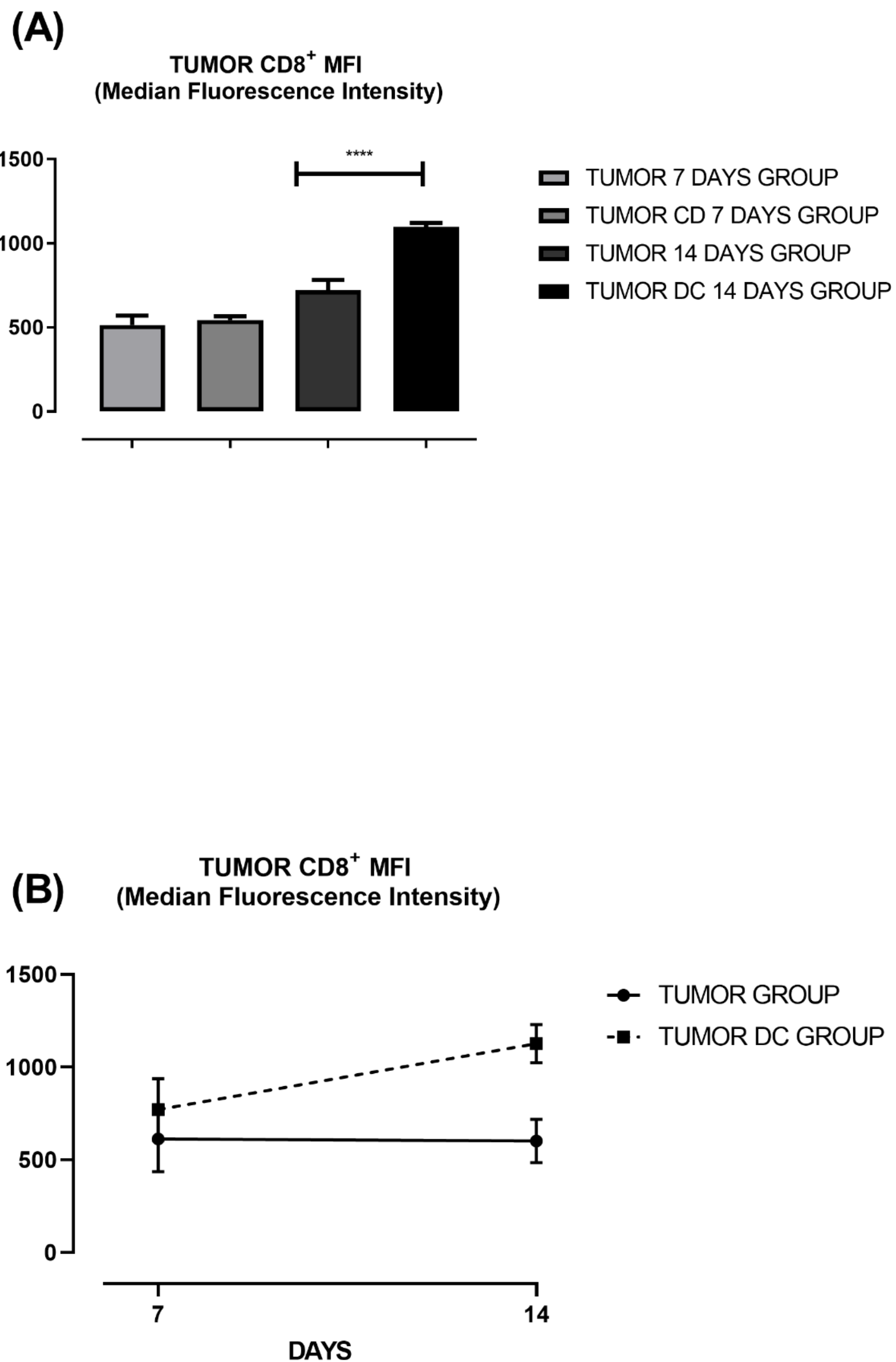

FIGURE 3 - Mean Fluorescence Intensity (MFI) of CD8 + cells in tumors using the immunofluorescence technique. (A) represents the MFI of CD8 + cells in the tumors of the tumor groups 7 days, tumor treated 7 days, tumor 14 days and tumor treated 14 days, respectively. (B) kinetic

representation involving the MFI of CD8 + cells in the tumor and tumor DC groups from 7 to 14 days after the first dose of dendritic cell immunotherapy. **** represents the value of p <0.0001.

