UNIVERSIDADE FEDERAL DO TRIÂNGULO MINEIRO

POLYANA BARBOSA SILVA

CARACTERIZAÇÃO FENOTÍPICA DAS CÉLULAS T_{DC} EM CAMUNDONGOS COM CÂNCER DE MAMA

UBERABA – MG

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Tese de apresentada ao Programa de Pós-Graduação em Ciências da Saúde, área de concentração Medicina Translacional, da Universidade Federal do Triângulo Mineiro, como requisito parcial para obtenção do título Doutor em Ciências da Saúde.

Orientador: Prof. Dr. Eddie Fernando Cândido Murta.

Coorientadora: Prof.ª. Dr.ª Márcia Antoniazi Michelin.

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Banca examinadora

Profº Dr. Eddie Fernando Cândido Murta
(Docente – Disciplina de Ginecologia e Obstetrícia)

Prof.ª Dr.ª Rosekeila Simões Nomelini
(Docente – Patologia Ginecológica-UFTM)

Prof.ª Dr.ª Virgínia Oliveira Crema
(Docente – Disciplina de Histologia-UFTM)

Prof.ª Dr.ª Hélio Humberto Angotti Carrara
(Docente – Disciplina de Mastologia, Ginecologia e Obstetrícia-FMRP-USP)

Prof.ª Dr.ª Alessandra da Cunha
(Docente – Disciplina Recursos Fisioterapêuticos-UNIUBE)

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Estuda sempre. A luz do conhecimento armar-te-á o espírito contra as armadilhas da ignorância.

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RESUMO

As células T_{DC} representam um subconjunto raro de células hematopoiéticas com características fenotípicas e funcionais de células dendríticas e linfócitos T. Neste trabalho, avaliamos a expressão de marcadores de superfície (CD4, CD8 e CD86), fatores de transcrição (Tbet, Foxp3, Gata3 e Rorγt), e citocinas (IFN-γ, TNF-α, IL-10, IL-12 e IL-17) em células T_{DC} αβ obtidas do baço, fígado, linfonodo, medula e tumor. Também identificamos e caracterizamos as células T_{DC} γδ e o efeito do tumor sobre essas células em camundongos saudáveis (controle) e induzidos ao desenvolvimento de câncer pelas células 4T1, por meio da técnica de citometria de fluxo. Observamos que as T_{DC} αβ são mais frequentes em linfonodos de ambos os grupos estudados 70.05 (52.78-87.32) e 67.02 (67.02 – 97.88) ($p<0.0001$). Notamos, diminuição da expressão de T_{DC} CD4 no fígado 1662 (1551-1662) ($p=0.0001$), T_{DC} CD8 no baço 764.7 (485.8 - 1467) ($p=0.0012$) e fígado 2078 (2078 – 2602) ($p=0.0028$) e T_{DC} CD86 no baço 3997 (1550 – 7700) e no fígado 2742 (2197 – 2742) ($p=0.0337$) grupo tumor induzido por 4T1. Diminuição de T_{DC} Tbet e T_{DC} Foxp3 baço 1427 (704,1 – 1517) ($p<0,0001$); 1016 (962,9 – 2265) ($p<0,0001$) e fígado 5338 (4804 – 5338) ($p=0,0001$); 5133 (5133–6077) ($p=0,0337$), respectivamente, T_{DC} Gata3 no fígado 3346 (3346 – 5101) ($p=0,0028$) e aumento de T_{DC} Rorγt no tumor 3340 (3223 – 3722) ($p<0.0001$) desse mesmo grupo. Evidenciamos diminuição de T_{DC} IFN-γ no baço 1554 (705.3 – 1885) ($p<0.0001$) e medula óssea 733.1 (733.1 – 1307) ($p=0.0002$), T_{DC} TNF-α no baço 1655 (2673 -403.8) e medula óssea 819.2 (819.2 – 1521) ($p<0.0001$), T_{DC} IL-10 no baço 1689 (914,3 – 2049) ($p<0.0001$), fígado 5219 (5219 – 5619) ($p<0.0001$) e medula óssea 894.0 (894.0 – 1712), IL-12 no baço 1841 (360.7 – 2728) e medula óssea 791.0 (688.8 – 791.0) ($p=0.0002$) e IL-17 no baço 578.5 (326.3 – 873.8) ($p<0.0001$) e fígado 1200 (1200 – 1988) ($p=0.0001$) de camundongos induzidos ao câncer de mama. Notamos, a presença de 26.53% de células T_{DC} γδ nos grupos controle e um percentual maior de células T_{DC} αβ no grupo tumor induzido por câncer de mama comparado as células T_{DC} γδ ($p<0.0001$). As proporções de citocinas IFN-γ, TNF-α, IL-12 e IL-17 produzidas pelas T_{DC} γδ são diminuídas em condições tumorais ($p<0.0001$). Alterações fenotípicas podem ser conduzidas pelo microambiente tecidual na presença do tumor, as células T_{DC} γδ possuem características imunológicas compartilhadas com as células T_{DC} αβ. Direcionamentos são necessários a entender a funcionalidade associados a possível imunoterapia antitumoral.

Palavras-chave: Câncer de mama; Células dendríticas; Sistema Imune; Linfócitos T; Resposta imune antitumoral.

ABSTRACT

T_{DC} cells represent a rare cluster of hematopoietic cells with phenotypic and functional characteristics of dendritic cells and T lymphocytes. In this work, we evaluate the expression of surface markers (CD4 e CD8), transcription factors (Tbet, Foxp3, Gata3 e Rorγt), and cytokines (IFN-γ, TNF-α, IL-10, IL-12 e IL-17) in T_{DC} αβ cells in liver, lymph node, bone marrow and tumour. We also identify and characterize T_{DC} γδ cells and the effect of the tumor on these cells in healthy mice (control) and induced to the development of cancer by 4T1 cells, through the flow cytometry technique. We note that T_{DC} αβ are more frequent in lymph nodes of both groups studied 70.05 (52.78-87.32) e 67.02 (67.02 – 97.88) ($p<0.0001$). We noticed, decreased expression of T_{DC} CD4 in the liver 1662 (1551-1662) ($p=0.0001$), T_{DC} CD8 in the spleen 764.7 (485.8 - 1467) ($p=0.0012$) and liver 2078 (2078 – 2602) ($p=0.0028$) e T_{DC} CD86 in the spleen3997 (1550 – 7700) and liver 2742 (2197 – 2742) ($p=0.0337$) 4T1-induced tumor group. Decrease of T_{DC} Tbet and T_{DC} Foxp3 spleen 1427 (704,1 – 1517) ($p<0,0001$); 1016 (962,9 – 2265) ($p<0,0001$) and liver 5338 (4804 – 5338) ($p=0,0001$); 5133 (5133–6077) ($p=0,0337$), respectively T_{DC} Gata3 in liver 3346 (3346 – 5101) ($p=0,0028$) and increased T_{DC} Rorγt in tumor 3340 (3223 – 3722) ($p<0.0001$) that same group. We have shown a decrease in T_{DC} IFN-γ in spleen 1554 (705.3 – 1885) ($p<0.0001$) and bone marrow 733.1 (733.1 – 1307) ($p=0.0002$), T_{DC} TNF-α in spleen 1655 (2673 -403.8) and bone marrow 819.2 (819.2 – 1521) ($p<0.0001$), T_{DC} IL-10 in spleen 1689 (914,3 – 2049) ($p<0.0001$), liver 5219 (5219 – 5619) ($p<0.0001$) and bone marrow 894.0 (894.0 – 1712), IL-12 in spleen 1841 (360.7 – 2728) and bone marrow 791.0 (688.8 – 791.0) ($p=0.0002$) and IL-17 in spleen 578.5 (326.3 – 873.8) ($p<0.0001$) and liver 1200 (1200 – 1988) ($p=0.0001$) breast cancer-induced mice. We do not, the presence of 26.53% T_{DC} γδ cells in the control groups and a higher percentage of cellsT_{DC} αβ in the breast cancer-induced tumor group compared to breast cancer cells T_{DC} γδ ($p<0.0001$). The proportions of citokines IFN-γ, TNF-α, IL-12 and IL-17 produced by T_{DC} γδ are decreased in tumour conditions ($p<0.0001$). Phenotypic lt can be conducted by microenvironment and tissue in the presence of the tumor, theT_{DC} γδ have immunological characteristics shared with the cells T_{DC} αβ. Directions are necessary to understand the functionality associated with possible antitumor immunotherapy.

Keywords: Breast cancer. Dendritic cells. Imune system. T lymphocytes. Antitumor immune response.

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LISTA ABREVIATURAS E SIGLAS

4T1	Linhagem tumoral murina mamária
APC	*Antigen Presenting Cells*
Batf3	*Basic Leucine Zipper ATF-Like Transcription Factor 3*
DC	*Dendritic cell*
CD11c	*Cluster of differentation 11c*
CD3	*Cluster of differentation 3*
CD4	*Cluster of differentation 4*
CD8	*Cluster of differentation 8*
CEUA	Comissão da Ética Uso em Animais
CHP	Complexo de Histocompatibilidade Principal
CMV	Citomegalovirus
DNA	Ácido desoxirribonucleico
FITC	Isotiocianato de Fluoresceína (do inglês, Fluorescein Isothiocyanate)
Flt3	Tirosino-quinase 3 Fms
Flt3L	Ligante Tirosino-quinase 3 Fms
Foxp3	*Forkhead box P3*
FSC	*Forward Scatter*
GATA3	Fator de transcrição da família GATA
HSC	*Hematopoietic stem cells*
IFN-γ	Interferon Gama
Ikaros	Fator de transcrição semelhante a *Kruppel*
IL-10	Interleucina 10
IL-12	Interleucina 12
IL-17	Interleucina 17
IPON	Instituto de Pesquisa em Oncologia
IRF8	Fator Regulador de Interferon 8/
NK	*Natural Killers*
OMS	Organização Mundial de Saúde
P	Probabilidade
PBS	Phosphate Buffered Saline
PE	Ficoeritrina (do inglês, Phycoerythrin)
PerCP-Cy 5.5	Complexo Proteína Peridinina-Clorofila (do inglês, Peridinin Chlorophyll Protein Complex) 5.5
RPMI	Roswell Park Memorial Institute médium
SSC	*Side Scatter*
Tbet	*T-box transcription factor*
TCR	Receptor de linfócito T
Tfh	Linfócito T *helper* folicular
Th1	Linfócito T *helper* 1
Th17	Linfócito T *helper* 17
Th2	Linfócito T *helper* 2
Th22	Linfócito T *helper* 22
Th9	Linfócito T *helper* 9
TLR	Receptores *Toll-like*
TNF-α	Fator de Necrose Tumoral alfa

Treg	Linfócito T regulatório
Zbtb46	*Zinc finger transcription factor zDC*

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1 INTRODUÇÃO

1.1 CÂNCER

O câncer é uma doença caracterizada pelo crescimento incomum e desordenado de células que sofreram alterações genéticas (DA SILVA et al., 2020). O crescimento atípico caracterizado por uma divisão celular rápida e descontrolada, resulta em células com características agressivas, determinando assim a formação de tumores, que podem invadir tecidos e órgãos comprometendo suas funções (INCA, 2019).

É por meio das mutações genéticas que há formação do câncer, ou seja, alterações no material genético celular, mais precisamente no DNA (ácido desoxirribonucleico), que recebe estímulos de forma errônea e contribuem para atividades desordenadas. Essa alteração pode ocorrer em genes denominados proto-oncogenes, que codificam proteínas responsáveis pela regulação da diferenciação e crescimento celular, passando assim a serem denominados oncogenes executores das células cancerosas (WINDISCH et al., 2019).

O processo de formação do câncer é denominado de carcinogênese ou oncogênese, onde células normais sofrem a influência de agentes cancerígenos ou carcinógenos como exposição à radiação ionizante, multiparidade, fumo, alcoolismo, histórico familiar e em casos específicos a influência da terapia de reposição hormonal, dentre outros, associados a predisposição genética e que são responsáveis por proporcionarem tais desordens no ciclo celular, levando ao excesso na taxa de proliferação e deficiência nas taxas de morte celular, resultando em desequilíbrio celular (DA SILVA et al., 2020).

Consequentemente, essas desordens contribuem para a geração do câncer em seus vários estágios, como no estágio de iniciação, em que predominam modificações em genes por agentes carcinógenos; estágio de promoção em que células geneticamente alteradas continuam recebendo estímulos dos oncopromotores e sofrem seleção clonal e o estágio de progressão que ocorre a multiplicação celular desgovernada e irreversível (INCA, 2019; WINDISCH et al., 2019).

Estimativas globais mostram que o câncer é uma das principais causas de mortes no mundo. Foram 9,6 milhões de mortes em 2018, sendo os cânceres mais incidentes, o de pulmão e mama (2,09 milhões de casos cada), colorretal (1,80 milhão de casos), próstata (1,28 milhão de casos), câncer de pele (não melanoma) (1,04 milhão de casos) e estômago (1,03 milhão de casos) (FENG et al., 2019). O número de mortes por essa doença possui propensão a um aumento em cerca de 630.000 mortes em 2020. Em países desenvolvidos, como os Estados

Unidos, estão estimados 1.806.590 novos casos de cânceres e 606.520 mortes para o ano de 2020 (SIEGEL; MILLER; JEMAL, 2019).

No Brasil, pais em desenvolvimento, foi estimado no biênio 2018-2019, a ocorrência de 600 mil novos casos de cânceres para cada um dos anos, incluindo câncer de pele não melanoma, dados que o posicionam, entre os países com maiores incidências de cânceres no mundo (INSTITUTO NACIONAL DO CÂNCER, 2018).

Os cânceres de próstata (68 mil) em homens e os de mama (60 mil) em mulheres serão os mais recorrentes no geral, no Brasil. Relacionado a distribuição por gênero temos, os para o sexo masculino, os cânceres de próstata (31,7%), pulmão (8,7%), intestino (8,1%), estômago (6,3%) e cavidade oral (5,2%) e para o sexo feminino, mama (29,5%), intestino (9,4%), colo do útero (8,1%), pulmão (6,2%) e tireoide (4,0%) os mais incidentes (MACIEL; MARIA; CARVALHO, 2020).

Estimativas acerca de novos casos de cânceres, atualmente, apresentam taxas de incidência integrada com a idade, com o objetivo de subtrair o impacto da distribuição etária populacional, fornecendo um diagnóstico mais preciso e atualizado de novos casos com informações impactantes e perfil da doença na população (INSTITUTO NACIONAL DO CÂNCER, 2018).

Mesmo com as atenções voltadas para o rastreamento e o diagnóstico precoce, os fatores de risco desta doença, ainda estão fortemente presentes na população brasileira, destacando o tabagismo, obesidade e o sedentarismo (INSTITUTO NACIONAL DO CÂNCER, 2018).

1.2 CÂNCER DE MAMA

Dentre os vários tipos existentes, a neoplasia mamária é a mais comum entre o público feminino no mundo e a maior causa de mortalidade entre as mulheres, depois do câncer de pele não melanoma, correspondendo a 28% dos novos casos a cada ano. De acordo com estimativas realizadas pelo Instituto Nacional do Câncer (INCA), no Brasil em 2019, foram esperados aproximadamente 59.700 mil casos novos de câncer de mama em mulheres (INSTITUTO NACIONAL DO CÂNCER, 2018).

Dados mundiais demonstram que idade, álcool, genética, histórico familiar, obesidade, dieta, sedentarismo e fatores endócrinos estão associados à patogênese dessa doença, por compor os fatores de risco. No Brasil, aumenta-se de forma progressiva, a incidência do câncer de mama em mulheres acima de 35 anos e também especialmente após os 50 anos de vida (DIAS XAVIER et al., 2019).

Dos casos de câncer de mama diagnosticados, 5% podendo chegar a 12% estão relacionados a mutações genéticas específicas, sendo mais comuns em indivíduos com histórico familiar (MERRITT et al., 2015). Além disso, durante o período reprodutivo, tem-se maior exposição ao hormônio estrógeno que associados a fatores genéticos e ambientais tem favorecido o desenvolvimento deste tipo de câncer (LI et al., 2017).

