Universidade Federal do Triângulo Mineiro

Instituto de Pesquisa em Oncologia

Programa de Pós-graduação em Ciências da Saúde

Tese de Doutorado

Desenvolvimento e avaliação da influência das vacinas de células dendríticas e células NK em modelo experimental induzido ao câncer de mama com células 4T1

Eduardo Arthur Rodovalho Alves

Uberaba

Janeiro 2020

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

Co-orientador: Prof. Dr. Eddie Fernando Cândido Murta.

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Janeiro 2020

Não se pode criar experiência. É preciso passar por ela.

George Sand

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"Todas as vitórias ocultam uma abdicação."

Simone de Beauvoir

Resumo

Introdução: Imunoterapias têm se tornado foco no tratamento de vários tipos de câncer, principalmente para o câncer de mama. A relevância em utilizá-la como tratamento, se faz, por intermédio da estimulação de células do sistema imune em reconhecer antígenos tumorais gerando respostas imunes específicas direcionadas contra estas células, impedindo a progressão tumoral. **Objetivos:** Objetivando avaliar o tratamento contra o câncer de mama induzido com células 4T1, em camundongos da linhagem Balb/C, utilizamos o protocolo de imunização com as vacinas de células dendríticas (DCs), e células Natural Killer (NK). **Materiais e Métodos:** Compuseram o estudo, camundongos do grupo controle, tumor sem tratamento, tumor tratados com vacina de células DCs e o grupo tumor tratados com vacina de células NK, o protocolo de tratamento foi realizado durante 3 semanas, ao término foram submetidos a eutanásia para remoção do baço e infiltrado tumoral. O material biológico foi preparado para realização da citometria de fluxo utilizando os marcadores de ativação das células NK, NKG2D, NKp46 e inibição Ly49G2, NKG2A/C/E, associado a cada um os marcadores para as citocinas, IL-10, IL-2, IL-12, IL-17, IFN-γ e TNF-α. **Resultados:** Os resultados demonstraram que as células NK frente a imunoterapia com células DCs, influenciaram diretamente na resposta antitumoral, uma vez que, observamos maior expressão dos receptores de ativação NKp46 e NKG2D, e menor expressão dos receptores de inibição Ly49G2 e NKG2A/C/E, com a produção das citocinas IL-2, IL-10, IFN-γ, TNF-α e IL-12, indutoras da ativação de células NK e promotoras da ativação de outras células do sistema imune. Para o tratamento com a vacina de células NK, nossos achados mostram um desbalanço na expressão dos receptores de ativação e inibição, onde uma alta expressão dos receptores de inibição como o Ly49G2 leva a uma interferência na eliminação de células alvo com baixa expressão de MHC classe I. **Conclusões:** Concluindo nossos achados, demonstramos que a imunoterapia com DC é capaz de alterar o perfil das células NK, influenciando-as diretamente na resposta antitumoral, devido à expressão reduzida do receptor de inibição e ao aumento da produção de citocinas como TNF-α e IFN-γ estimulando o sistema imunológico a eliminar as células tumorais, porém, na imunoterapia com células NK a expressão aumentada do receptor de inibição Ly49G2, e a migração desse perfil fenotípico para o microambiente tumoral, impede que células NK sejam ativadas em associação a presença da IL-10, gerando um estado de dormência e contribuindo para a progressão do tumor.

Palavras-chave: Células NK, Sistema Imune, Imunoterapias, Câncer de mama.