Lymphonode MHCI vs Tumor CD8

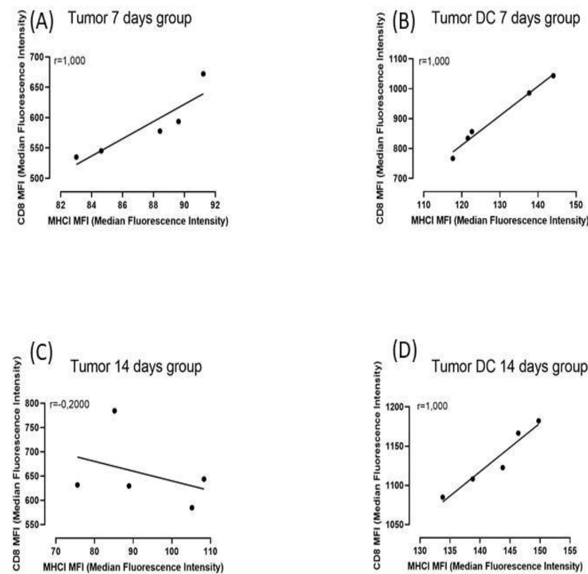

FIGURE 4 - Spearman correlation showing the correlation between tumor development and the presence of CD8 + in the tumors of the experimental groups. (A) represents the correlation between the tumor volume and the presence of CD8 + in the tumors of the tumor group 7 days. (B) represents the correlation between the tumor volume and the presence of CD8 + in the tumors of the DC tumor group 7 days. (C) represents the correlation between the tumor volume and the presence of CD8 + in the tumors of the tumor group 14 days. (D) represents the correlation between the tumor volume and the presence of CD8 + in the tumors of the DC tumor group 14 days.

The presence of transcription factors of cd4+ t lymphocyte and tumor volume

T-bet, the main transcription factor responsible for CD4+ T lymphocytes differentiation into lymphocytes of Th1 profile, demonstrated its highest expression in spleens from the Control group dendritic cells, where it is 6 times more expressed in the Control group dendritic cell when compared to Control group. In a comparison of T-bet in the Tumor group and Treated Tumor group, we noticed a higher final T-bet expression in the Tumor group CD compared to the Tumor group.

GATA3, the transcription factor responsible for the differentiation of CD4 lymphocytes into Th2 profile cells, is about 10 times more expressed in the tumor group without treatment in 14 days while the same transcription factor is about 8 times more

expressed in the tumor group 7 days . We note that the greatest expression of GATA3 is found in tumor groups without treatment.

RORγt is the main transcription factor responsible for the differentiation of CD4 + T lymphocytes into Th17 profile lymphocytes. Our results showed that the greatest expression of this transcription factor was also found in the tumor groups without treatment both in 7 and 14 days. In the tumor group 7 days the transcription factor is about 8 times more expressed when compared to the tumor group and in the tumor group 14 days the same transcription factor is about 10 times more expressed when compared to the control group.

FOXP3, an essential transcription factor for the differentiation of T lymphocytes into Treg lymphocytes, is about 20 times more expressed in the tumor group 14 days when compared to the tumor group. In the 7-day tumor group, the same transcription factor is about 5 times more expressed when compared to the control group. When we analyze the kinetics of FOXP3 in the groups at 7 and 14 days we noticed that the greatest increase in the expression of this transcription factor is in the tumor group, going from about 5 times more expressed in the tum or group in 7 days to about 20 times more expressed in the tumor group in 14 days.