1.3 MODELO EXPERIMENTAL DE TUMOR DE MAMA COM LINHAGEM 4T1

A linhagem celular do carcinoma mamário 4T1 compreende uma das quatros subpopulações do tumor 410.4, que foi absolutamente isolada de um câncer de mama espontâneo de camundongo Balb/c por Fred Miller e colaboradores (DUPRÉ; REDELMAN; HUNTER, 2007; PULASKI; OSTRAND-ROSENBERG, 2000; TAO et al., 2008; YOSHIMURA et al., 2013).

A fim de avaliar e compreender a biologia dos tumores de mama tem-se utilizado o modelo experimental 4T1, devido à sua grande propensão a metástases espontâneas, a partir do tumor primário de uma glândula mamária, em múltiplos e diferentes locais, incluindo os nódulos linfáticos, sangue, fígado, pulmão, cérebro e ossos. Essa linhagem celular é altamente tumorigênica e invasiva e, morfologicamente, o tumor apresenta proliferação epitelial maligna e com elevado índice mitótico, apoptótico e vários vasos sanguíneos ligados diretamente a progressão tumoral (PULASKI; OSTRAND-ROSENBERG, 2000).

O tumor formado apresenta diversas características que permitem utilizar este modelo experimental para estudo do câncer de mama humano. Características a ele designadas, por possuírem facilidade das células 4T1 serem transplantadas na glândula mamária, de modo que o tumor se desenvolva na própria glândula. Outra característica é que, semelhante ao que se observa no estágio avançado do câncer de mama humano, essas células possuem a capacidade de gerar metástases nos mesmos sítios do câncer de mama em humanos, e possuem um comportamento similar ao subtipo molecular triplo negativo, sendo um dos mais agressivos (KAUR et al., 2012; PULASKI; OSTRAND-ROSENBERG, 2000).

1.4 RESPOSTA IMUNE ANTITUMORAL

As alterações genéticas e epigenéticas causadas nas células tumorais podem ser reconhecidas pelo sistema imune, por meio da vigilância imunológica, e assim desempenhar respostas antitumorais eficazes (REIS et al., 2018).

Por sua vez, o sistema imune exerce um papel fundamental de preservação da integridade do organismo, promovendo proteção contra microrganismos patogênicos e desenvolvendo a defesa contra o câncer (CANDEIAS; GAIPL, 2016). O monitoramento do crescimento tumoral depende de procedimentos interligados entre as respostas imunes inata e adaptativa relacionadas a vigilância imunológica, a qual é responsável pela seleção de vias imunorreguladoras diferentes contra células tumorais (REIS et al., 2018).

Células do sistema imune inato trabalham em conjunto com as células do sistema imune adaptativo. O desenvolvimento das células neoplásicas é monitorado pela primeira linha de defesa, composta pela imunidade inata. O estresse causado por esse mecanismo, resulta na ativação de células importantes no combate tumoral, como as células *natural killer* (NK), macrófagos, neutrófilos e células dendríticas (DCs, do inglês *Dendritic Cells),* consideradas as células apresentadoras de antígenos profissionais (APCs, do inglês *Antigen Presenting Cells),* que juntamente com moléculas de superfície coestimuladoras, irão atuar de forma positiva para uma resposta imune adaptativa ativa (VAHIDIAN et al., 2019). A reposta imune adaptativa responde a antígenos resultando na ativação e expansão clones de linfócitos B e T (MIRZA et al., 2019).

A resposta imune adaptativa humoral é comandada pelos linfócitos B, que podem atuar como APCs no microambiente tumoral e ainda se diferenciarem em plasmócitos, sob estímulos, produtores de anticorpos específicos e contribuir para a sobrevivência e proliferação de linfócitos T (LIU et al., 2019b). A participação dos linfócitos B, na resposta antitumoral, podem promover ou suprimir o desenvolvimento tumoral, o que depende dos estímulos criados no microambiente tumoral. Por meio da sua capacidade de reconhecer e regular a apresentação antigênica, essas células influenciam a atividade de outras células do sistema imune, principalmente aquelas que expressam os receptores Fc (GUN et al., 2019).

A imunidade celular é mediada por células T, mais precisamente, pelos linfócitos T auxiliares (T $CD4^+$) e os linfócitos T citotóxicos (T $CD8^+$) (LIU et al., 2019b).

O crescimento tumoral é principalmente monitorado por esses linfócitos, os quais linfócitos T auxiliares exercem papéis importantes contra as células tumorais, auxiliando e potencializando a ação de outras células imunes (SHANKARAN et al., 2001) e os linfócitos T

citotóxicos que se ligam as células-alvo tumorais destruindo-as por mecanismos de liberação de grânulos citotóxicos e ativação de receptores de morte programada, além da ação de mediadores antitumorais como a citocina *Interferon*- γ (IFN-γ) (GUN et al., 2019).

1.4.1 Células dendríticas

As células dendríticas (DCs, do inglês *Dendritic Cells)* são reconhecidas como células apresentadoras de antígenos profissionais (APCs, do inglês *Antigen Presenting Cells).* São células relacionadas a primeira linha de defesa, ou seja, ao sistema imune inato, estando diretamente relacionado ao princípio de modulação da resposta imune contra microrganismos, autoimunidade e resposta imune antitumoral (BANCHEREAU et al., 2000; SCHLITZER et al., 2015; STEINMAN; COHN, 1973).

Este tipo celular tem origem em precursores hematopoiéticos pluripotentes (HSC, do inglês *Hematopoietic stem cells*) da medula óssea que durante a fase de hematopoese, estes precursores diferenciam-se em progenitores mieloides comuns dando origem aos monócitos, macrófagos, megacariócitos, eritrócitos e granulócitos, e progenitores linfoides comuns que dão origem aos linfócitos T e B (SCHRAML; REIS E SOUSA, 2015).

As células dendríticas são classificadas em células dendríticas convencionais (cDCs) e plasmocitoides (pDCs) (LU et al., 2009).

As cDCs se originam de um progenitor comum das células dendríticas na medula óssea e migram para os órgãos linfoides periféricos. Possuem como fatores de transcrição o PU.1, Ikaros, IRF8, RelB e Batf 3 para seu desenvolvimento, porém nenhum deles pode ser usado para definir exclusivamente uma linhagem de células dendríticas (MAK et al., 2011; SATPATHY; MURPHY; WUMESH, 2011).

As pDCs são caracterizadas pela expressão de CD11c, CHP II (Complexo de Histocompatibilidade principal do tipo II), CD45R, Siglec-H e *Bst2*. Além disso, são conhecidas por exercer a função de resposta imune antiviral por meio da síntese de IFN-α (MITCHELL; CHINTALA; DEY, 2018).

As células dendríticas maduras expressam em sua superfície moléculas coestimulatórias (CD80 e CD86) que se ligam ao receptor CD28 nos linfócitos T virgens (BANCHEREAU; STEINMAN, 1998) e resultam na promoção de sinais de reconhecimento pelo receptor de linfócitos T (TCR), aumentando assim a sobrevida e proliferação dessas células (HOTBLACK et al., 2018).

As células dendríticas são heterogêneas e diferem-se em localização, vias migratórias e função imunológica. Funcionalmente, elas são capazes de capturar, processar e apresentar antígenos a linfócitos T, além disso desempenham um papel relevante à tolerância imunológica, memória e diferenciação dos linfócitos T auxiliares (T CD4), em subtipos, Th1, Th2, Th9, Th17, Th22, Treg e Tfh (HANAHAN; WEINBERG, 2011). Quando estimuladas com receptores do tipo Toll-*like*, há produção das citocinas IL-12 e TNF-α, importantes para uma resposta imune antitumoral (WACLECHE et al., 2018).

1.4.2 Linfócitos T

Os linfócitos T possuem origem em precursores hematopoiéticos pluripotentes, porém sua maturação acontece no timo, em que precursores dessas células passam por processo conduz à geração de linfócitos T maduros $CD4^{+}CD8^{-}$ (Linfócitos T auxiliares) e $CD4^{-}CD8^{+}$ T (Linfócitos T citotóxico) (LOVE; BHANDOOLA, 2011).

O mecanismo de vigilância e eliminação acontece por reconhecimento de células imunes a antígenos tumorais endógenos via molécula do complexo principal de histocompatibilidade I (CHPI) apresentados e reconhecidos por linfócitos citotóxicos $TCD8^{+}$ e por reconhecimento, processamento e apresentação por APCs, por exemplo, macrófagos, células dendríticas e linfócitos B, via moléculas de CHP classe II à linfócitos T auxiliares $TCD4^{+}$ (LIU et al., 2019b).

1.4.2.1 Linfócitos T auxiliares

Os linfócitos T são capazes de se diferenciar após reconhecimento antigênico e sinais fornecidos por uma APC. Sendo assim, fenótipos de linfócitos T auxiliares são formados e determinados pela natureza e concentração do antígeno, microambiente, citocinas e moléculas coestimulatórias, dentre outros aspectos (KIDD, 2003).

Assim que recebe tais estímulos, essas células desempenham a função de produzir citocinas e estimular os linfócitos B a produzirem anticorpos. Além disso, são diferenciados em fenótipos identificados como Th1, Th2, Th17, Th9, Th22, Treg e Tfh, cada um secretando um perfil diferente de citocinas (KIDD, 2003) (Figura 1).

O subtipo Th1, sua diferenciação é induzida por citocinas secretadas por DCs e macrófagos, como IL-12, IL-18, IFN-α e IFN-β. A resposta resultante por Th1 depende do fator de transcrição Tbet e da molécula STAT4 que serão ativadas frente a estímulos antigênicos e

assim produzirem citocinas como IFN-γ, IFN-α, IFN-β e IL-2 para desempenharem o papel de estimular a imunidade celular frente a patógenos intracelulares e participarem da patogênese das doenças autoimunes (KAIKO et al., 2008).

O perfil Th2 é induzido por patógenos extracelulares e alérgenos, pelo efeito de citocinas como IL-4, IL-25, IL-33 e IL-11 secretadas por mastócitos, eosinófilos e células NKT. Sendo assim, há uma indução de ativação intracelular dos fatores de transcrição STAT-6 e GATA-3 e consequentemente secreção de citocinas do fenótipo Th2, como IL-4, IL-5, IL-9, IL-13, IL-10 e IL- 25, resultando em troca de classe de imunoglobulina para IgE que irá ativar o sistema imune inato (HO; TAI; PAI, 2009).

A diferenciação em Th9 acontece pela estimulação das citocinas TGF-β e IL-4 (LI; ROSTAMI, 2010). Este perfil produz as citocinas IL-9 e IL-10 e não expressam citocinas ou fatores de transcrição dos subconjuntos Th1, Th2 ou Th17. Funcionalmente é responsável pelo crescimento dos mastócitos e a secreção de IL-1β, IL-6, IL-13 e TGF-β (MA; TANGYE; DEENICK, 2010).

O perfil Th17, é ativado a partir da combinação das citocinas IL-6, IL-21, IL-23 e TGF-β. A IL-6, que irá agir no fator de transcrição Rorγt ativando-o a produzir citocinas como IL-17A e IL-17F. Estas citocinas por sua vez, principalmente a IL-17, irão se ligar a receptores de células mesenquimatosas para promover a liberação de mediadores inflamatórios (LIU et al., 2019a).

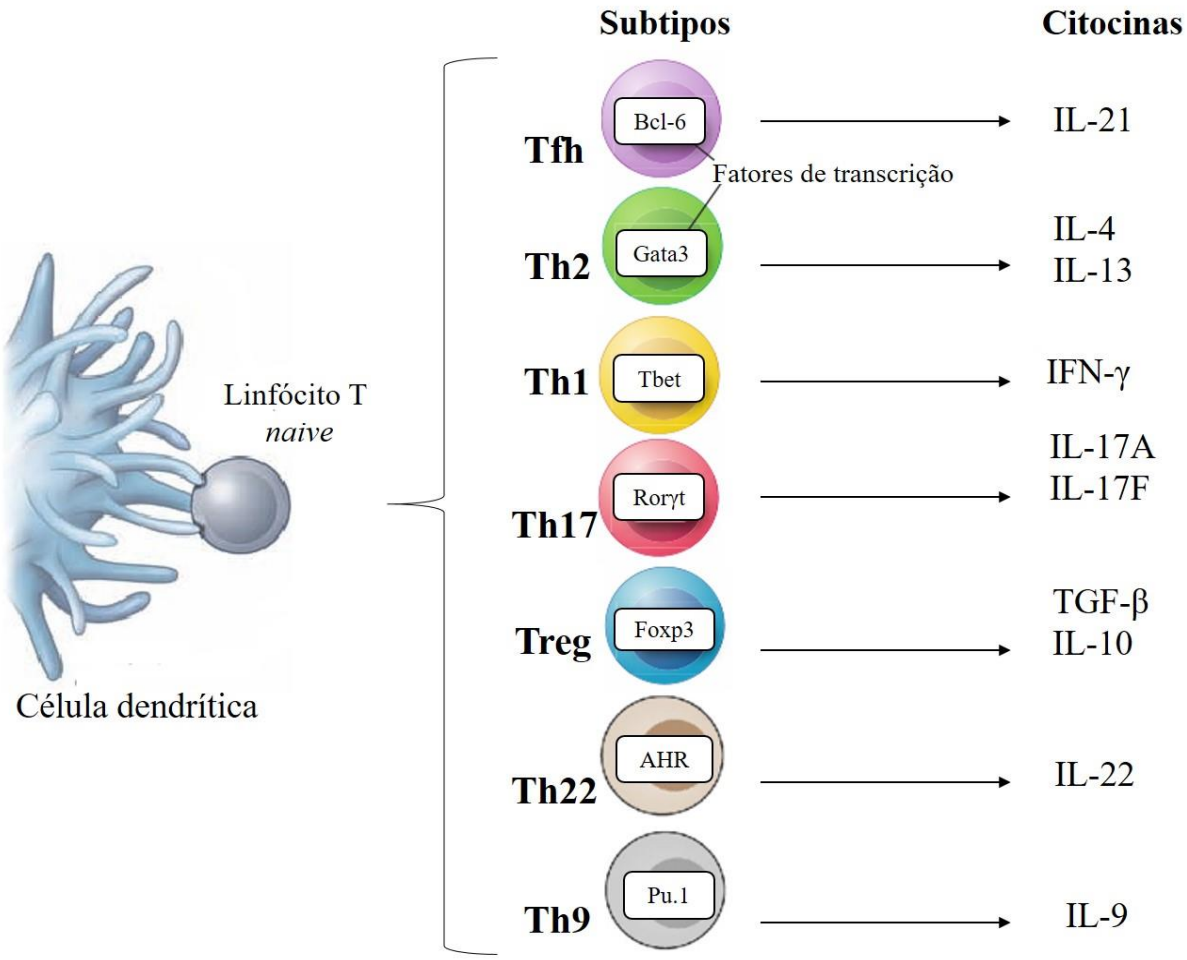
Os linfócitos Th22 são estimulados pelas citocinas IL-6 e TNF-α com a participação de DC plasmocitoide. Sendo assim, ativa-se o fator de transcrição AHR que está relacionado a secreção de citocinas como a IL-22 e TNF-α envolvidos na angiogênese e fibrose (EYERICH et al., 2009).

Os linfócitos T auxiliares foliculares (Tfh) foram descobertas recentemente e denominadas como células T do centro germinativo auxiliadoras das células B a produzirem anticorpos. Para essa célula se desenvolver é necessária a ação das citocinas IL-6, IL-12 e IL-21, a qual terá a função de auxiliar a transformação de células B plasmócitos produtores de anticorpos com diferentes isotipos e produção de células B de memória (DENG et al., 2019; GONG; ZHENG; ZHOU, 2019).