Abstract

Introduction: Immunotherapies have become a focus in the treatment of several types of cancer, mainly for breast cancer. The relevance of using it as a treatment is done through the stimulation of cells of the immune system to recognize tumor antigens, generating specific immune responses directed against these cells, preventing tumor progression. **Objectives:** In order to evaluate the treatment against breast cancer induced with 4T1 cells, in Balb / C mice, we used the immunization protocol with dendritic cell (DC) vaccines and Natural Killer (NK) cells. **Materials and Methods:** The study comprised mice from the control group, untreated tumor, tumor treated with DC cell vaccine and the tumor group treated with NK cell vaccine, the treatment protocol was carried out for 3 weeks, at the end of which they were submitted to euthanasia to remove the spleen and tumor infiltrate. The biological material was prepared for flow cytometry using the cell activation markers NK, NKG2D, NKp46 and Ly49G2, NKG2A/C/E inhibition, associated with each one of the markers for cytokines, IL-10, IL-2 , IL-12, IL-17, IFN-γ and TNF-α. **Results:** The results demonstrated that the NK cells in the face of immunotherapy with DC cells, directly influenced the antitumor response, since we observed greater expression of the activation receptors NKp46 and NKG2D, and less expression of the inhibitors of Ly49G2 and NKG2A/C/E, with the production of cytokines IL-2, IL-10, IFN-γ, TNF-α and IL-12, inducing the activation of NK cells and promoting the activation of other cells of the immune system. For treatment with the NK cell vaccine, our findings show an imbalance in the expression of activation and inhibition receptors, where a high expression of inhibitory receptors such as Ly49G2 leads to an interference in the elimination of target cells with low expression of MHC class I. **Conclusions:** Concluding our findings, we demonstrate that immunotherapy with DC is able to alter the profile of NK cells, directly influencing the antitumor response, due to the reduced expression of the inhibitor receptor and the increase in the production of cytokines such as TNF-α and IFN- γ stimulating the immune system to eliminate tumor cells, however, in immunotherapy with NK cells the increased expression of the Ly49G2 inhibitory receptor, and the migration of this phenotypic profile to the tumor microenvironment, prevents NK cells from being activated in association with the presence of IL -10, generating a state of numbness and contributing to the progression of the tumor.

Keywords: NK cells, Immune System, Immunotherapies, Breast cancer.

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Lista de Abreviaturas

IARC – International Agency for Research on Cancer

GLOBOCAN – Global Cancer Observatory

WHO – World Health Organization

INCA – Instituto Nacional do Câncer

S I – Sistema Imune

MHC – Complexo principal de histocompatibilidade

HLA – Antígeno Leucocitário Humano

CD4 – Linfócitos T auxiliares

CD8 – Linfócitos T citolíticos

Th1 – Subtipo de linfócito auxiliar do perfil 1

Th2 – Subtipo de linfócito auxiliar do perfil 2

Treg – Linfócitos T regulatórios

IL – Interleucina

TNF-α – Fator de necrose tumoral alfa

IFN-γ – Interferon gama

NK – Células Natural Killer

NKT – Células natural killer expressando receptor de célula T

CD – Cluster of Diferentiation (Diferenciação)

ADCC – Citotoxicidade Dependente de Anticorpos

GMCSF – Fator estimulador de colônia grânulo-monocítica

RAG – Gene de ativação de recombinação

APC – Célula apresentadora de antígeno

HPSC – Célula tronco hematopoiética

DC – Célula dendrítica

Lista de Siglas

NKp – Receptor de citotoxicidade natural (NCR)

NKG2D – Receptor de ativação da família dos receptores de lectina Tipo C

KIR – Conjunto de receptor de célula NK semelhante a imunoglobulina

Ly49G2 – Receptor inibitório de células NK da família dos receptores de lectina Tipo C

NKG2A/C/E – Receptor inibitório de células NK da família dos receptores de lectina Tipo C

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Introdução

1- Introdução:

O câncer tem uma alta incidência no mundo, em 2018 a estimativa de novos casos de câncer é de 18.078.957 segundo IARC e GLOBOCAN. Dentre estes casos, 2.088.849 mil mulheres apresentaram o câncer de mama. Devido esta incidência, a atenção perante o indivíduo e o tratamento, dependem de medidas necessárias para o combate ao câncer, somado aos fatores de risco (INTERNATIONAL AGENCY FOR RESEARCH ON CANCER (IARC), 2018; WORLD HEALTH ORGANISATION, 2018).