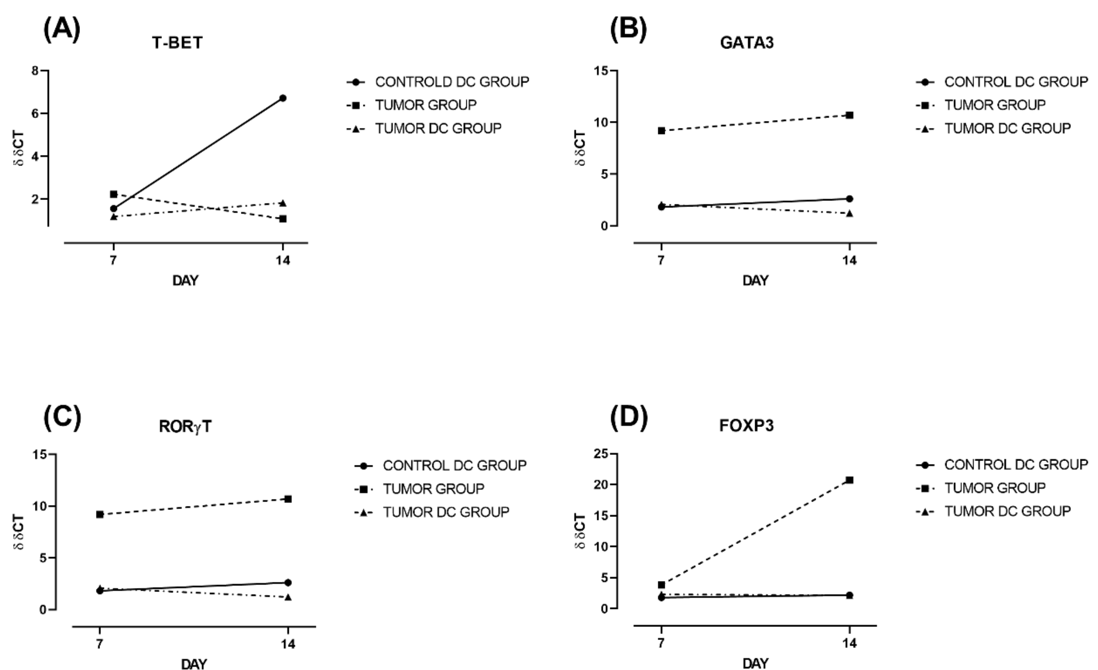

FIGURE 5 - graphical representation of the kinetics involving the main transcription factors of T lymphocytes from 7 to 14 days after the first dose of immunotherapy with dendritic cells. (A) graphical

representation of the δδCT of the transcription factor T-bet in the control groups DC, tumor and tumor DC from 7 to 14 days. (B) graphical representation of the δδCT of the transcription factor GATA3 in the control groups DC, tumor and tumor DC from 7 to 14 days. (C) graphical representation of the δδCT of the transcription factor RORγt in the control groups DC, tumor and tumor DC from 7 to 14 days. (D) graphical representation of the δδCT of the FOXP3 transcription factor in the DC, tumor and DC tumor groups from 7 to 14 days.

Given the aforementioned molecular data relating immunotherapy to a better anti-tumor immune response, we also measured the tumors of animals in the experimental groups. Our results show that in 7 days there is no difference between the tumor volume of the tumor group and the tumor volume of the tumor group treated with DC. At 14 days there is a great difference between the tumor volume of the two groups analyzed. The tumor volume is higher on average in the tumor group when compared to the tumor group treated with DC with a value of $p < 0.0001$, a statistically significant result.