Os linfócitos T reguladoras (Treg) possuem os marcadores de superfície $CD4^{+}CD25^{+}$ e expressam constitutivamente o fator de transcrição $Foxp3^{+}$, que é essencial para o seu desenvolvimento e são estimuladas pela presença das citocinas de IL-10 e TGF-β. Os linfócitos

Tregs possuem papel centralizado a mecanismos de auto tolerância e homeostase imune. São células capazes de se infiltrar no estroma tumoral e por lá exercerem imunossupressão celular (SAKAGUCHI et al., 2010).

Figura 1 – Subtipos de linfócitos T auxiliares



Fonte: Adaptado de Schulz et al., 2009

1.4.2.2 Linfócitos T citotóxicos

Os linfócitos T $CD8^+$ desempenham suas funções efetoras por meio de sua citotoxicidade, capaz de destruir diretamente as células infectadas por vírus ou células malignas (COX; KAHAN; ZAJAC, 2013) pela indução da apoptose da célula alvo, liberação de grânulos

citolíticos e ligantes para receptores de morte como FasL (KUMAR; CONNORS; FARBER, 2018) .

Além dessas ações desempenhadas por essas células, esses linfócitos secretam as citocinas IFN-γ e TNF-α, importantes na defesa contra infecções virais e no controle da proliferação de células tumorais (COX; KAHAN; ZAJAC, 2013).

1.4.2.3 Receptores de linfócitos T

O receptor de célula T, denominado TCR, é composto de duas cadeias peptídicas da superfamília das imunoglobulinas, com uma região variável e uma região constante que a se maturarem sofrem recombinação gênica, dando origem à quatro polipeptídios de receptores de antígenos peculiares (α, β, γ e δ) que rearranjados formam dois heterodímeros diferentes (α: β e γ: δ) (GUN et al., 2019).

No sangue periférico humano, há aproximadamente 95% de células T pertencentes as subunidades de TCR α/β enquanto que a cadeia de TCR γ/δ compreende a 5% (VAN DER MERWE; DUSHEK, 2011).

As cadeias do TCR α/β, possuem uma grande diversidade de repertórios, os quais se associam a moléculas do complexo de histocompatibilidade principal (CHP), na superfície das células apresentadoras de antígenos ou células alvo, o que resulta em alterações bioquímicas nas porções citoplasmáticas do complexo CD3 e consequentemente os sinais de ativação são estabelecidos (VAN DER MERWE; DUSHEK, 2011).

Funcionalmente o TCR α/β possui o papel importante para o sistema imunológico relacionado ao reconhecimento de proteínas antigênicas e neoantígenos mutados provenientes das células tumorais (LIU; BIOLOGY; MARDIS, 2018).

O TCR γδ representa um subconjunto relevante e não menos importante de linfócitos. Nesses linfócitos as cadeias γ e δ apresentam uma variabilidade menor quando comparado ao TCR α/β (ZARIN et al., 2014). Suas funções são peculiares no que se diz respeito ao reconhecimento antigênico e a cinética de resposta imune. Possuem propriedades inatas e adaptativas, com pequena diversidade de repertórios, não são restritas ao complexo de CHP e são capazes de reconhecer antígenos não proteicos, como glicoproteínas e fosfolipídios. Combatem de forma eficaz infecções, possuem efeitos antitumorais por meio da ação de proteínas citolíticas combatem de forma eficaz as células tumorais e em alguns casos possuem efeitos protetores tumorais (LEGUT; COLE; SEWELL, 2015).

1.5 CÉLULAS T_{DC}

A eficácia de uma resposta imune, seja ela contra antígenos virais, bacterianos ou tumorais, se faz imprescindível a interação de mecanismos associados aos sistemas imunes inato e adaptativo, ou seja, é fundamental a comunicação entre dois tipos celulares correspondidos as células dendríticas e os linfócitos T, para que se inicie e estabeleça uma resposta mais específica (BANCHEREAU et al., 2000; MOSER; LEO, 2010).

Progenitores de células T e células dendríticas estão compreendidos na medula óssea, os quais passaram por processo de diferenciação de acordo com os estímulos a eles destinados, com isso características peculiares tomam conta de subtipos bem diferenciados. Algumas análises funcionais desses dois tipos celulares permitiram serem observados como similares, não havendo o distanciamento no âmbito da funcionalidade como foi até hoje descrito (LIU; NUSSENZWEIG, 2010).

Um tipo celular em especial, descoberto por Kuka e colaboradores (2012), as células $T_{DC,}$ assim denominadas, são células T αβ policlonais, que possuem características inatas de células dendríticas e adaptativas de linfócitos T. A combinação desses dois tipos celulares coloca as células T_{DC} em posição intermediária a ações efetoras da imunidade inata e adaptativa. Assim, essas células necessitam do timo para completarem seu desenvolvimento, bastante semelhante ao desenvolvimento dos linfócitos T, mas também possuem propriedades das células dendríticas (KUKA; ASHWELL, 2013; KUKA; MUNITIC; ASHWELL, 2012).

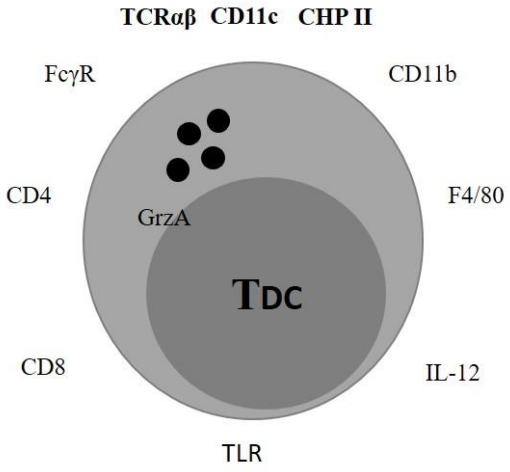
O tamanho e a morfologia das células T_{DC} são bastante semelhantes aos linfócitos T. Essas células ainda expressam altos níveis de CD11c, de moléculas de apresentação de antígeno (CHPII) e o receptor de linfócito T (TCR αβ). Além disso, são responsáveis pela expressão em sua superfície dos marcadores CD11b, F4/80 e FcRγ (Figura 2) (KUKA; MUNITIC; ASHWELL, 2012).

Essas características sugerem que talvez elas possam ser um novo subtipo de células T. Funcionalmente, são capazes de fornecer funções para si mesma indicando que talvez elas não precisem de uma APC convencional para se ativarem, sendo autossuficientes para a apresentação antigênica, e regulada positivamente por moléculas coestimulatórias, assim como ocorre nos DCs convencionais (KUKA; ASHWELL, 2013; KUKA; MUNITIC; ASHWELL, 2012)

Estudos complementares mostraram que quando as T_{DC} são estimuladas com agonistas de TLRs elas produzem IL-12, uma citocina normalmente produzida por células dendríticas e

importante para a polarização do perfil Th1. A IL-12 produzida pelas T_{DC} podem aumentar, em sua expressão, quando associada ao IFN-γ recombinante (KUKA; ASHWELL, 2013).

Figura 2 – Desenho esquemático das células T_{DC}



Fonte: Do autor, 2020.
Nota: GrzA: Granzima A; TLR: Receptor do Toll-like.

2 JUSTIFICATIVA

O câncer de mama triplo negativo está entre os subtipos moleculares mais agressivos, representando, em média, 15% dos casos de câncer de mama no mundo e que ainda não dispõe de uma terapia-alvo, ou seja, não foi possível ainda desenvolver um tratamento específico e direcionado para esse tipo de câncer. Este tipo se caracteriza pela negatividade para os três tipos de receptores, ou seja, dificilmente irão responder a tratamentos hormonais (negativo para estrogênio e progesterona) e também a tratamentos com drogas específicas para o receptor HER2, diminuindo e limitando métodos gerais de tratamento.

O sistema imunológico possui um papel importante diante o reconhecimento e combate à progressão do câncer. Células com características específicas contribuem para os mecanismos de defesa desempenhado por ele.

Os linfócitos T e as células dendríticas desempenham um papel fundamental na resposta imune, inclusive antitumoral. A produção e secreção de citocinas produzidas por esses tipos celulares auxiliam e potencializam a cascata celular a resposta imune eficaz.

Há alguns anos um novo tipo celular foi descrito na literatura, as células $T_{DC.}$ que possuem características de linfócitos T e de células dendríticas. Assim, para elucidar o fenótipo das células T_{DC}, torna-se importante a investigação dessas células. Visto que o estudo pioneiro trouxe a descoberta desse tipo celular em camundongos e em humanos saudáveis, assim como pós infecções por citomegalovírus (CMV), estudos como este justificam-se em elucidar a fenotipagem dessas células em uma resposta imune antitumoral sistêmica e/ou local e também o fenótipo de células TCR γδ, podendo ser inclusive utilizadas futuramente como alvo terapêutico ou de prognóstico.

3 HIPÓTESES

- **Hipótese A**

Hipótese nula: T_{DC} αβ e γδ ausentes no tecido

Hipótese alternativa: T_{DC} αβ e γδ presente nos tecidos

- **Hipótese B**

Hipótese nula: T_{DC} αβ e γδ presentes e sem diferenças fenotípicas

Hipótese alternativa: T_{DC} αβ e γδ presente e com alterações fenotípicas

- **Hipótese C**

Hipótese nula: não há supressão fenotípica de T_{DC} αβ e γδ

Hipótese alternativa: há supressão fenotípica de T_{DC} αβ e γδ

4 OBJETIVOS

4.1 OBJETIVO GERAL

Este estudo tem por objetivo caracterizar o fenótipo das células T_{DC} frente a uma resposta imune antitumoral em camundongos com câncer de mama induzido por células 4T1.

4.2 OBJETIVOS ESPECÍFICOS

- Avaliar o perfil de fenotípico células T_{DC}, através da análise dos marcadores de superfície, CD4, CD8, CD11c, TCRαβ, CHPII, dos fatores de transcrição, Foxp3, GATA3, Tbet e RoRγT, e das citocinas IFN-γ, IL-12, IL-10, IL-17 e TNF-α, obtidas do baço, linfonodos sentinelas, fígado, medula óssea e tumor de camundongos com câncer de mama induzido por células 4T1 e camundongos saudáveis.

- Identificar o repertório de células T_{DC} γδ de células esplênicas e o efeito do tumor sobre essas células, utilizando os marcadores de superfície CD4, CD8, TCRγδ e CHPII e citocinas IFN-γ TNF-α, IL-12 e IL-17 em camundongos com câncer de mama induzido por células 4T1 e camundongos saudáveis.

- Comparar os perfis de células T_{DC} αβ e T_{DC} αβ γδ entre os grupos controle e induzido por células 4T1 frente a uma resposta antitumoral.

5 MATERIAIS E MÉTODOS

5.1 ANIMAIS

Foram utilizados 30 camundongos BALB/c fêmeas, com idades entre 6 e 8 semanas, mantidos do biotério setorial do laboratório de Pesquisa em Oncologia (IPON) da Universidade Federal do Triângulo Mineiro. Os animais, durante o período experimental de 28 dias, foram divididos em dois grupos, sendo o grupo 1: Controle (sem inoculação de células tumorais) e o grupo 2: Tumor induzido (inoculação de células tumorais da linhagem 4T1). Cada grupo composto por 15 animais, acondicionados em gaiolas plásticas (5 animais por gaiola) sob um ciclo 12h claro/escuro a 21± 3°C, com comida e água disponíveis *ad libitum*. Após o período experimental, os mesmos foram eutanasiados por meio da superdosagem de 50mg/kg cetamina e 15mg/kg de xilazina.

Este estudo foi desenvolvido seguindo os princípios éticos e com recomendações da Comissão de Ética no Uso de Animais da Universidade Federal do Triângulo Mineiro, com protocolo de aprovação sob o número 379/2016 – CEUA.

5.2 INDUÇÃO TUMORAL

Os animais sofreram processo de aleatorização simples antes da experimentação. Os camundongos são colocados nas gaiolas, e uma pessoa que não participa do trabalho distribui de forma aleatória, em outras duas gaiolas, separando assim os grupos controle e o que irá receber a inoculação das células tumorais. Os denominados como controles não foram inoculados com células da linhagem tumoral 4T1 correspondente ao câncer de mama. Já o grupo denominado tumor induzido, recebeu $2x10^5$ células no último par de mamas, na glândula mamária esquerda dos camundongos a serem estudados. As células tumorais da linhagem citada, foram mantidas em cultura no meio RPMI (Roswell Park Memorial Institute médium) e armazenada em estufa umidificada (Water Jacket Incubator 3110, Thermo Fisher Scientific, Marietta, OH) a 37º C e 5% de CO_2. Após o período de cultura, as células foram lavadas com solução salina a 0,9% e centrifugadas a 290xg, a 4ºC por 10 minutos e posteriormente inoculadas nos camundongos do grupo tumor induzido.

5.3 CARACTERIZAÇÃO DE CÉLULAS IMUNES POR CITOMETRIA DE FLUXO

5.3.1 Análise das células T_{DC} αβ

Baço, fígado, linfonodo sentinela, medula óssea e tumor foram coletados, passaram pelo processo de ruptura mecânica, exceto a medula óssea, e $1x10^6$ células foram colocados em tubos próprios para a técnica de citometria para receberem a marcação extracelular com os seguintes anticorpos: Receptor de células T αβ - anticorpo monoclonal TCRβ anti-mouse hamster (FITC); Linfócitos T auxiliares ($CD4^+$) – anticorpo monoclonal CD4 anti-mouse (PE-Cy 5); Linfócitos T citotóxicos ($CD8^+$) – anticorpo monoclonal CD8a anti-mouse (PerCP-Cy 5.5); Molécula de adesão (CD11c, células dendríticas totais) – anticorpo monoclonal CD11c anti-mouse hamster (APC); Moléculas coestimulatórias (CD86) – anticorpo monoclonal CD86 anti-mouse (APC) e Molécula de apresentação de antígeno (CHPII) – anticorpo monoclonal IA anti-mouse (PE).

Posteriormente a marcação extracelular, as células passaram por um processo de incubação por um período de 30 minutos em temperatura de 4º C ao abrigo da luz. Em seguida, sofreram lavagens com PBS (solução salina tamponada com fosfato) com o objetivo de remover anticorpos em demasia. Após este processo os tubos que receberam a marcação intracelular foram permeabilizados, fixados e incubados por 20 minutos com solução permeabilizadora (BD Cytofix/Cytoperm™), lavadas com solução tampão (BD Perm/Wash™ Buffer) para receberem a marcação intracelular.

Para a marcação intracelular, utilizamos para fatores de transcrição os anticorpos monoclonais anti-mouse Tbet (Alexa Fluor® 488), anti-mouse Foxp3 (Alexa Fluor® 488), anti-mouse Gata3 (PE) e anti-mouse Rorγt (PE). Para citocinas utilizamos os anticorpos monoclonais: anti-mouse IFN-γ (FITC), anti-mouse TNF-α (PE), anti-mouse IL-10 (FITC), anti-mouse IL -12 (PE) e anti-mouse IL-17A (PerCP-Cy 5.5).

5.3.2 Análise das células T_{DC} γδ

O baço foi coletado passou pelo processo de divulsão mecânica e $1x10^6$ células contatas na câmera de *Neubauer* foram colocados em tubos próprios para a técnica de citometria para receberem a marcação celular com os anticorpos extracelulares para células T_{DC} γδ os anticorpos monoclonais anti-mouse TCR-γδ, anti-mouse CD11c (APC) e anti-mouse IA (CHPII - PE); Linfócitos T auxiliares ($CD4^+$) – anticorpo monoclonal CD4 anti-mouse (PE-Cy 5); Linfócitos T citotóxicos ($CD8^+$) – anticorpo monoclonal CD8a anti-mouse (PerCP-Cy 5.5)

e citocinas anti-mouse IFN-γ (FITC), anti-mouse TNF-α (PE) e anti-mouse IL -12 (PE) da BD Biosciences ™).