No Brasil, o câncer de mama é o primeiro mais frequente, isto, se desconsiderarmos os tumores de pele não melanoma. Em 2018 a estimativa para os novos casos de cancer de mama feminino é de 59.700 novos casos com um incidência de 29,5% da população brasileira (INCA, 2018).

Para que haja a formação e sustentação do câncer, Hanahan e Weinberg (2000) propuseram que alterações fundamentais na fisiologia celular, que direcionam a transformação de uma célula normal, em célula cancerígena como; a autossuficiência em sinais de proliferação, insensibilidade a sinais inibidores do crescimento, evasão da apoptose, potencial de se replicar ilimitado, angiogênese sustentada, invasão tecidual e metástase. Para contemplar estas “capacidades”, são necessários diferentes mecanismos moleculares, intrínsecos às células e tecidos, onde o acúmulo dessas alterações, contribui para o crescente desenvolvimento do câncer (HANAHAN; WEINBERG, 2000). Atualmente uma grande evidência que leva a indução da tumorigênese, são de que, tecidos submetidos a inflamação crônica geralmente exibem grande incidência de câncer (QUAIL; JOYCE, 2013).

Considerando que células tumorais, apesar de serem capazes de evadir do sistema imune, sua eliminação pode ser promovida através da ativação de mecanismos efetores tornando as células capazes de reconhecerem alvos antigênicos em células tumorais. Para isto, conhecer o microambiente tumoral e suas características específicas, podem auxiliar no desenvolvimento de novos tratamentos, capazes de erradicar as células tumorais (GARCÍA-HERNÁNDEZ; TING KOH; KAST, 2006).

1.1- Células NK e a resposta imune:

Kiessling, Klein e Wigzell (1975), ao realizar experimentos em camundongos descobriram uma terceira classe de linfócitos com morfologia diferente a dos linfócitos T e B e com grande atividade citolítica contra as células tumorais de uma linhagem

leucêmica chamando-as de “killers cells” (células assassinas). Estas células são provenientes de células progenitoras hematopoiéticas $CD34^+$ da medula óssea (HPCs), e possuem morfologia de linfócitos grandes granulares por possuírem um repertório heterogêneo de receptores que reagem a diferentes estímulos como: produtos microbianos, citocinas, quimiocinas e moléculas expressas na superfície de células infectadas e neoplásicas, por isso tornaram-se alvo de vários estudos (LANIER, 1998).

As células NK representam um importante componente do sistema imune inato capaz de eliminar células transformadas e infectadas com vírus, sem a necessidade de prévia sensibilização imunológica. No entanto, recentes descobertas sobre a função dessas células levaram à compreensão que sua participação não se restringe a imunidade inata, mas podem atuar em respostas imunes adaptativas aumentando a possibilidade de serem utilizadas na imunoterapia adotiva contra o câncer. Ao contrário das células T, as NKs dependem da mediação dos receptores através da recombinação somática RAG (MULLER et al., 2015).

Estas células são caracterizadas pela expressão simultânea de vários receptores inibidores e ativadores da superfamília da imunoglobulina e das lectinas. A Interação de receptores inibitórios com moléculas do complexo principal de histocompatibilidade de classe I, conhecido também por MHC I do inglês Major Histocompatibility Complex, sobre células normais, induzem sinais inibidores que impedem a atividade citotóxica das NKs. Na ausência de sinais negativos ou o aumento dos níveis de ligantes de ativação nas células alvo, elas se tornam ativadas e matam as células alvo através de granzimas e perforinas, juntamente com a ação de ligantes do receptor de morte ou citocinas liberadas, como o interferon-γ (IFN-γ) (SEREN BERNARDONE, 2008).

A interação das células NK com outras células do sistema imune como as células dendríticas, tem demonstrado a indução na resposta de células T em infecções primárias, e o sistema de resposta que envolve a interação das duas células são os receptores de reconhecimento padrão (PRRs) como os receptores Toll-Like (TLRs) que reconhecem padrões moleculares associados aos patógenos (PAMPs) desencadeando a resposta imune inata. Entretanto quando células dendríticas imaturas (iDC) são ativadas por TLR elas se diferenciam em células dendríticas maduras (mDC) expressando altos níveis de MHC de classe I e II, bem como moléculas co-estimulatórias CD80 e CD86 necessárias para a estimulação de células T naive (PEREZ-MARTINEZ et al., 2011).