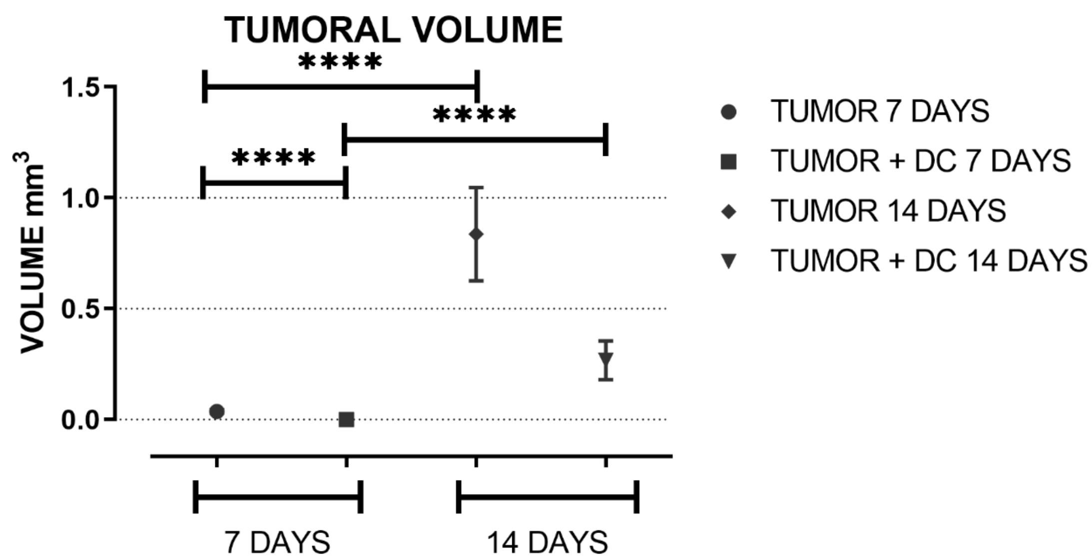

FIGURE 6 - Tumor development in experimental groups induced to develop breast tumor with 4T1 cells in 7 and 14 days. Balb/c mice were induced to develop breast tumor from 4T1 cells. Part of these animals was treated with dendritic cell immunotherapy, the so-called DC tumor group and another group received no treatment at all. The graph shows the development of tumors in these groups 7 days after the first dose of dendritic cell immunotherapy and 14 days after the first dose of dendritic cell immunotherapy. **** represents the value of $p < 0.0001$.

DISCUSSION

In lymphocyte activation, it is known that CD3 associates to TCR and aids the activation of T lymphocytes. Our results showed that in lymph nodes, the lymphoid organ activates lymphocytes, there is a higher MFI of CD3 in the Tumor group when compared to the Treated Tumor group 7 days. In 14 days, MFI is similar in the Tumor group and Treated Tumor group, thus demonstrating there are CD4 or CD8 T lymphocytes differentiation in lymph nodes of the Treated group in 14 day. In a prospective analysis, it was found that nonresponsive patients to CD3 in the lymph nodes had a greater recurrence of head and neck tumors when compared to the group of responsive patients [18], thus demonstrating the importance of the molecule in the immune response to tumors.

CD8 is associated with cytotoxic T lymphocytes, therefore showing cytotoxic function against tumor cells [19]. It is known that the presence of tumor infiltrate of cytotoxic T lymphocytes is a sign of good prognosis in the course of several types of cancers [1,20,21]. In the analyzed tumors, our results showed the highest MFI of CD8 T lymphocyte in the Treated Tumor group 14 days when compared to other groups. By analysing the tumors in 7 days, we noticed that the infiltrate of CD8 T lymphocytes is similar in both the Tumor group and in the Treated Tumor group with dendritic cell vaccine. It demonstrates that 14 days are sufficient for CD8 T lymphocytes differentiation and activation by dendritic cell immunotherapy.