Após a marcação intracelular, as células foram incubadas por 30 minutos de acordo com protocolo pré-estabelecido e então lavadas com solução permeabilizadora Perm / Wash Buffer (BD Perm / Wash ™) com objetivo de remover anticorpos em excesso. Posterior a esta etapa, as células foram ressuspensas em 50μL de PBS e lidas no citômetro de fluxo BD FACSCalibur™ e os dados analisados pelo *Flowing Software*.

5.3.3 Estratégia de *gating*

As células coletadas dos baços, fígados, linfonodos sentinelas, medula óssea e tumores de camundongos do grupo controle e do grupo tumor induzido foram inicialmente delimitados por tamanho e granulosidade (FSCxSSC). Sendo assim, foi desenhada uma determinação dupla positiva para a marcação CD11c e IA (CHPII) e a partir dessa delimitação foi traçado um gráfico com a marcação para o TCRαβ para determinar as células T_{DC} αβ e TCR γδ para determinar as células T_{DC} γδ. Dentro dessas populações analisamos os marcadores fenotípicos de interesse para cada trabalho realizado, como marcadores de superfície, fatores de transcrição e citocinas.

5.4 ANÁLISES ESTATÍSTICAS

As análises estatísticas e os gráficos foram elaborados no *GraphPad Prism* 5.0 (GraphPad Software). Os testes *Kolmogorov-Smirnov* foram utilizados para verificar a normalidade das variáveis. Para o artigo 1, os dados não normais utilizamos o teste Mann-Whitney para comparar o grupo controle e o tumor induzido por 4T1 e o *Kruskal-Wallis* e *Dunn's post hoc tests* para comparar a expressão das células T_{DC} nos microambientes teciduais do baço, fígado, linfonodo sentinela, medula óssea e tumor. No artigo 2, foi utilizado o teste de Mann-Whitney para comparação entre grupos. Os resultados foram expressos em valores de mediana (mínimo e máximo). Diferenças foram consideradas estatisticamente significativas quando p menor que 5% ($p<0,05$).

6 RESULTADOS

6.1 ARTIGO 1

TÍTULO: **Phenotypic differences of tecidual T_{DCs} obtained from breast cancer mice**

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Phenotypic differences of tecidual T_{DCs} obtained from breast cancer mice

Polyana Barbosa Silva[1], Millena Prata Jammal[1,3], Márcia Antoniazi Michelin.[1,2] Eddie Fernando Cândido Murta[1,3]

[1] Reseach Institute of Oncology (IPON) - Federal University of The Triângulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil.

[2] Discipline of Immunology, Federal University of The Triângulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil.

[3]Discipline of Gynecology and Obstetrics, Federal University of The Triângulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil.

Corresponding author: E-mail address: eddiemurta@mednet.com.br. The Oncology Research Institute (IPON)/Titular Professor Eddie Fernando Cândido Murta (PhD), Departament of Gynecology and Obstetrics, (UFTM), Avenida Getulio Guarita, sn, CEP 38025-440, District Abadia, Uberaba-MG, Brazil; Tel: (+55 34) 3318-5326; Fax: (+55 34) 3318-5342

Abstract

Objective: To evaluate T_{DC} expression by flow cytometry for surface markers (CD4, CD8 and CD86), transcription factors (Tbet, Foxp3, Gata3 and Rorγt), and cytokines (IFN-γ, TNF-α, IL-10, IL-12 and IL-17) in spleen, liver, lymph node, bone marrow and tumor of 4T1 induced and healthy mice. *Results:* T_{DC} are more frequent in lymph nodes in the control and tumor groups, compared to the other environments studied ($p<0.0001$). When we compare the expression of surface markers between control and 4T1 induced groups we noted decreased CD4 T_{DC} expression in liver ($p=0.0001$), and the same with CD8 T_{DC} expression in spleen ($p=0.0012$) and liver ($p=0.0028$), as well as the expression of CD86 T_{DC} in spleen and liver ($p=0.0337$), in the 4T1-induced tumor group. When comparing transcription factors, there was a decrease T_{DC} Tbet and T_{DC} Foxp3 in spleen and liver ($p=0.0001$); and the same with T_{DC} Gata3 in liver ($p=0.0028$), and increase in T_{DC} Rorγt in bone marrow in the tumor group ($p <0.0001$). Regarding cytokines, we found decreased IFN-γ T_{DC} in spleen ($p<0.0001$) and bone marrow ($p=0.0002$), and the same with TNF-α T_{DC} in spleen and liver ($p<0.0001$), as well as the expression of IL-10 T_{DC} in spleen ($p<0.0001$), liver ($p<0.0001$) and bone marrow ($p<0,001$), of IL-12 T_{DC} in spleen and bone marrow ($p<0,001$), and IL-17 T_{DC} in spleen and liver ($p<0,001$) in the 4T1-induced tumor group in all comparisons. Phenotypic changes may be driven by the tissue microenvironment in the presence of the tumor. Directions are needed to understand the functionality associated with possible antitumor immunotherapy.

Key words: T_{DC} cells, breast cancer, tissue microenvironment, antitumor immune response.

Introduction

T_{DC} cells represent a rare and newly discovered subset of hematopoietic cells (1). Phenotypic characteristics refer to the nomenclature of this cell type, as they have the T-cell receptor (TCRαβ) marker as well as conventional dendritic cells (CD11c and MHC II)(2).

T lymphocytes, functionally important for the immune system, have in their structure the TCR receptor, consisting of an alpha and beta chain or a gamma and delta chain. This receptor recognizes major histocompatibility complex (CHP) molecules present in almost all nucleated cells, thus enabling T cell activation leading to a multitude of immune responses (3).

CD11c and MHC II surface markers are expressed by dendritic cells (DCs), ie professional antigen presenting cells, derived from pluripotent hematopoietic progenitors in the bone marrow (4,5)

DCs constitute about 1% of mononuclear leukocytes present in peripheral blood, and their localization in other tissues provides the function of immune system sentinels, continuously monitoring antigens (6).

In healthy mice 7% of splenic DCs express the TCRαβ receptor, a different characteristic from conventional DCs. These cells have similar characteristics in their origin and development to conventional DCs and T cells, being called T_{DC} cells. In addition, they are self-sufficient for antigen presentation and, when stimulated with lipopolysaccharides (LPS), are capable of producing IL-12 cytokine and exhibiting cytotoxic gene expression. (1).

Understanding and exploring the phenotypic characteristics of T_{DC} cells regarding the expression of surface markers, transcription factors and cytokines in the spleen, liver, lymph node, bone marrow and tumor in healthy conditions and in the presence of tumor cells in mice is of utmost importance.

However, few studies directed to this characterization have been performed. Thus, the objectives of the present study were to verify the frequency of T_{DC} cell expression in the tissues of the 4T1 cell-induced control and tumor groups, as well as to compare such cell frequency between the tissues of both groups, by evaluating the phenotypic characteristics of related T_{DC} cells. surface markers (CD4, CD8 and CD86), transcription factors (Tbet, Foxp3, Gata3 and Rorγt), and cytokine expression (INF-γ, TNF-α, IL-10, IL-12 and IL-17) in spleens, livers, sentinel lymph nodes, bone marrow and tumors obtained from healthy mice and 4T1-induced breast cancer.

Materials and Methods

Animals and experimental groups

For this study, 30 8-week-old female Balb/c mice were obtained from the Oncology Research Institute (IPON) of the Federal University of Triangulo Mineiro in Uberaba, Minas Gerais. The animals were divided into two groups: control (n=15), without tumor cell inoculation, and 4T1-induced tumor group (n=15), which were inoculated with breast cancer tumor cells of the 4T1 cell line. During the experimental phase, mice were kept in plastic cages under a 12h light / dark cycle at 21 ± 3°C, with food and water available ad libitum. After 28 days they were euthanized by an overdose of 50mg/kg ketamine and 15mg/kg xylazine. This study was approved by the Animal Use Ethics Committee (CEUA) of the Federal University of Triangulo Mineiro, under number 317.

Tumor induction

The 4T1 cells were cultured in RPMI medium in a humidified greenhouse (Water Jacket Incubator 3110, Thermo Fisher Scientific, Marietta, OH) at 37°C and 5% CO_2. For tumor induction, cells were washed with 0.9% saline and centrifuged at 290xg at 4°C for 10 minutes. Subsequently, when removing the supernatant, the cells were counted and $2x10^5$ cells were injected into the left mammary gland of the last pair of breasts of the 4T1 mouse group (Figure 1).

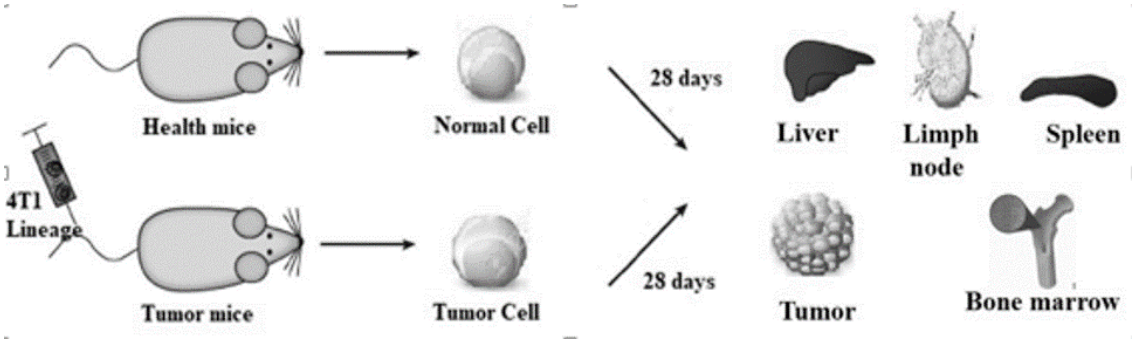

Fig. 1. Representation of tumor induction with 4t1 cell line in balb/c mice

T_{DC} cell characterization

T_{DC} cells were characterized according to immunophenotypic analysis by flow cytometry, performed by Kuka et al. (2012) by simultaneous expression of TCRαβ (T lymphocyte receptor), CD11c (adhesion molecule) and MHC II (Molecule Histocompatibility Complex) (1).

To characterize T_{DC} cells in different tissues, we performed the phenotypic analysis with surface markers with the same methodology as the authors cited above, that is, using the same gate strategy, revealing differences between the control and 4T1-induced tumor groups. Surface molecules represented by CD4 (helper T lymphocytes), CD8 (cytotoxic T lymphocytes) and CD86/B7-2 (costimulatory molecules) were analyzed, as were helper T lymphocytes, transcription factors (Tbet, Gata3, Rorγt and Foxp3) and cytokine expression (INF-γ, TNF-α, IL-10, IL-12 and IL-17).

Flow cytometry

After 28 days of the experimental period, the 4T1-induced Control and Tumor mice were euthanized and the spleens, livers, sentinel lymph nodes and tumors were removed and subjected to a mechanical rupture process to obtain suspended cells. Bone marrow cells were extracted from the femur and tibia of the mice of the studied groups as well. The suspended cells were homogenized and washed with 0.9% saline and centrifuged at 290xg for 10 minutes at 4°C. The supernatant was collected and the cell pellet counted and distributed at 1x106 cells in each cytometry tube.

The obtained tissue cells were incubated for 30 minutes at 4°C in the dark with extracellular monoclonal antibodies listed in Table 1. They were then washed with PBS (phosphate buffered saline) in order to remove unattached antibodies. After this procedure, cells in the tubes that would receive the intracellular labeling were permeabilized, fixed and incubated for 20 min with permeabilizing solution (BD Cytofix / CytopermTM) and then washed with buffered solution (BD Perm / WashTM Buffer) and intracellularly labeled.

For intracellular labeling we used monoclonal antibodies (Table 1) for transcription factors and cytokines, which were incubated for 30 min according to the pre-established protocol and then washed with Perm/Wash Buffer solution (BD Perm / Wash ™) to remove excess antibodies. Subsequently, they were resuspended in 50μL of PBS for reading using a BD FACSCalibur™ cytometer.

The collected data were analyzed and the average fluorescence intensities were determined by Flowing Software.

The gating strategy used was primarily size and granularity delimitation (FSCxSSC). In lymphocytes, positive populations were selected simultaneously for $CD3^+$ and IA^+ (MHCII). An FSCx TCRαβ graph was plotted from this selection. Within each of these populations, a dot plot was used to delineate T_{DC} cells and thus average fluorescence intensity by histograms for surface markers, transcription factors and cytokines of interest.

Statistical analysis

For statistical data analysis we used the GraphPad Prism 6.0 software (GraphPad Software). The Mann-Whitney test was used to compare the 4T1-induced tumor and control groups and the Kruskal-Wallis and Dunn's post hoc tests to compare TDC cell expression in tissue microenvironments: spleens, livers, sentinel lymph nodes, bone marrow and tumors. Results were expressed as medians (minimum and maximum values). Differences were considered statistically significant when p was less than 5% ($p < 0.05$)

Results

T_{DC} cells predominate in healthy and tumor-induced 4T1 mouse lymph nodes

The representative cytometric cell flow profile revealed a predominant T_{DC} cell infiltrate ($TCR\alpha\beta^+CD11c^+MHCII^+$) in the lymph nodes (Figure 2) of healthy mice (control) and 4T1 cell-induced breast cancer animals.

The frequency of $_{TDC}$ cells was significantly higher in the sentinel lymph nodes of the control group 70.05 (52.78-87.32) and 4T1-induced tumor group 67.02 (67.02 - 97.88) ($p < 0.0001$) compared to the other environments studied: lymph node versus spleen (*$p < 0.05$), lymph node versus liver (**$p < 0.001$), lymph node versus bone marrow (***$p < 0.0001$) of the control group; and lymph node versus spleen (*$p < 0.05$), lymph node versus liver (***$p < 0.0001$), lymph node versus bone marrow (*$p < 0.05$), lymph nodes versus tumor (** p <0 , 0001) of the 4T1-induced tumor group.

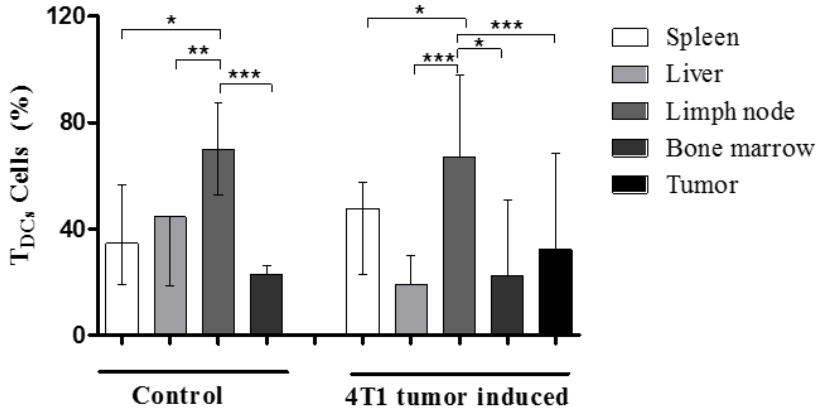

Fig. 2. Expression of T_{DC} cells in tissues of control and 4T1 tumor induced. Percentual of two independent experiments (n=15) of flow cytometric analyses to identify TCRαβ$^+$ CD11c$^+$ MHC II$^+$ T_{DCs} cells in spleen, liver, lymph node, bone marrow and tumor of control and 4T1 tumor induced. The results were analyzed by Kruskal-Wallis and Dunn's post hoc tests to compare the percentage of T_{DCs} cells in organs (represent by median with range). Differences were considered statistically significant at p <0.05 (5%). *p<0,05; **p<0,001; ***p<0,0001.

The tissue microenvironment determines the immunosuppressive phenotype of T_{DC} cells in the 4T1 cell-induced tumor group.