Receptores ativadores e inibidores, e, a interação célula-célula são cruciais para induzir respostas imunes efetivas, e a produção de citocinas são essenciais para orquestrar esta resposta (PAUL; LAL, 2017).

A cerca dos receptores de ativação que são os responsáveis, por conferir as células NK o disparo de suas funções especificas, temos importantes receptores como os NCRs (receptores de citotoxicidade natural) que são o NKp30, NKp44, NKp46 o NKG2D receptor da família das lectinas tipo-C sendo os principais em desencadear respostas direcionadas as células tumorais (JAMES; COHEN; CAMPBELL, 2013).

Os receptores expressos na membrana das células NK é que conferem estímulos inibitórios e ativatórios, como por exemplo os receptores inibitórios KIRs (receptor semelhante a imunoglobulina de células assassinas) em humanos e os receptores da família Ly49 em camundongos que se ligam ao MHC classe I mantendo a tolerância das células NK, para células hospedeiras saudáveis (CHENG et al., 2013).

As citocinas que são cruciais para que elas completem o ciclo de ativação, após a ligação dos receptores de ativação NKp46 ou NKp30 as células NK produzem IFN-γ. Em contribuição para aniquilar células alvo as células NK ativadas produzem IFN-γ e TNF-α, o TNF-α liberado por células NK no microambiente tumoral estimula as células T CD4 a polarizar-se para o perfil Th1 acelerando o desenvolvimento de macrófagos e células T CD8 nos tumores (CHESTER; FRITSCH; KOHRT, 2015).

O interferon que possui capacidade viral antiproliferativa e imunomoduladora, produzido também por células NK, demonstrou em estudos com camundongos e humanos que as células NK podem participar da resposta imune adaptativa modulando DCs através da produção de IFN-γ promovendo a produção de interleucina 12 (IL-12) e a polarização ao perfil Th1 (MARTÍN-FONTECHA et al., 2004).

1.2- Células Dendríticas e a resposta imune a tumores:

As células DCs fazem parte da imunidade inata sendo conhecidas como células profissionais apresentadoras de antígenos (APCs), e possuem uma múltipla população celular de grande heterogeneidade. São produzidas pela medula óssea e podem ser geradas "*in vitro*" a partir de monócitos circulantes ou de células HPSCs. Algumas das subpopulações de DCs são restritas aos tecidos linfoides, e outras estão localizadas nos

tecidos periféricos conhecidas como DCs sentinelas que possuem um enorme potencial de migração e atuação nas infecções (PALUCKA; BANCHEREAU, 2012).

A eficácia das DCs em estimular linfócitos B e T são características peculiares a estas células. Os linfócitos B, precursores das células secretoras de anticorpos, podem reconhecer diretamente o antígeno através dos seus receptores de células-B. Os Linfócitos T, no entanto, precisam que o antígeno seja processado e apresentado a eles por uma célula apresentadora de antígeno (APC). Os receptores de antígenos das células T (TCRs) reconhecem fragmentos de antigênicos ligados a moléculas do MHC na superfície de uma APC. As proteínas de ligação de peptídeos são de dois tipos, MHC classe I e MHC de classe II, as quais estimulam respectivamente, as células T citolíticas (CTL) e células T auxiliares. Antígenos intracelulares, quebrados em peptídeos no citosol da APC, se ligam a moléculas do MHC de classe I e são reconhecidas pelas CTLs, que, uma vez ativados, podem eliminar diretamente a célula alvo. Antígenos extracelulares que são processados pela via endocítica da APC são geralmente apresentados por moléculas de MHC classe de II para as células T-helper, produzindo efeitos imunoreguladores (MAILLIARD et al., 2003).