CTLA4 is associated with a poor prognosis in the development of cancer. It is known that CTLA4 is a co-stimulatory molecule and has an immunosuppressive role through several mechanisms, such as: competition with CD28 during the lymphocyte activation process [22]. Checkpoint blockers are already well described and even used as a treatment for cancer, such as ipilimumab, which is an antibody that blocks the binding site of CTLA4, thus preventing it from exerting its immunosuppressive function [23]. A study demonstrated that CTLA4 blockade combined with immunotherapy prolonged CD8 T lymphocyte cell activity without the need to stimulate proliferation with IL-2 [24]. Our results demonstrated that in an analysis of tumor development versus CTLA4 MFI in lymph nodes, there is a positive correlation in the Tumor group 14 days, which means that the greater the tumor volume, the greater the MFI of CTLA4. However, in the Treated Tumor group 14 days there is a negative correlation negative, which means the greater the tumor volume, the lower the MFI of CTLA4 in the lymph nodes. As previously

shown, the dendritic cell vaccine induces a fight against the tumor by decreasing its volume in the Treated groups.

Due to its immunosuppressive role, it is known that CTLA-4 is associated with a poor prognosis in several types of cancers [25]. CTLA-4 blockade is already used as immunotherapy, so it was suspected that blocking this molecule could also potentiate the immune response. Suspicion confirmed in a work published in 2019 [26]. This result is in agreement with the work published in 2020 [27], and we can correlate these data with our results which demonstrate that the dendritic cell vaccine has the potential to inhibit CTLA-4 MFI due to the fact that the tumor group treated with dendritic cells present higher CTLA-4 MFI in 7 days and in 14 days MFI has decreased, thus increasing the potential for tumor destruction by dendritic cells.

It is well described that lymphocytes are activated in the secondary lymphoid organs by dendritic cells. In the case of CD8 T lymphocytes, after interaction of the MHCI of the antigen presenting cells with their TCR, they migrate to the infection site to execute their effector function, which is to destroy cells that have known antigen in their MHCI [28]. CD8 T lymphocytes are part of the adaptive immune response, therefore more specific, one of the reasons why this cell type is essential in the immune response against cancer [29].

Several studies demonstrate that the tumor infiltrate of CD8 T lymphocytes and the presence of this cell type is a sign of a good prognosis for the cancer patient [30,31]. Our results demonstrated in the analyzed lymph nodes of the tumor group in 14 days that the lower the MFI of MHCI in the lymph nodes, the lower the MFI of CD8 lymphocytes in the tumor with a negative correlation demonstrating the inability of the immune system to fight the tumor without stimulation. When we analyzed the tumor group treated with dendritic cell immunotherapy, we obtained a strong correlation with $r = 1$ when we analyzed the CD8 MFI in tumors vs MHCI MFI in lymph nodes, demonstrating that immunotherapy positively stimulated T lymphocyte activation CD8 in 14 days. Result that is repeated in the tumor group treated when MFI analysis of TCR in lymph nodes vs MFI of CD8 in tumors.

Among the transcription factors that act during an immune response to cancer, we can mention the T-bet, which is the main transcription factor related to the cell profile Th1, a profile responsible for an ideal immune response against tumor cells[32]. Among the

transcription factors described in the early 2000s [33], it is now known that the expression of t-bet by helper T lymphocytes induces the production of cytokines such as IFN-γ and IL-12. IFN- γ induces greater differentiation in T lymphocytes in Th1 lymphocytes and inhibition of Th2 profile, in macrophages it induces differentiation in M1, increases the maturation of dendritic cells and the expression of costimulatory molecules and inhibits the differentiation of regulatory T lymphocytes [34]. Our results demonstrated that the behavior of T-bet in immunotherapy with dendritic cells increased from 7 to 14 days in the treated tumor group and in the tumor group the opposite behavior, suggesting that from 14 days on we can start to notice improvements in the behavior of the immune system in combating tumor cells. Complementing these results, we note what is well described in the literature, that T-bet has an opposite behavior or an equilibrium behavior with GATA3[35] : from 7 to 14 days T-bet increases in the treated tumor group and GATA3 decreases in the same group and in the tumor group, T-bet decreases and GATA3 increases from 7 to 14 days.