We evaluated surface markers (CD4, CD8 and CD86) and found in healthy mice (control group) increased expression of the CD4 molecule (helper T lymphocytes) in T_{DC} cells in the liver compared to the other organs analyzed with median of 1662 (1551-1662) (p = 0.0001) (Figure 3a). When looking at the 4T1-induced Tumor group, we noted that the tumor has the highest CD4$^+$ T_{DC} expression in tumor, with median of 2621 (1243 - 4962) (p = 0.0001) (Figure 3a). When comparing groups, we observed in the 4T1-induced tumor group there was a significant decrease in CD4$^+$ T_{DC} compared to the control group with median of 1662 (1551 - 1662) (p=0.0001) in the livers of the mice studied (Figure 3a).

Liver-resident T_{DC} express significantly high levels of the CD8$^+$ marker in the control group, with median *of* 3593 (2372 - 3593) (p = 0.0089) (Figures 3b). In the 4T1-Induced Tumor group we found high CD8$^+$ T_{DC} in lymph node, with median of 2911 (1181 - 4342), but reduced in bone marrow 488.5 (488.5 - 2678) (p <0.0001) (Figures 3b). Comparing the control and 4T1-induced tumor groups, we noted low levels of CD8$^+$ T_{DC} cells in spleen, with median of 764.7 (485.8 - 1467) and liver with 2078 (2078-2602) (p=0.0012 and p=0.0028, respectively) in the 4T1-induced tumor group. compared to the control group (Figure 3b).

In the analysis of the organs studied, we observed that in the control group, the spleen showed higher expression of $CD86^+$, with median of 3997 (1550 - 7700) T_{DC} cells ($p < 0.0001$) and bone marrow showed lower expression of these cells, with 398.0 (398.0 - 829.9) ($p < 0.0001$) (Figures 3c). In the 4T1-induced Tumor group we found higher $CD86^+$ T_{DC} expression in tumor, with median of 3395 (1757-3604) ($p = 0.0021$) (Figure 3c). By analyzing the relationship of $CD86^+$ T_{DC} cells between control and 4T1-induced Tumor we identified low levels in spleen, with median of 3997 (1550-7700) and liver with 2742 (2197-2742) of the 4T1-induced tumor group ($p=0.0012$ and $p=0.0337$, respectively) (Figure 3c).

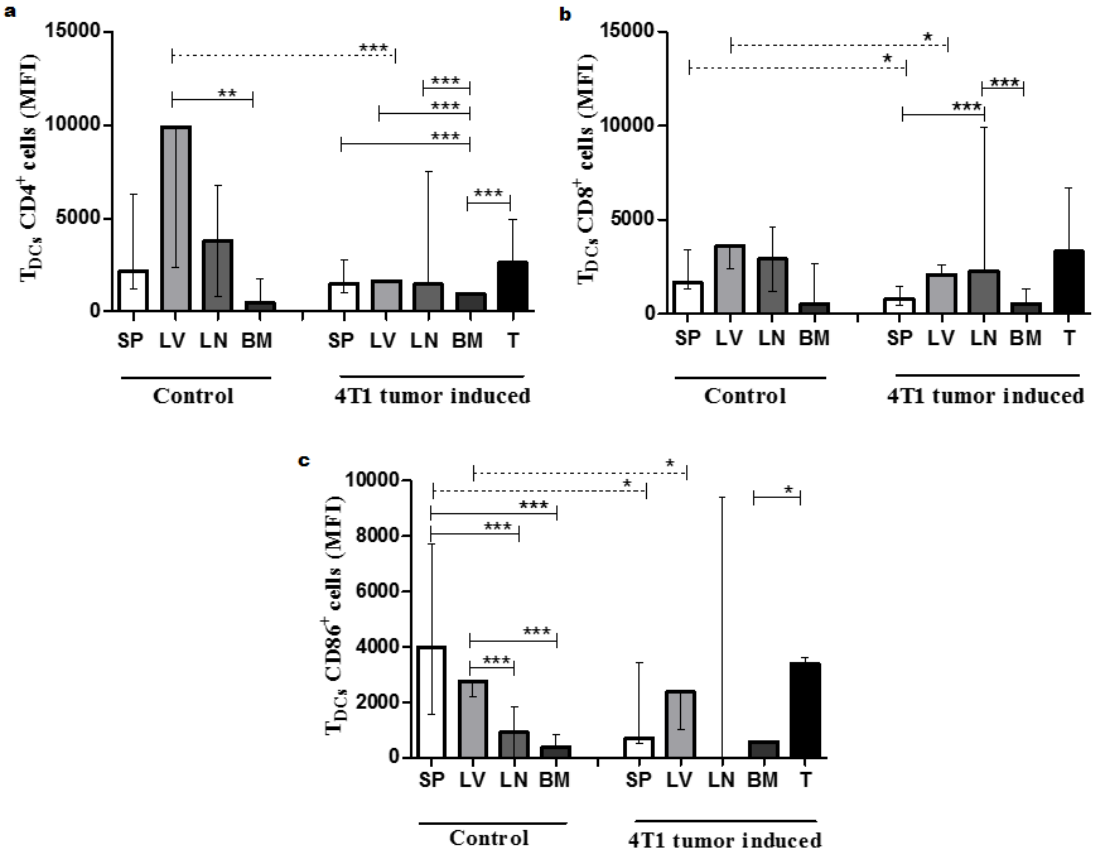

Fig. 3. The tissue microenvironment determines the T_{DC} cells phenotype positive for CD4, CD8 and CD86 control mice and 4T1 tumor induced. Mean fluorescence intensity of two independent experiments (n=15) (median with range) in flow cytometric analyses to identify T_{DCs} $CD4^+$, $CD8^+$ and $CD86^+$ cells in spleen, liver, lymph node, bone marrow and tumor of control and 4T1 tumor induced. (a) Mean fluorescence intensity of T_{DC} $CD4^+$ cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. (b) Mean fluorescence intensity of T_{DC} $CD8^+$ cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. (c) Mean fluorescence intensity of T_{DC} $CD86^+$ cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. The results were analyzed by and Kruskal-Wallis and Dunn's post hoc tests to compare the mean fluorescence instensity of T_{DCs} cells in organs statistical differences represented by solid line) and Mann-Whitney test to compare control and 4T1 tumor induced groups (statistical differences represented by dashed line). Differences were considered statistically significant at $p < 0.05$ (5%). *p<0,05; **p<0,001; ***p<0,0001. BM: Bone Marrow, LN: Limp node, LV: Liver, MFI: Mean fluorescence intensity, T: Tumor.

Different tissue microenvironments induce the positivity of transcription factors in T_{DC} cells

We investigated Tbet expression levels in T_{DC} cells in the various tissues studied (spleen, liver, lymph node, bone marrow and tumor). We observed that in the control group high levels of T_{DC} $Tbet^+$ levels were found in the sentinel lymph nodes, with a median of 6425 (6153 - 6697) ($p < 0.0001$), while in the 4T1-induced tumor group, we found greater expression of this transcription factor in liver, with a median of 5338 (4804 - 5338) ($p < 0.0001$). Comparing control and 4T1-induced tumor groups, we found that the presence of T_{DC} $Tbet^+$ in 4T1-induced breast cancer animals were decreased in spleen, with a median of 1427 (704.1 - 1517) ($p < 0.0001$) and liver with a median of 5338 (4804 - 5338) ($p = 0.0001$) compared to the control group (Figure 4a).

Analyzing the organs, we found that the presence of T_{DC} cells positive for the transcription factor Foxp3, showed higher expression in the sentinel lymph nodes of the control group, with a median of 5404 (4371 - 6436) ($p < 0.0001$) and also of the 4T1 induced tumor group, with a median of 5165 (5165 - 7473) ($p < 0.0001$). Evaluating between the 4T1-induced tumor and control groups, we noted a decrease in Foxp3+ T_{DC} in the 4T1-induced tumor group in spleen, with a median of 1016 (962.9 - 2265) ($p < 0.0001$) and in liver with a median of 5133 (5133 - 6077) ($p = 0.0337$) (Figure 4b).

$Gata3^+$ T_{DC} cells showed significantly higher levels in the liver of the control mice, with a median of 5788 (4700-5788) ($p < 0.0001$) and the 4T1-induced tumor group, with a median of 3346 (3346 - 5101) ($p < 0.0001$), compared to the spleen, lymph node, boné marrow and tumor. However, comparing the control group with the 4T1-induced tumor group we found a

lower expression of these cells in the liver of the 4T1-induced tumor group, with a median of 3346 (3346 - 5101) compared to the 5788 (4700- 5788) control group (p = 0, 0028) (Figure 4c).

Comparing organs, we found in the lymph node of the control group a higher level of T_{DC} expression Rorγt$^+$, with a median of 5589 (1373 - 9805) (p=0.0010) and in the group of mice induced by 4T1, the tumor has a higher expression, with a median of 3340 (3223 - 3722) (p <0.0001) (Figure 4d).

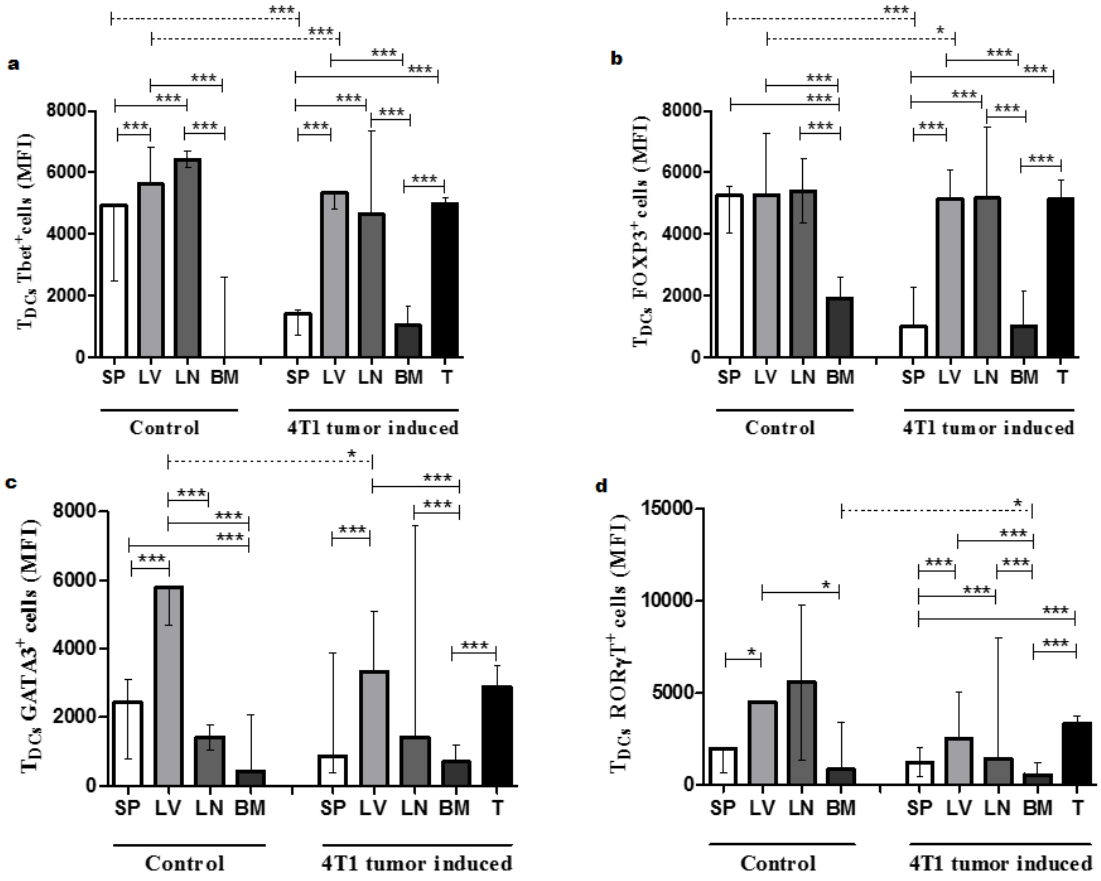

Fig. 4. Different tissue microenvironments induce positivity of transcription factors in T_{DC} cells Tbet$^+$, Foxp3$^+$, Gata3$^+$ and Rorγt$^+$ cells in control and 4T1 tumor induced mice. Mean fluorescence intensity of two independent experiments (n=15) (median with range) in flow cytometric analyses to identify to identify T_{DC} Tbet$^+$, Foxp3$^+$, Gata3$^+$ and Rorγt$^+$ cells in spleen, liver, lymph node, bone marrow and tumor of control and 4T1 tumor induced mice. (a) Mean fluorescence intensity of T_{DC} Tbet$^+$ cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. (b) Mean fluorescence intensity of T_{DC} Foxp3$^+$ cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. (c) Mean fluorescence intensity of T_{DC} Gata3$^+$ cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. (d) Mean fluorescence intensity of T_{DC} Rorγt$^+$ cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. The results were analyzed by Kruskal-Wallis and Dunn's post hoc tests to (statistical differences represented by solid line) compare the mean fluorescence instensity of T_{DCs} cells in organs. The Mann-Whitney test to compare control and 4T1 tumor induced mice groups (statistical differences represented by dashed line). Differences were considered statistically significant at p<0.05 (5%). *p<0,05; **p<0,001; ***p<0,0001. BM: Bone Marrow, LN: Limp node, LV: Liver, MFI: Mean fluorescence intensity, T: Tumor.

Increased levels of proinflammatory cytokines in T_{DCs} from different tissue microenvironments

The positive IFN-γ anti-inflammatory cytokine in T_{DC} was higher in the liver tissue microenvironment of control mice, with a median of 7010 (7010 - 7293), data also found in the 4T-induced tumor group, with a median of 7590 (6335 - 7590) ($p < 0.0001$). In the comparison between the two groups studied, we found that in the 4T1-induced tumor group, the expression of IFN-γ^+ T_{DC} significantly decreased in the spleen, with a median of 1554 (705.3 - 1885) ($p<0.0001$) and bone marrow, 733.1 (733.1 - 1307) ($p = 0.0002$) (Figure 5a).

We evaluated the expression of cytokine IL-10 in T_{DC} in the tissues studied, and higher expression was found in the control mouse liver with a median of 8777 (6999 - 8777) ($p < 0.0001$) and in the 4T1-induced tumor group lymph node with 6791 (6791 - 8162) ($p < 0.0001$). The results found showed that when tumor cells were involved, IL-10^+ T_{DC} expression decreased significantly in spleen, with a median of 1689 (914.3 - 2049) ($p < 0.0001$), 5219 (5219 - 5619) ($p < 0.0001$) in liver, and 894.0 (894.0 - 1712) ($p = 0.0002$) in bone marrow (Figure 5b).

The IL-12^+ T_{DC} evaluated were more evident in the liver of both study groups, control the median was 6153 (6153 - 7415) ($p = 0.0022$) and 4T1-induced tumor the median was 5300 (5300 - 7415) ($p < 0.0001$). Again, we note that the presence of tumor cells induces a significant decrease in tumor cell expression, particularly in the spleen with a median of 1841 (360.7 - 2728) and bone marrow with a median of 791.0 (688.8 - 791.0) of 4T1-induced breast cancer mice ($p = 0.0002$) (figure 5c).

Represented by Figure 5d, tumor necrosis factor (TNF-α) expressed in T_{DC} was found to be highest in the liver of both groups, with control with a median of 5303 (5303 - 5454) ($p = 0.0001$) and 4T1-induced tumor with a median of 4494 (4494 - 5032), respectively. Findings observed in the comparison between both groups, we found significant decrease in TNF-α^+ T_{DC} levels in spleen, with a median of 1655 (403.8 – 2673) and bone marrow 819.2 (819.2 - 1521) ($p < 0.0001$).

We also analyzed the expression of IL-17 + T_{DC} and observed elevated levels in the liver of the control group, with a median of 4793 (4783 - 5717) mice ($p = 0.0026$) and the 4T1-induced tumor lymph node with a median of 4776 (1590 - 4776) ($p < 0.0001$). We found that in the presence of tumor cells this immunosuppressive cytokine was decreased in the spleen, with a median of 578.5 (326.3 - 873.8) ($p < 0.0001$) and liver cancer-induced mice with 1200 (1200 - 1988) ($p = 0.0001$) (Figure 5e).