A imunidade inata é compreendida em células, citocinas e proteínas, junto ao sistema complemento que auxilia na opsonização de patógenos por células fagocíticas e induz respostas inflamatórias para combater infecções. Estas células utilizam uma variedade de receptores de reconhecimento padrões, que reconhecem proteínas partilhadas entre agentes patogênicos, por exemplo, o lipopolissacarídeo bacteriano (LPS), hidratos de carbono, e o RNA viral de cadeia dupla. A imunidade adaptativa de acordo com sua evolução, precisa ser instruída e regulada pelas APCs, e as células DCs são as principais capazes de induzir respostas imunes primárias, permitindo o estabelecimento da memória imunológica (BANCHEREAU et al., 2000).

Várias moléculas, como o CD40, TNF-R, e IL-1R podem ativar DCs e provocar a transição de DCs imaturas em DCs maduras apresentadoras de antígenos. A maturação de DC é um processo contínuo iniciado na periferia onde estas células encontram o antígeno, ou, através da produção de citocinas inflamatórias que posteriormente conclui-se na interação das DCs maduras apresentando seus antígenos para as células T. O processo de maturação está associada com vários acontecimentos coordenados, tais como: a perda de receptores de endocitose fagocíticos; regulação positiva de moléculas co-estimuladoras CD40, CD58, CD80 e CD86; a alteração na morfologia, e a mudança

nos compartimentos lisossomais com regulação negativa do CD68 e regulação positiva de DCs associadas a proteína de membrana lisossomal (DC-LAMP); e mudança nos compartimentos do MHC de classe II (BANCHEREAU et al., 2000).

Devido a este grande potencial das células dendríticas, de reconhecer e apresentarem antígenos as células T efetoras, evidentemente tornarem-se alvo de estratégias imunoterápicas em combate as células cancerígenas por induzir respostas imunes efetoras contra estas (PALUCKA; BANCHEREAU, 2013).

A imunoterapia com células DCs possui um futuro promissor, cujo princípio básico consiste em estimular a apresentação de patógenos tumorais, acarretando em uma resposta eficiente, de células T citotóxicas contra as células alvo (PALUCKA; BANCHEREAU, 2012).

Ao longo dos tratamentos que envolvem as células DCs, uma nova abordagem para o tratamento do câncer surgiu como ferramenta de estudo utilizando as células NK, desde então discussões sobre estratégias imunoterapêuticas envolvendo estas células são realizadas em busca de novos horizontes para a imunoterapia contra o câncer (HU et al., 2019).

1.3- Imunoterapias no tratamento do câncer:

O conceito imunoterapia contra o câncer foi introduzido no final do século XIX, quando William B. Coley observou a redução tumoral pela injeção de produtos bacterianos em volta do tumor. Desde então observações como a maior incidência de cancer em pacientes imunodeprimidos, e uma maior identificação de antígenos e linfócitos específicos do tumor, estimularam pesquisas visando induzir respostas imunes específicas (LESTERHUIS; HAANEN; PUNT, 2011).

Um dos objetivos da imunoterapia é estimular respostas imunes vigorosas na eliminação das células cancerígenas, uma vez que, em pacientes com câncer o sistema imune é suprimido (ZHOU, 2014).

Com a descoberta das imunoterapias ao longo dos anos várias abordagens biotecnológicas de tratamento foram desenvolvidas. Anticorpos monoclonais utilizados na terapia do câncer são obtidos por técnicas avançadas que geram anticorpos otimizados para combater ou inibir vias de sinalização das células tumorais alvo (SCOTT; ALLISON; WOLCHOK, 2012).

Pacientes com câncer usualmente apresentam um perfil imunossupressor com baixas contagens de células T, e consequentemente no microambiente tumoral o infiltrado celular de linfócitos T citolíticos é diminuído. Portanto, observa-se o desenvolvimento tumoral pela predominância de linfócitos T auxiliares tipo 2 (Th2) e macrófagos M2 tolerantes ao tumor (GAO et al., 2015).