It has been shown that for the development of regulatory T cells, the activation of the FOXP3 transcription factor is sufficient in mice [36]. It is known that for the activation of the proinflammatory Th17 profile, the main transcription factor is RORγT [37]. The profile of Th17 cells plays a controversial role in the immune response to tumors, sometimes fighting tumor development, and other times contributing to tumor development [38]. However, the role of the regulatory T profile has been well described and it is known that cells of this profile have a pro-tumor role in the immune response to cancer, with its markers being successful targets in immunotherapies against cancer [23,39]. Our results demonstrated that in 14 days of dendritic cell immunotherapy the gene expression of FOXP3 did not change in the treated tumor group, but in the tumor group without treatment there was a great increase in the genetic expression of FOXP3. Considering RORγT, our results showed that in 14 days in the treated tumor group there was a decrease in the genetic expression of this factor, but in the tumor group without treatment there was, as well as FOXP3, a great increase.

CONCLUSION

Our results demonstrate that 14 days of immunotherapies with dendritic cells are sufficient for modulation of the immune system. The levels of great prognostic markers such as T-bet and infiltrate of CD8 T lymphocytes increase when compared to bad prognostic markers such as FOXP3, CTLA4 among other studied markers. In practice, we note these results by assessing the tumor volume where we notice a significant difference in tumor volume between the tumor group and the treated tumor group. In general, immunotherapy of dendritic cells gives the treated mice a good prognosis in the fight against cancer and this immunotherapy should be studied more thoroughly, so that in the near future it can be used as a base treatment in most diverse cancers.

1

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6. CONSIDERAÇÕES FINAIS

Descritas em 1974 as células dendríticas que fazem a ponte entre a resposta imune inata e adaptativa. Com relação as imunoterapias de células dendríticas, no início do ano 2000 vários grupos iniciaram pesquisas sobre o potencial das células dendríticas em combater vários tipos de canceres.

As células dendríticas apresentam a capacidade de modular a resposta imunológica frente ao estímulo recebido. Caso o antígeno fagocitado por elas seja um parasita elas modulam a resposta imune para combate a parasitas, nesta resposta são ativados linfócitos T do perfil Th2. Caso o antígeno fagocitado seja um imunógeno com potencial inflamatório significativo são ativados linfócitos de perfil Th17, por fim caso o antígeno seja um antígeno intracelular são ativados linfócitos do perfil Th1. Os linfócitos Th1 modulas a resposta imune para o combate dos antígenos intracelulares e ativam células que reconhecem células infectadas como linfócitos T CD8, células NK e a produção de citocinas que potencializam a ação destas células como IL-12 e IFN-γ.

Ao longo dos anos 2000 vários trabalhos demonstraram a eficácia da imunoterapia com células dendríticas em combate a vários tipos de câncer. Algumas poucas pesquisas foram feitas na tentativa de entender em qual momento a imunoterapia com células dendríticas modulava a resposta imune em combate ao tumor. São necessários mais estudos com a intenção de entender e verificar melhor outros parâmetros da resposta imunológica que são alterados durante a imunoterapia de células dendríticas

7. CONCLUSÕES

Demonstramos com este trabalho que as células dendríticas já conhecidas pelo seu potencial em combate aos tumores tem a capacidade de modular a resposta imunológica em favor ao combate tumoral em 14 dias. Em 7 dias pode-se perceber a mudança de alguns parâmetros da resposta imunológica, porem em 14 dias a mudança de parâmetros importantes são evidentes.

Demonstramos mais uma vez que a imunoterapia com células dendríticas é eficaz contra o desenvolvimento tumoral além de demonstrar a mudança de parâmetros da resposta imunológica.