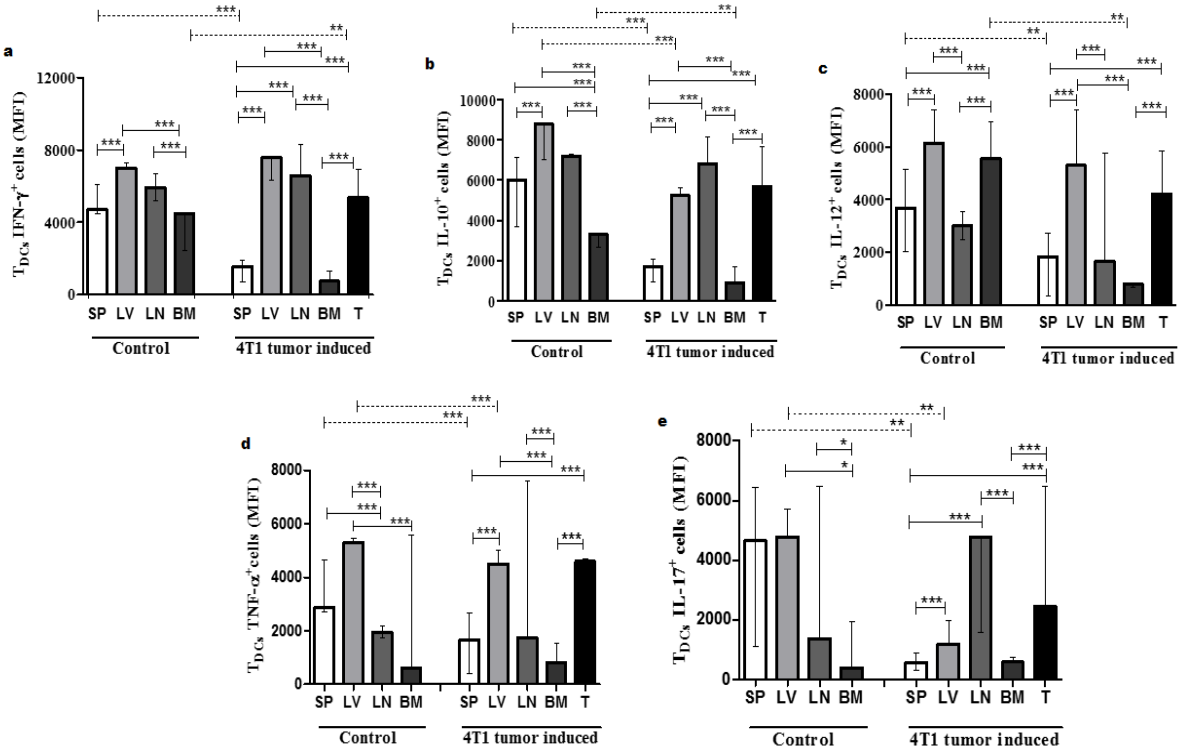

Fig. 5. Increased proinflammatory cytokine levels in T_{DC}s IFN-γ^+, IL-10^+, IL-12^+, TNF-α^+ and IL-17^+ from different tissue microenvironments from control and 4T1 tumor induced mice. Mean fluorescence intensity of two independent experiments (n=15) (median with range) in flow cytometric analyses to identify to identify T_{DC}s IFN-γ^+, IL-10^+, IL-12^+, TNF-α^+ and IL-17^+ cells in spleen, liver, lymph node, bone marrow and tumor of control and 4T1 tumor induced mice. (a) Mean fluorescence intensity of T_{DC} IFN-γ^+ cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. (b) Mean fluorescence intensity of T_{DC} IL-10^+ cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. (c) Mean fluorescence intensity of T_{DC} IL-12^+ cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. (d) Mean fluorescence intensity of T_{DC} TNF-α^+ cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. (e) Mean fluorescence intensity of T_{DC} IL-17^+ cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. The results were analyzed by Kruskal-Wallis and Dunn's post hoc tests to (statistical differences represented by solid line) compare the mean fluorescence intensity of T_{DC}s cells in organs. The Mann-Whitney test to compare control and 4T1 tumor induced mice groups (statistical differences represented by dashed line). Differences were considered statistically significant at $p<0.05$ (5%). *p<0,05; **p<0,001; ***p<0,0001. BM: Bone Marrow, LN: Limp node, LV: Liver, MFI: Mean fluorescence intensity, T: Tumor.

Discussion

Kuka et al. (2012), pioneers in the discovery and characterization of T_{DC} cells, reported that approximately 7% of mouse DCs expressed on their surface the TCR αβ receptor, with 0.04% present in the spleen, a differentiated characteristic related to conventional αβ T cells (1).

Recent studies have shown that some subpopulations of macrophages have the expression combinatorial receptor TCRαβ (7). This immune receptor is present in macrophages in autoimmune diseases (8), in diseases such as atherosclerosis (9) and in the tumor microenvironment playing an anti-inflammatory role (10). A study carried out with human neoplasms (colon cancer, esophageal cancer, liver carcinoma, melanoma) demonstrated that 40% of macrophages express TCRαβ in the tumor microenvironment and more than 30% in experimental breast cancer induced in mice with adenocarcinoma cells breast (10).

In our studies, we used a 4T1 murine mammary adenocarcinoma cell line. This experimental model is widely used in the evaluation and better understanding of tumor biology. It is a highly tumorigenic and invasive cell line, that is, spontaneous metastases are observed in various organs (11). According to the literature, research uses this line because it allows to evaluate the immune response to malignant neoplasms effectively.(12–15).

The mouse mammary carcinoma 4T1 was originally isolated as subpopulation 410.4 derived from a spontaneously arising mammary tumour in BALB/c fC3H mice (16,17). The 6-thioguanine-resistant 4T1 tumour metastasizes via the hematogenous route to liver, lungs, bone and brain, making it a good model of human metastatic breast cancer (18). 4T1 grows progressively and causes a uniformly lethal disease, even after excision of the primary tumour (19).

According to the experimental design used, the parameters related to the animals' weight were not evaluated. The mice used in our study were born, raised and fed under the same conditions, according to international standards for the management of experimental animals (20). Thus, the animals showed no obvious signs of obesity or malnutrition. The time required for the study was not enough for the malignancy to result in weight changes due to obesity or cachexia.

In lymph nodes, this same characterization study found that 0.06% of lymph node DCs had the TCR αβ marker (1). In view of this, this study verified the presence of these cells in different tissue microenvironments such as the spleen, liver, lymph node, bone marrow and tumor in healthy conditions and in the presence of breast cancer in mice induced by the 4T1 cell line.

Lymph nodes are frameworks that specialize in interception between innate and adaptive immunity. Professional antigen presenting cells (APCs) such as dendritic cells (DCs) and lymphocytes are brought to this organ for antigens (21). T_{DC} are believed to perform the same mechanism (1).

As a result, we found that T_{DC} are present in larger amounts in lymph nodes when studying the expression of these cells in the other environments studied ($p<0.0001$). Regarding the comparison between healthy and breast cancer-induced mice, no significant differences were observed related to the expression of these cells.

Taken together, these results suggest that T_{DC} cells play a previously unknown role in the presentation or recognition of antigens on lymph nodes. In addition, we also demonstrate that the presence of neoplastic cells has no influence on the frequency of T_{DC} cells.

Following the phenotypic characterization line of T_{DC} in different tissue microenvironments, we analyzed molecules that characterize helper T lymphocytes (CD4) and cytotoxic T lymphocytes (CD8) and dendritic cells with the presence of the B7-2 costimulatory molecule (CD86).

In healthy mice we found helper T_{DC} ($CD4^+$ T_{DC}) and cytotoxic T_{DC} ($CD8^+$ T_{DC}) cells in greater quantity in the liver compared to the other organs ($p <0.0001$). In contrast to animals with breast cancer, these markers were more evident in the tumor and sentinel lymph nodes, so we can infer from the regulatory cells, suppressed by the tumor conditions present there ($p <0.0001$).

Our results corroborate the study by Rad et al. (2015) which indicated that the ratio of helper and cytotoxic T lymphocytes was somewhat different in peripheral blood, tumor microenvironment and lymph nodes, resulting in lymph node as tissue. with the largest amount of these lymphocytes followed by peripheral blood and tumor tissues (6,22).

Another hypothesis would be that these cells may be in a stage of lymphocyte or dendritic cell maturation, thus not being able to migrate to the tumor microenvironment, thus inferring the characterization of a greater presence of these cells in the lymph node compartments. Studies with mouse models and human samples have shown that metabolic dysregulation caused by hepatic tumor precursor non-alcoholic fatty liver disease (DHGNA) causes selective loss of intrahepatic T-lymphocytes, but this change was not found with regard to hepatic cytotoxic T lymphocytes in the presence of tumor cells, which consequently accelerate the processes of hepatocarcinogenesis (23).

The liver is considered an immunological organ formed by a complex histological structure composed of 70% hepatocytes, 16 to 22% intrahepatic lymphocytes and 30% non-parenchymal cells (24) of which the hepatic macrophages corresponded 80 to 90% (24,25).

Recent studies have demonstrated the presence of several types of lymphocytes residing in the liver. We find in the microenvironment of this organ CD8 memory lymphocytes, invariant natural killer cells (iNKT), mucosa associated T cells (MAIT) and γδT cells, in addition to

innate lymphoid cells (ILCs) and NK that can remodel phenotypic and functional regulatory characteristics transcriptional (25).

Functionally, these lymphocytes recognize a wide variety of harmful signals, playing the role of sentinels contributing to immune surveillance in the face of infectious and non-infectious responses in the liver (25).

Regarding T_{DC} cells positive for the B7-2 molecule (T_{DC} $CD86^{+}$), we observed the presence of significant spleen in healthy mice and breast cancer-induced tumor ($p < 0.0001$). ($p<0,0001$).

The CD86 costimulatory molecule is related to the B7/ CD28 family and the tumor necrosis factor (TNF) family, which are involved in triggering the cell-mediated immune response, and in later stages, by activating T-receptor-bound T lymphocytes (TCR) (26). Thus, the T_{DC} were characterized and studied because they have a functional TCR and are self-sufficient for antigen presentation. (1). Our results indicate that the B7-2 molecule may be present in splenic T_{DC} and favor antigen presentation by T_{DC}. The immune system cells present in the spleen include various subsets of T cells, B and DCs, and other cell types. (27).

Studies by Slits and colleagues (2016) analyzed subsets of myeloid cell-derived suppressor cells (MDSC) in the hepatic parenchyma of mice with hepatocellular carcinoma, which may cause phenotypic changes in efficient defensive cells called Kupffer cells, and found that these cells expressed less CD86 and MHCII costimulatory molecule in the livers of these mice (28).

In order to verify the T_{DC} cell profile, we evaluated the transcription factors Tbet, Gata3, Rorγt and Foxp3. Changes in these transcription factors were found in TDCs from different tissues in both groups.

Our results demonstrated that Tbet transcription factor in TDCs is present more in lymph nodes of healthy mice and liver of mice with breast cancer ($p<0,0001$). Tbet is considered a chief regulator of Th1 lymphocytes (29). Kachler and colleagues (2018) observed increased expression of Tbet and Foxp3 transcription factors in pulmonary helper T lymphocytes ($TCD4^{+}$) of lung cancer mice, accompanied by increased production of TGF-β, immunosuppressive cytokine. In our study, we observed an increase in Foxp3 expression in T_{DC} in sentinel lymph nodes ($p < 0.0001$) and a decrease in spleen and liver in mice with breast cancer ($p<0,0001$).

In Gata3 T_{DC}, we found a marked presence in the liver ($p < 0.0001$) in both healthy and breast cancer mice. Gata3 transcription factor is a major determinant of Th2 lymphocyte polarization (30) and studies suggest that expression of this marker in breast cancer is highly

related to luminal transcription of this cancer (31), in part because it causes an inflammatory process and favors tumor growth (32).

Wei et al. (2017) reported that Gata3 expression was negatively regulated in the presence of gastric cancer mainly in humans, and was still associated with tumor size, stage in which it was present and episodes of metastasis (33). In our study, we also identified a remarkable expression of T_{DC} Rorγt cells in the tumors of mice with experimental breast cancer ($p < 0.0001$), thus assuming that the high levels of this transcription factor are related to a Th17 profile that can influence the tumor microenvironment in its promotion and also regulate the activity of neighboring stromal cells (34). The results are similar to those observed in the study by Wang et al. (2016), who reported in their studies that the transcription factor Rorγt was strongly expressed in human prostate cancer tumors (35).

With these results we can establish that the expression of transcription factors in T_{DCs} cells varies according to the tissue in which they are found, and this variation can often be due to the escape mechanism that tumor cells acquire for an ineffective immune response.

By evaluating the phenotypic and functional characteristics of T_{DCs} cells related to expression of INF-γ, TNF-α, IL-10, IL-12 and IL-17 cytokines in tissues obtained from healthy mice and 4T1-induced breast cancer, we have seen that IFN-γ expression in TDC cells was suppressed in lymph nodes obtained from mice with experimental breast cancer. ($p<0,0001$).

IFN-γ cytokine plays an important role in immune response to infectious agents and tumors (36). Studies by Tanner and colleagues (2016) have suggested that immune system failures responsible for carcinogenesis may contribute to tumor development and progression; furthermore, mice with colorectal cancer demonstrated that helper T lymphocytes produced less IFN-γ due to mutations caused by tumor cells to immune cells (37).

We evaluated the expression of tumor necrosis factor alpha (TNF-α) in T_{DC} cells in the tissues studied and found a higher amount of this cytokine in the liver of healthy and breast cancer mice ($p<0,0001$). TNF-α is a cytokine with proinflammatory effect comprised in inflammatory circumstances (38), but also present in advanced tumors, responsible for cachexia and even involved in escape mechanisms (39). Studies by Kastl et al. (2014) have shown that hepatocellular carcinoma (HCC) is enriched by high levels of TNF-α responsible for NF-κB activation, favoring the migration of immune cells to the site (40).

We found that T_{DC} cells constitute a cellular repertoire, which is IL-10 producing, and in mice with experimental breast cancer, IL-10^{+} T_{DC} were reduced in all studied organs, being more evident in sentinel lymph nodes ($p<0,0001$).

Studies related to IL-10 cytokine have shown that many cells have the ability to produce it, including immune system cells such as monocytes/macrophages, dendritic cells, B lymphocytes, regulatory T cells, CD4 T lymphocytes, CD8 T and NK cells. (41)

In addition to IL-12, we also studied the production of IL-17 by T_{DC} in different tissue microenvironments. Our results consisted of the decrease in IL-17^+ T_{DC} cells in the lymph node, spleen and liver obtained from mice with experimental breast cancer ($p < 0.0001$). IL-17 is a cytokine produced by Th17 profile T helper cells which is associated with down regulation of immune cells and tumor promotion to various immune responses. (42).

Studies of mice with cervical cancer by Yang et al. (2016) showed that lymphocyte expression of this cytokine may reduce the size of tumors in transplanted mice, and association with tumor cells may increase lymphocytic infiltration into the tumor tissue microenvironment (43).

Overall, these data show that although there were differences in tissue microenvironment, the phenotype of cytokine expression in T_{DC} showed detectable differences in the presence of tumor cells, thus inferring the action of the tumor evasion mechanism provided by the presence of tumor cells.

Among the limitations of this study, it should be mentioned that the methodology did not include performing cell sorting or cDNA microarray analysis in order to provide a better evaluation of gene expression of T_{DC} cells, which would have allowed us to achieve more.

The main objectives have been elucidated, allowing a further explanation of the importance and functionality of these cells. This study is unprecedented in the analysis of T_{DC} cells, with respect to the surface markers (CD4, CD8 and CD86), transcription factors (Tbet, Foxp3, Gata3 and Rorγt) and cytokine expression (INF-γ, TNF-α, IL-10, IL-12 and IL-17 in different mouse organs (spleen, liver, lymph node, bone marrow and tumor) under the influence of breast cancer tumor cells.