A utilização de imunomoduladores como tratamento do câncer tem sido muito empregado atualmente pela capacidade de estimular resposta imunes efetoras do próprio hospedeiro a reconhecer os antígenos tumorais e combater as células infectadas (NELSON; BALLOW, 2003).

A terapia celular adotiva (ACT) é outro tipo de imunoterapia que envolve principalmente o isolamento e expansão in vitro de células T específicas aos tumores, seguida por reinfusão para o paciente com câncer. Esses esforços também se estenderam ao uso de células natural killer, por exibirem uma rápida e potente imunidade contra tumores sólidos, metástase e câncer hematológico (GUILLEREY; HUNTINGTON; SMYTH, 2016; OISETH; AZIZ, 2017).

Células da imunidade inata e adaptativa que previnem ativamente o desenvolvimento neoplásico, processo chamado de imunovigilância do câncer que confere ao indivíduo modulação da resposta contra alterações celulares e o reconhecimento de antígenos tumorais ou alterações teciduais, são de suma importância para impedir a progressão do câncer (KIM, 2007).

As células do sistema imune inato, que incluem monócitos, macrófagos, células DCs e células NK, medeiam respostas imediatas de curta duração liberando citocinas que lisam diretamente células neoplásicas ou células infectadas por vírus, e capturam debris das células tumorais mortas e os eliminam. Contudo, a imunidade inata é a ponte para a ativação da imunidade adaptativa (CLOUGH, 2018).

As células do sistema imune adaptativo, incluem os linfócitos T e linfócitos B, que intermedeiam respostas de longa duração antígeno-específicas tornando células eficazes de memória. Apesar destas respostas imunes serem um meio de eliminar as células cancerígenas, elas podem desenvolver mecanismos para burlar a imunovigilância. Os tumores têm a capacidade de criar mecanismos para se proteger, através de um ambiente imunoprivilegiado. A produção de citocinas imunossupressoras como Interleucina-10 (IL-10) e o fator de crescimento transformante beta (TGF-β), para

suprimir a resposta imune antitumoral adaptativa ou direcionar a respostas imune para um perfil Th2 com menor capacidade de eliminar células tumorais, podendo também alterar a expressão de IL-6, IL-10, fator de crescimento epitelial vascular ou fator estimulante de colônia granulo-monocítica (GM-CSF), prejudicando assim a função de células dendríticas (DC) pela inativação ou supressão da maturação destas células. Além disto, em alguns casos as células T reguladoras (Tregs) induzem a supressão da resposta por células T auxiliares ($CD4^+$) e citotóxicos ($CD8^+$) específicas do tumor (CHENG et al., 2013).

Diante das discussões relativas ao câncer, as células tumorais para serem eliminadas dependem de respostas imunitárias adequadas, e imunoterapias celulares atualmente, estão sendo muito utilizadas objetivando estimular o sistema imune a desenvolver uma resposta eficaz contra o tumor sem haver o comprometimento de células vizinhas, evitando lesões teciduais, limitando suas funções (BELLATI et al., 2013).

Estudos que utilizam de células autólogas estimuladas *ex-vivo*, e reinfundidas ao indivíduo com câncer, tornou-se uma realidade devido ás condições atuais quanto ao manuseio destas células e a empregabilidade para fim de tratamento, uma vez que, os efeitos colaterais causados pela quimioterapia são um agravante, nas imunoterapias estes sintomas não são observados (OISETH; AZIZ, 2017).

Dos estudos realizados em nosso laboratório, Rodrigues et. al 2011, demonstrou que a vacinação com células dendríticas aumentou o perfil de células $TCD4^+$ expressando as citocinas IL-2, IFN-γ, IL-12, TNF-α e IL-10 ao início do tratamento, com similar efeito para células $TCD8^+$ expressando IL-2, concluindo que a imunoterapia com células DC pode estimular respostas imunes durante o período do tratamento (RODRIGUES et al., 2011).