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579 # 9. ANEXO 1 – CEUA (certificado protocolo 402)

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 cinética da influência da vacina de células dendríticas no infiltrado tumoral e de linfonodos em camundongos fêmeas de linhagem Balb/C tumor induzidos por linhagem de células 4T1", registrada com o nº **402**, 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 reunião de 03/02/2017.

Finalidade	( ) Ensino (x) Pesquisa Científica
Vigência da autorização	03/02/2017 à 01/02/2021
Espécie/Linhagem/Raça	Camundongos isogênicos Balb/c
Nº de animais	88
Peso/idade	20-30 gramas / 8 semanas
Gênero	Fêmeas
Origem	Biotério do IPON - UFTM

Profª. Drª. Selma Sanches Dovichi
Coordenadora substituta da CEUA

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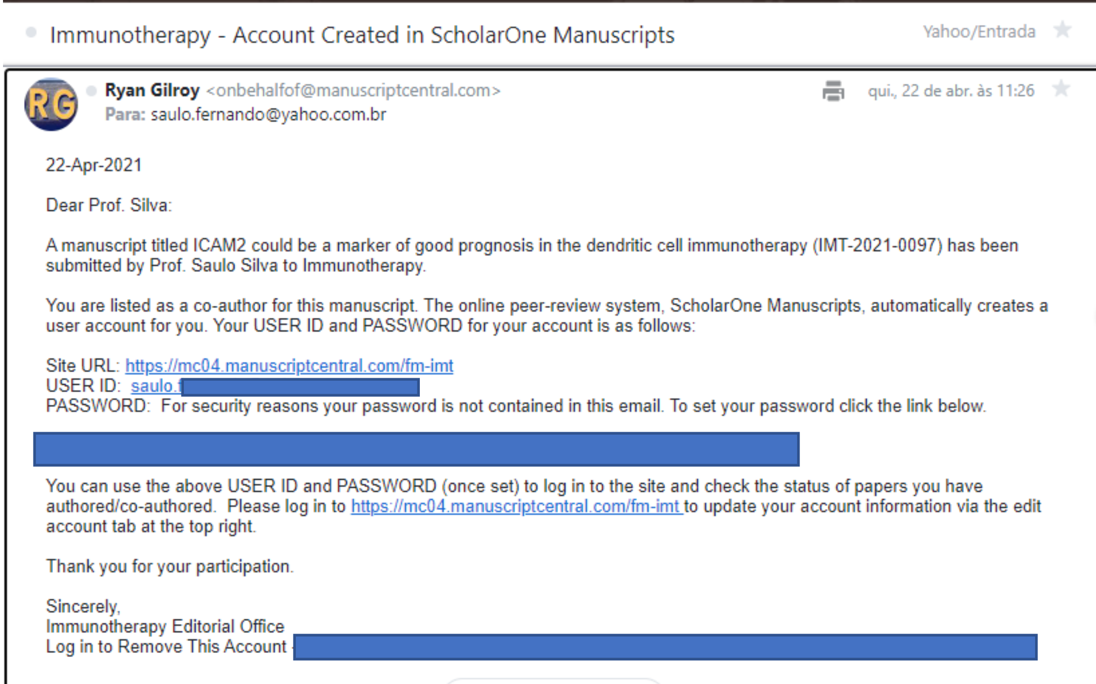
584 # 10. ANEXO 2 – Comprovante Submissão Artigo 1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22-Apr-2021

Dear Prof. Silva:

A manuscript titled ICAM2 could be a marker of good prognosis in the dendritic cell immunotherapy (IMT-2021-0097) has been submitted by Prof. Saulo Silva to Immunotherapy.

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600 **11. ANEXO 3 – Comprovante de submissão e aceite do artigo 2 referente a tese de**
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Após análise pelos pareceristas externos, foi tomada uma decisão sobre o artigo submetido à revista Brazilian Journal of Development,

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A decisão é: Aceitar.

Estamos à disposição caso tenha dúvidas, por email ou pelo telefone/WhatsApp: (41) 3534-7401.
Abraços.
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