Conclusion

T_{DC} cells showed a decrease in several surface markers, transcription factors and cytokines in the studied tissue microenvironments obtained from animals with 4T1 cell-induced cancer. In the lymph nodes the presence of T_{DC} cells was quite evident, but it is in the liver that we found that these cells can play an immunoactivity role, as evidenced by positive T lymphocyte markers characteristic of the Th1 profile, by the expression of Tbet and also the

production of cytokines favorable to effective antitumor immune response such as TNF-α, INF-γ and IL-12.

Taken together, these results reveal that the tissue microenvironment provides strong indications for a phenotypic adequacy of T_{DC}, raising the question of whether phenotypic alterations can be driven by the tissue microenvironment in the presence of the tumor and thus result in functional differences in relation to the tumor expression of these markers against an immune response.

Future research is needed to address crucial issues related to T_{DC} cell immaturity, which will certainly provide additional information on the expression process and functionality related to possible cancer immunotherapy.

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Conflict interest

The authors declare no conflicts of interest.

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Table 1: Antibody reagentes for flow cytometry

Reagent	Supplier	Cat. No/lot no.	Isotype	Clone
FITC-anti-mouse TCRβ	BD Biosciences	553170	Hamster IgG2	H57-597
PE-Cy 5-anti-mouse CD4	BD Biosciences	553050	Rat (DA) IgG2a, κ	RM4-5
PerCP-Cy 5.5-anti-mouse CD8a	BD Biosciences	551162	Rat (LOU) IgG2a, κ	53-6.7
APC-anti-mouse CD11c	BD Biosciences	550261	Hamster IgG1, λ2	HL3
APC-anti-mouse CD86	BD Biosciences	553768	Rat (LOU) IgG2a, κ	GL1
PE-anti-mouse MHC Class II (I-A)	BD Biosciences	553548	Mouse (SJL) IgG2b, κ	MAS-32.1
ALEXA FLUOR®488-anti-mouse Tbet	BD Biosciences	561266	Mouse IgG1, κ	O4-46
ALEXA FLUOR®488-anti-mouse Foxp3	BD Biosciences	560407	Rat IgG2b	MF23
PE-anti-mouse Gata3	BD Biosciences	560074	Mouse (BALB/c) IgG1, κ	L50-823
PE-anti-mouse Rorγt	BD Biosciences	562607	Mouse IgG2a, κ	Q31-378
FITC-anti-mouse IFN-γ	BD Biosciences	554411	Rat IgG1, κ	XMG1.2
PE-anti-mouse TNF-α	BD Biosciences	554419	Rat IgG1	MP6-XT22
FITC-anti-mouse IL-10	BD Biosciences	554466	Rat IgG2b	JES5-16E3
PE-anti-mouse IL-12	BD Biosciences	554479	Rat IgG1	C15.6
PerCP-Cy™5.5-anti-mouse IL-17A	BD Biosciences	560666	Rat IgG1	TC11-18H10

Fonte: BD Biociences

6.1 ARTIGO 2

TÍTULO: **Immunological characteristics between T_{DC} αβ and T_{DC} γδ cell in mice spleen with breast cancer-induced**

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Immunological characteristics between T_{DC} αβ and T_{DC} γδ cell in mice spleen with breast cancer-induced

Polyana Barbosa Silva[1], Millena Prata Jammal[1,3], Márcia Antoniazi Michelin[1,2], Eddie Fernando Cândido Murta[1,3]

[1] *Reseach Institute of Oncology (IPON) - Federal University of The Triângulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil.*
[2] *Discipline of Immunology, Federal University of The Triângulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil.*
[3]*Discipline of Gynecology and Obstetrics, Federal University of The Triângulo Mineiro (UFTM), Uberaba, Minas Gerais, Brazil.*

Corresponding author: The Oncology Research Institute (IPON)/Titular Professor Eddie Fernando Cândido Murta (PhD), Departament of Gynecology and Obstetrics, (UFTM), Avenida Getulio Guarita, sn, CEP 38025-440, District Abadia, Uberaba-MG, Brazil; Tel: +553433185326; Fax:+553433185342 e-mail address: eddiemurta@mednet.com.br.

Conflict of Interests:

The authors have no conflict of interests to declare.

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Abstract

Objectives: to clarify the possible role antitumoral of γδ T_{DC} cells and αβ T_{DC} cells in an experimental model of breast cancer. **Materials and methods:** 30 spleens of Balb / c mice analyzed by flow cytometry, separated between the control group (n = 15) and tumor group induced by 4T1 (n = 15). **Results:** presence of a percentage of 26.53% of γδ T_{DC} in the studied mice, control group ($p < 0.0001$), the proportion of αβ T_{DC} lower in splenic cells than γδ T_{DC}. However, these two cell types are reduced in tumor conditions ($p < 0.0001$) and proportion of IFN-γ, TNF-α, IL-12 and IL-17 cytokines produced by γδ T_{DC} higher than those produced by αβ T_{DC}, but decreased under conditions of tumor-related immune system response ($p < 0.0001$). **Conclusion:** Healthy mice induced to the 4T1 breast tumor presented T_{DC} with γδ TCR repertoire. These cells express cytotoxic T helper and T lymphocyte molecules, producing anti-tumor proinflammatory cytokines.

Keywords: Immunology. Breast neoplasms. Immunotherapy. T-lymphocytes. Receptors.

Introduction

A new type of immune cell has been described, and these new cells have characteristics of innate and acquired immunity (1). The T_{DC} cells were identified in mice and humans, as cells that express T cell receptors (TCRαβ) specific of T lymphocytes, and simultaneously express the CD11c markers and major histocompatibility complex type II (MHCII or HLA in humans), found in innate cells, mainly in dendritic cells. These molecules in the same cell confer unique characteristics and properties, behave functions of dendritic cells (DCs) that do not need to be activated by antigen-presenting cells (APCs). When stimulated by specific receptors, such as the family of Toll-like receptors, it can produce cytokine IL-12, process, and present antigens (1).

The T lymphocytes respond in a specific manner to pathogens and cancer cells by the recognition specific of antigens due to TCR in their membrane, similar to the role of the immunoglobulins in B cells. The TCR consist of two polypeptide chains, approximately 90-99% of all T cells have the αβ TCR, but a minority has chains γδ (2,3). Both cells originate from common thymic precursors, but the biological roles and molecular understanding of these two subsets differ substantially. The T lymphocytes that express αβ TCRαβ depends on the presentation of antigens in a defined HLA molecule to be activated, and usually are tolerant to self-peptides. By the other way the γδ T lymphocytes do not rely on the recognition of classic HLA molecules, and the identification of infection of cancer cells are made by ubiquitous changes observed across many individuals, which allows these cells not to undergo the rejection process, and consequently can be transferred more easily between individuals. Different of αβ T cells that have their biological role well- characterized in cancer immune surveillance, the protective role of γδ cells during tumor development has only been increasingly reported over the past two decades.

The presence of tumor-infiltrating γδ T lymphocytes has been associated with good prognosis in patients with melanoma (4) and gastric cancer (5), and high levels of these types of circulating lymphocytes have been associated with reduced cancer risk and increased 5-year-disease-free and with increased survival after bone marrow transplant with acute leukemia (6).

The ability antitumoral of γδ T lymphocytes is associated with their synthesis of interferon γ (IFN-γ), and tumor necrosis factor-alpha (TNF-α), as well as their cytotoxic potential. And other studies have also reported the role of IL-17-produced by γδ T cells, mainly when they act together with immunogenic cell death-inducing chemotherapeutic drugs (7).

To clarify whether T_{DC} cells could also have the chains γδ, and the possible antitumor role of this new cell population, we investigated if the T_{DC} population comparing both αβ TCR and γδ TCR T_{DC}, and their cytokines in an experimental model of breast cancer induced by 4T1 cells.

Materials and methods

Animals

Thirty female BALB/c mice were used, aged between 6 and 8 weeks, kept in the sectoral vivarium of the Oncology Research laboratory (IPON) of the Federal University of Triangulo Mineiro (UFTM). During the 28-day experimental period, the animals were divided into a control group (healthy mice) and a 4T1-induced group (inoculation of breast tumor cells of the 4T1 strain). Each group composed of 15 animals, was conditioned in plastic cages under a 12h light / dark cycle at 21 ± 3 ° C, with food and water available ad libitum. After the experimental period, the animals were euthanized by overdosing 50mg / kg ketamine, and 15mg / kg xylazine and the spleens were removed for the study. This study was approved by the Animal Use Ethics Committee of the Federal University of Triangulo Mineiro, under number 379/2016 - CEUA / UFTM.

Tumor induction

The animals were selected at random, which the group induced by 4T1 were inoculated with 2x105 cells in the last pair of breasts, in the left mammary gland. Tumor cells of the strain mentioned above are cells isolated from the spontaneous tumor of Balb / c mice, with high proliferative, invasive, and tumorigenic power. The cells were maintained in culture in RPMI medium (Roswell Park Memorial Institute Medium) and incubated at 37ºC and 5% CO2 (Water Jacket Incubator 3110, Thermo Fisher Scientific, Marietta, OH) After the culture period, the cells were washed with 0.9% saline solution and centrifuged at 290xg, at 4ºC for 10 minutes and then inoculated in the mice of the tumor group induced by 4T1.

Characterization of immune cells by flow cytometry

The spleens of the mice in the control group and induced by 4T1 were disclosed, filtered, and washed with saline solution, and after counting in a Neubauer chamber, $1x10^6$ cells were placed in tubes suitable for the flow cytometry technique. The cells were then labeled with

extracellular anti-γδ TCR antibodies (T lymphocyte receptor), anti-CD11c (adhesion molecules), and anti-IA (CHPII-antigen-presenting molecule), anti-CD4 (helper T lymphocytes) and anti-CD8 (cytotoxic T lymphocytes) all antibodies acquired from BD Biosciences ™. After the 30-minute incubation, the cells were washed and prepared to receive the intracellular antibody labels for anti-IFN-γ, anti-TNF-α, IL-12, and IL-17 pro-inflammatory cytokines. To block nonspecific binding, we used the anti-mouse IgG2b, anti-rat IgG2a, and anti-rat IgG2b isotypes. The cells were read on the BD FACSCalibur™ cytometer and the data analyzed using Flowing software.

The gating strategy used was the delimitation by size and granularity (FSCxSSC) of the spleen cells of the control group and induced by 4T1. Subsequently, the double-positive labeling of CD11c and IA (MHCII) was limited and, thus, the γδ TCR labeling traced the γδ T_{DC} cells. Within this population of γδ T_{DC}, we analyzed the phenotypic and cytokine markers of interest.

Statistical Analysis

Statistical analyzes and graphs were prepared using GraphPad Prism 5.0 (GraphPad Software). The Kolmogorov-Smirnov tests were used to verify the normality of the variables. Non-normal samples were analyzed by the Mann-Whitney test, both for comparison between control groups and tumor-induced from both profiles and for comparison of αβ T_{DC} and γδ TDC cell expression. The data obtained were represented with corresponding median, minimum and maximum values. The difference found between the groups was considered statistically significant when $p < 0.05$.

Results

The flow cytometry profile shows the comparison of αβ T_{DC} (TCRαβ+CD11c+ MHCII+) and γδ T_{DC} (TCRγδ+CD11c+MHCII+) cell infiltrates in the spleen of healthy mice induced by breast cancer by 4T1 cells (Figure a, b e c). When analyzing the frequency of the γδ T_{DC} cell profile (figure a), we found a significant decrease in the 4T1-induced tumor group, with a median of 18.11 (17.21 - 19.01) compared to the control group (26.53, 23.62 - 29.99) ($p < 0.0001$). We compared the frequencies of both αβ T_{DC} and γδ T_{DC} cells and found statistical significance in the tumor-induced group of both cell profiles, that is, there is a higher amount

of αβ T_{DC} cells (47.74, 22.97 - 57.36) than γδ T_{DC} cells (18.11, 17.21 - 19.01) in the spleen of the tumor-induced mice group 4T1 ($p < 0.0001$).

We analyzed the mean fluorescence of auxiliary T lymphocyte (CD4) and cytotoxic T lymphocyte (CD8) markers present in the αβ T_{DC} and γδ T_{DC} cells of both groups (figure b), we observed that the CD8 αβ T_{DC} cells showed a decrease in the group tumor induced by 4T1 (764.7, 485.8 - 1467) concerning the control group (1650, 1292 - 3418) ($p = 0.0012$). Regarding CD4 γδ T_{DC} cells, we found a significant decrease in the tumor-induced group 4T1 (1873, 1421 - 2325) compared to the control group (2350, 2140 - 2561) ($p = 0.0009$), as well as for CD8 γδ T_{DC} cells, a significant decrease in the tumor group induced by 4T1 (329.0, 292.3 - 692.4) compared to the control group (1630, 1370 - 1889) ($p < 0.0001$). When comparing both cell profiles, we found a decrease in the tumor-induced group of CD8 γδ T_{DC} cells (329.0, 292.3 - 692.4) compared to CD8 αβ T_{DC} cells (764.7, 485.8 - 1467) ($p < 0001$).

The mean fluorescence intensity of the cytokines produced by αβ T_{DC} and γδ T_{DC} cells were analyzed (figure c). We note that for IFN-γ αβ T_{DC} cells there was a decrease in the induced group 4T1 (1554, 705.3 - 1885) compared to the control group (4720, 4488 - 6120) ($p < 0.0001$), as well as for TNF-α αβ T_{DC} decrease in the 4T1 induced group (1655, 403.8 - 2673) compared with the control group (2877, 2716 - 4658) ($p < 0.0001$). Regarding IL-12 αβ T_{DC} cells, we observed a decrease in the tumor group induced by 4T1 (1841, 360.7 - 2728) compared to the control group (3686, 2028 - 5163) ($p = 0.0002$) as well as for L-17 αβ T_{DC} cells, which also observed a decrease in the tumor group induced by 4T1 (578.5, 326.3 - 873.8) compared to the control group (4666, 1117 - 6436) ($p < 0.0001$).

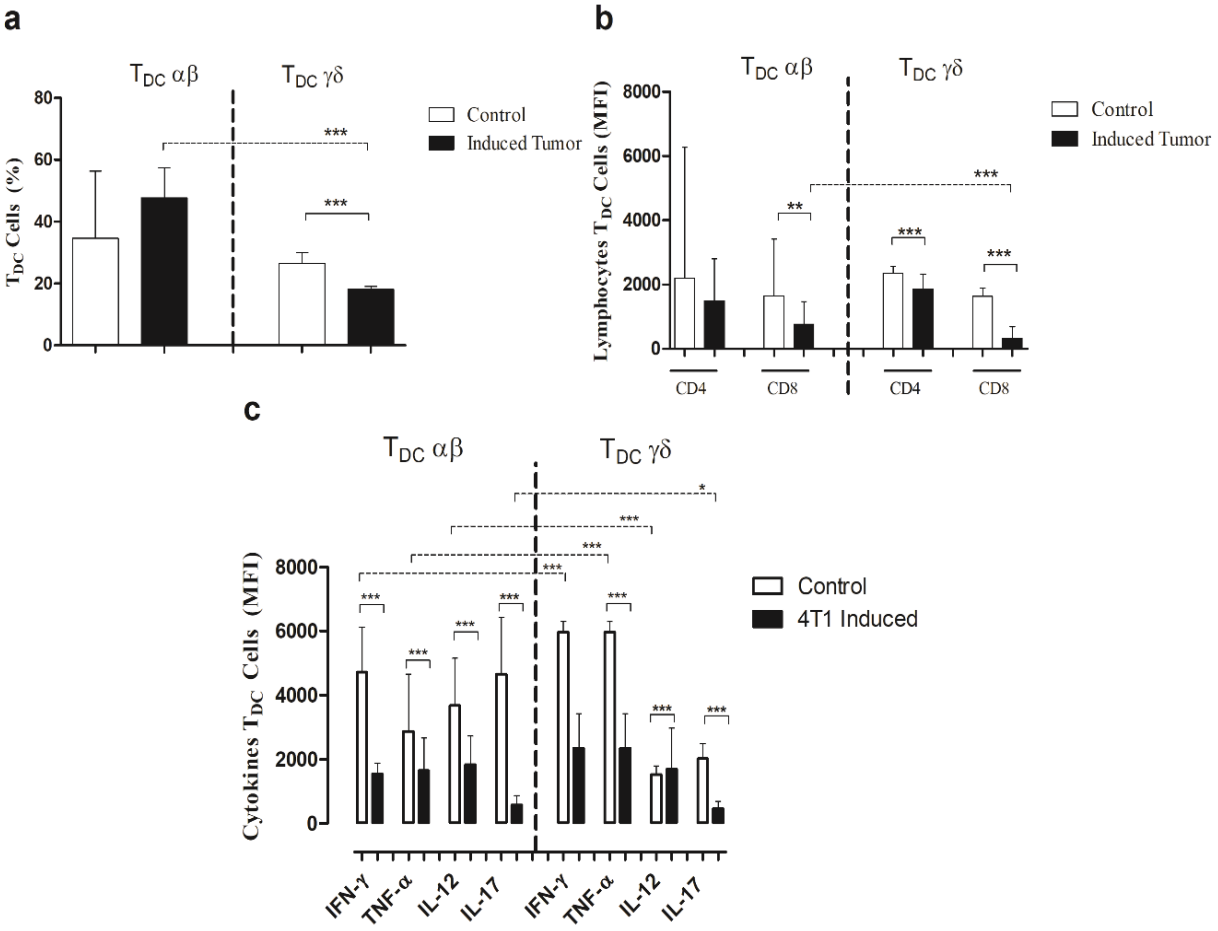
Regarding the cytokines produced by γδ T_{DC} cells, we found for IFN-γ γδ T_{DC} cells a decrease in the induced group 4T1 (2349, 1261 - 3429) compared to the control group (5972, 5649 - 6297) ($p < 0.0001$) and about IL-17 γδ T_{DC} cells, we observed a decrease in the tumor group induced by 4T1 (468.3, 307.1 - 692.2) compared to the control group (2026, 1563 - 2489) ($p < 0.0001$).

Finally, we compared the profiles of αβ T_{DC} and γδ T_{DC} cells, and found an increase in the IFN-γ γδ T_{DC} cells control group (5972, 5649 - 6297) compared to IFN-γ αβ T_{DC} cells (4720, 4488 - 6120) ($p = 0.0005$). We also found an increase in the TNF-α γδ T_{DC} cells control group (5972, 5649 - 6297) compared to TNF-α αβ T_{DC} cells (2877, 2716 - 4658) ($p < 0.0001$) and an increase in TNF-α γδ T_{DC} cells in the tumor group induced by 4T1 (2349, 1261 - 3429) in relation to TNF-α αβ T_{DC} cells (1655, 403.8 - 2673) ($p = 0.0157$). There was a decrease in IL-12 γδ T_{DC} cells in the control group (1526, 1290 - 1793) compared to IL-12 γδ T_{DC} cells (3686, 2028 - 5163) ($p < 0.0001$). When comparing IL-17 γδ T_{DC} cells, we found a decrease in the

tumor group induced by 4T1 (468.3, 307.1 - 692.2) in relation to IL-17 αβ T_{DC} cells (578.5, 326.3 - 873.8) (p = 0.0157).

Discussion

Kuka and colleagues (2012) described the cells T_{DC} ($TCR\alpha\beta^{+}CD11c^{+}MHCII^{+}$), as a cell



subtype with properties common to polyclonal T αβ cells and dendritic cells.

Figure 1: Comparison of Immunological characteristics between T_{DC} αβ e T_{DC} γδ cell in control and 4T1 tumor-induced 4T1 mice group. (a) Flow cytometric analyses to identify frequency T_{DC} αβ e T_{DC} γδ cells in the spleen of control and 4T1 tumor-induced. (b) Mean fluorescence intensity of T_{DC} αβ $CD4^{+}$ / T_{DC} γδ $CD8^{+}$ and T_{DC} γδ $CD4^{+}$ / T_{DC} γδ $CD8^{+}$ cells in spleen, of control mice and 4T1 tumor-induced. (c) Mean fluorescence intensity of T_{DC} αβ IFN-γ, T_{DC} αβ TNF-α, T_{DC} αβ IL-12, and T_{DC} αβ IL-17 and T_{DC} γδ IFN-γ, T_{DC} γδ TNF-α, T_{DC} γδ IL-12 and T_{DC} γδ IL-17 cells in the spleen of control and 4T1 tumor-induced. Representative graphs of two independent experiments, n=15 each (median with range). The results were analyzed by and Mann Whitney test to compare the mean fluorescence intensity of subtypes T_{DC} αβ e T_{DC} γδ cells (statistical differences represented by the dashed line). Differences were considered statistically significant at p <0.05 (5%). *p<0,05; **p<0,001; ***p<0,0001.

These rare cells have a morphological similarity to dendritic cells that express intermediate levels of CD11c and present MHC class II antigenic molecules. Besides, these cells are also characterized by the expression of co-stimulatory molecules (CD80, CD86) and lymphocyte surface markers (CD3, CD4 e TCR α/β) (1).

The frequency of αβ T_{DC} cells described by Kuka and collaborators (2012) is approximately 0.04% in the spleen of healthy mice. In our study, we identified an average of 34.64% in healthy mice and 47.74 in the group that was induced to the tumor. We verified the presence of the cell profile for γδ T_{DC} ($TCR\gamma\delta^{+}CD11c^{+}MHCII^{+}$) in the same conditions, and we found the presence of a percentage of 26.53% of γδ T_{DC} in the control group and 18.11% in mice induced to breast cancer ($p<0.0001$).

The study reports that 1 to 4% of all T cells present in the Thymus, secondary lymphoid organs and lungs of adult mice are γδ T lymphocytes. In mucous membranes, such as the intestinal, there are 25 to 40% of this cell type, where the most significant amount is concentrated (8).

According to the analysis of cell clonotypic and phenotypic diversity carried out on cells, γδ T discovered that there are several different subtypes of γδ T cells, each with different phenotypic and functional properties, some with more innate characteristics and others with more adaptive originated from development neonatal and with a high degree of cellular plasticity evident in both peripheral blood and solid tissues, results of responses mainly to acute viral infections (9)

In our studies, we saw the effect of a systemic immune response under the influence of tumor cells, which decreased both αβ T_{DC} and γδ T_{DC} cells. However, when comparing these two cell profiles, αβ T_{DC} and γδ T_{DC}, we saw that there is a higher amount of αβ T_{DC} in the 4T1 induced tumor group than γδ T_{DC} ($p <0.0001$).

The αβ T cell repertoire is higher in T lymphocytes, clearly, most of the time, they have protective antitumor activity and especially in the specific targeting of tumor-related antigens, mainly related to human melanoma tumors (10).

A study with human blood samples from 38 patients diagnosed with breast cancer compared to healthy controls, showed that the proportion of γδ T cells in the circulating blood of healthy controls is 1.6 times greater than in breast cancer patients (11). Data that corroborate with our study, since γδ T_{DC} cells and are present in more significant quantities in the control group ($p <0.0001$).

Concerning γδ T cells, in an antitumor immune response, pioneering studies on the immunoprotected role of these cells in mice were performed in murine models with skin cancer, which were chemically induced by carcinogens or by subcutaneous transfer of melanoma tumor lineage, from this, relevant roles of γδ T in antitumor immunity have been described, with mechanisms mediated by the NKG2D receptor by dendritic epidermal T cells (DETCs)Vγ5+residing in tissues (12,13).

Studies comparing tumor progression in mice with deficient γδ T cells (due to genetic inactivation of the TCRδ receptor) versus mice with sufficient γδ T cells (wild) have firmly established the protective role of γδ T cells during tumor development (10) because it was found that γδ T cells prevented the progression of chemically induced papilloma to cutaneous squamous cell carcinomas. In contrast, αβ cells, on the contrary, seemed to favor tumor progression (14); also, more studies reported that γδ T cells were also protective against spontaneous B cell lymphomas (15), prostate cancer (16) and in the transplantable model of melanoma B16-F0 (17).

Besides, some studies show in the context of infections by cytomegalovirus and malaria, that γδ T cells can be activated later, in the form of direct cytotoxicity, by the action of granzyme B and through stimulating effects such as the secretion of cytokines IFN-γ and TNF-α, or by the direct presentation of antigen (18).

γδ T cells, unlike αβ T lymphocytes, most do not exhibit CD4 or CD8 coreceptors, so antigen recognition is not restricted to antigen-presenting molecules (8). Thus, we compared the expression of αβ T_{DC} and γδ T_{DC} cells related to helper T lymphocyte (TCD4) and cytotoxic (TCD8) markers, which found that the proportion of CD8 γδ T_{DC} is less expressed in splenic cells than CD8 αβ T_{DC}, but these two cell types are decreased in tumor conditions ($p<0.0001$).

A recently conducted study compared subsets of γδ T lymphocytes in 40 patients with Chron's disease, demonstrated a significant decrease in this cell population, concluding that this condition can affect the immune responses against this disease (19). We believe that with cancer, the suppressive conditions provided by it can have the same result with αβ and γδ T_{DC} leading to a deficiency of this mechanism.

The central cytokines produced by γδ T_{DC} presented a higher proportion of this cell type than those produced by αβ T_{DC}. That is, there is a higher production of the cytokines IFN-γ, TNF-α, IL-12 in the control group and lower IL-17 γδ T_{DC} in the group of mice with breast cancer. However, this condition has decreased when there is a systemic immune response related to tumors ($p<0.0001$).

We infer that γδ T_{DC} cells are similar to the mechanisms exerted by γδ T cells. Studies show that this cell type is an important precursor source of IFN-γ and TNF-α, which inhibits tumor growth and angiogenesis. Also, the study carried out with the combination of concanavalin A (ConA) and IL-2, demonstrated the potential for polarization and plasticity of γδ T_{DC} cells, which induced the intense proliferation of these cells and the consequent production of IL-12 e IL- 18 (20).

In inflammatory conditions, a situation observed in some cancers and infections, they favor the polarization of γδ T cells towards an IL-17 producing phenotype (21). A recent study of transcriptome sequencing in approximately 18 thousand tumor masses in humans, revealed that among tumor-infiltrating leukocytes, γδ T cells were strongly associated with a good prognosis (22) In our study we saw that γδ T_{DC} in a systemic immune response is suppressed concerning αβ T_{DC} cells in tumor conditions ($p = 0.0157$).

A specific type of γδ T cells (γδ T $CD27^+$) from mice secrete the IFN-γ cytokine, responsible for inhibiting tumor angiogenesis and improving the expression of MHC class I by tumor cells, thus promoting efficiency in the responses of $CD8^+$ T cells (23). In our studies, we saw that γδ T_{DC} cells expressed IFN-γ in more significant quantities in healthy mice ($p<0.0001$). In the study with an adoptive model of transfer of γδ T cells, in mice, against melanoma B16-F0, it was observed that a specific subtype of γδ T $V\gamma4^+$ (but not $V\gamma^+$ T cells) had the protective function, dependent on its high eomesodermin expression and IFN-γ production (24).

Even though IFN-γ is the main cytokine produced by mouse γδ T cells, IL-17 is very involved in the protective responses of γδ T cells in some cancer models (25). IL-17-producing γδ T cells cooperated in mediating bladder cancer regression (26). In another study, IL-17-producing γδ T cells are associated with chemotherapeutic agents (such as doxorubicin) in various models of epithelial tumor transplantation and demonstrated a better antitumor response (27). In our study, we identified that IL-17-producing γδ T_{DC} are less frequent in the breast cancer-induced group compared to IL17-producing T_{DC} αβ ($p <0.0001$). Thus, it can be inferred that in a systemic antitumor response, these cells may be suppressed by tumor escape mechanisms to antitumor immune responses.

Among the limitations of this study, it should be mentioned that the methodology did not include the performance of cell sorting or sorting analysis and molecular biology, in order to provide a better assessment of the gene expression of T_{DC} cells, which would allow us to achieve more

Therefore, according to the data found in this study, we can conclude that γδ T_{DC} has immunological characteristics shared with conventional effector αβ T_{DC} cells. The healthy mice induced to the 4T1 breast tumor presented T_{DC} with γδ TCR repertoire. These cells express T helper and cytotoxic T lymphocyte molecules, producing antitumor proinflammatory cytokines, suggesting that the T_{DC} γδ could have an antitumor role, and even be used in the future in antitumor immunotherapy. However, new studies investigating its function in other tumor types is necessary.

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7 CONSIDERAÇÕES FINAIS

O sistema imune atua como sentinela com grande potencial de reconhecimento e combate a progressão do câncer. Células importantes e com características especificas são capazes de desempenharem mecanismos de defesa imunológica eficazes.

As células T_{DC} pertence a esse quadro de células peculiares com características imunológicas desempenhadas frente a tumores, o qual foi evidenciado que por mecanismo de escape, suprimem esse tipo celular, em todos os aspectos fenotípicos e produção de citocinas, sejam elas do repertório de TCR αβ ou TCR γδ.

O desenvolvimento do presente estudo possibilitou uma análise das células T_{DC} com a importância desse estudo se tornar base para pesquisas futuras. Os avanços na imunologia podem ser racionalmente aplicados à prevenção e imunoterapia de tumores por meio de uma possível imunoterapia celular levando a destruição do câncer.

8 CONCLUSÕES

As células T_{DC} mostraram estar diminuídas em vários marcadores de superfície, fatores de transcrição e citocinas nos microambientes teciduais estudos segundo dados obtidos dos animais induzidos ao câncer de mama pelas células 4T1. Nos linfonodos, a presença de células T_{DC} foi bastante evidenciada, mas é no fígado que vimos que essas células podem desempenhar um papel imunoativador pela presença de marcadores linfocíticos favoráveis e característicos ao perfil Th1, pela expressão do marcador de transcrição Tbet e também pela produção de citocinas favoráveis à resposta imune antitumoral eficiente, como o TNF-α, IFN-γ e a IL-12.

Identificamos que as células T_{DC} possuem um repertório de TCR γδ, embora menor que o repertório αβ. Diante disso, encontramos diminuição da expressão dos marcadores de linfócitos CD4 e CD8, bem como a produção de citocinas IFN-γ, TNF-α e IL-12, frente a uma resposta sistêmica antitumoral.

As T_{DC} γδ possuem características imunológicas semelhantes as células T_{DC} αβ efetoras convencionais e divergem na característica linfocitária efetora mais evidente em T_{DC} γδ.

Tomados em conjunto, esses resultados sugerem que o microambiente tecidual fornece fortes indicações de direção para uma adequação fenotípica das células T_{DC}, corroborando a hipótese de que alteração fenotípicas podem ser causadas pelo microambiente tecidual na presença do tumor e, portanto, resultam em diferenças funcionais das T_{DC}.

Pesquisas futuras são necessárias para abordar questões cruciais relacionadas, tanto a maturidade celular quanto a funcionalidade dos repertórios de células T_{DC}, seja T_{DC} αβ e/ou T_{DC} γδ, que certamente fornecerão informações adicionais sobre o processo de expressão e a funcionalidade relacionada a possível imunoterapia contra o câncer.

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ANEXO: PROTOCOLO APROVAÇÃO CEUA

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

CERTIFICADO

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

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

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

Universidade Federal do Triângulo Mineiro
Comissão de Ética no Uso de Animais
R. Conde Prados, nº 191 - Bairro Abadia Uberaba/MG CEP 38025-280
(34) 3700-6802 E-mail: ceua@uftm.edu.br

Uberaba, 13 de novembro de 2018

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

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

Senhora Prof.ª Drª Márcia Antoniazi Michelin,

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

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

À consideração superior.

Aldo Rogelis Aquiles Rodrigues
Coordenador da CEUA

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

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