Em um outro estudo Cunha et. al 2016, avaliou que a vacinação com DC é capaz de estimular a secreção de citocinas como IFN-γ e IL-12 inibindo a secreção de TGF- e IL-10 nos linfonodos dos camundongos tratados (DA CUNHA; ANTONIAZI MICHELIN; CÂNDIDO MURTA, 2016).

A propósito, protocolos de tratamento utilizando atualmente, a combinação de dois tipos de imunoterapia celular, foram capazes de intensificar respostas específicas de forte impacto para as células transformadas, além da obtenção de resultados promissores no tocante ao combate do câncer. Questões como estas são de suma importância para o

mundo que necessita, em relação aos pacientes com câncer, uma medida efetiva para o tratamento e remissão completa da doença (ROSENBERG, 2014).

Um estudo correlacionando a interação entre células NK e DCs, demonstrou que a ativação de DCs por células NK resulta na diferenciação de uma DC capaz de induzir respostas mais eficientes em linfócitos T citolíticos (CTL), aumentando a capacidade de destruir células modificadas (COOPER, 2004).

1.4- Justificativa:

No instituto de Pesquisa em Oncologia da Universidade Federal do Triângulo Mineiro (IPON-UFTM), estudos envolvendo o tratamento com a vacinação por células DCs são desenvolvidos para melhor adequação do protocolo de tratamento visando estimular o sistema imune a desenvolver respostas ainda mais específicas contra células neoplásicas.

Dentre as pesquisas envolvendo a vacinação com células dendríticas realizadas no laboratório do IPON, os resultados são promissores e demonstram que a imunoterapia induz uma ativação do perfil Th1 e a produção de citocinas pró-inflamatórias sistemicamente gerando potentes respostas antitumorais (ALEIXO; MICHELIN; MURTA, 2014; MATIAS et al., 2013).

A vacinação com células NK desenvolvida atualmente pelo nosso grupo, tem como objetivo complementar o tratamento, e auxiliar no estímulo de respostas ainda mais vigorosas, o protocolo que desenvolvemos é o primeiro a utilizar células tronco hematopoiéticas (HPSCs) para diferenciação.

Sobre estes aspectos, estudar o sistema imune sob diferentes aspectos, desde a pesquisa básica experimental à clínica de pacientes oncológicos, focado em compreender e entender melhor como o sistema imune atua na progressão ou regressão tumoral. Portanto, obter maior precisão no tratamento envolve o desenvolvimento de protocolos eficazes e, para isso, a pesquisa experimental proporciona condições de avaliação ampla quanto a tratamentos utilizando imunoterapias. Estudos experimentais "*in vivo*" e pré-clínicos de fase I e II demonstraram que a vacinação com células dendríticas potencializa a efetividade da resposta por células NK em vários tipos de câncer levando à regressão tumoral, indicando fortemente que o aumento da atividade lítica das células NK, é induzido pela vacinação com DCs. Previamente, decidimos avaliar as células NK, na

imunoterapia com células dendríticas, e com células NK isoladamente, devido estas, atualmente serem muito estudadas, por potencializar respostas imunes antitumorais, ou, em outras condições bloquear determinadas vias de ativação, impedindo que células adotivas tenham maior poder e efetividade em eliminar células cancerígenas.

Objetivos

2- Objetivo Geral:

Avaliar a efetividade da ativação e atividade de células NK na imunoterapia com células DCs e com células NK isoladamente, em camundongos com tumor de mama induzidos por células 4T1.

2.1- Objetivos Específicos:

1- Desenvolver um protocolo de diferenciação de células hematopoiéticas (HPSCs), obtidas a partir da medula óssea de camundongos Balb/C, em células NK, para produção da vacina, nos quais os animais com câncer de mama induzidos com células 4T1 foram tratados;

2- Avaliar a eficácia do tratamento com a vacina de células DCs e células NK, por citometria de fluxo, através dos marcadores de ativação NKp46, NKG2D e inibição Ly49G2 e NKG2A/C/E das células NK, nos esplenócitos e infiltrados tumorais dos camundongos;

3- Avaliar e comparar a eficácia do tratamento através da produção das citocinas intracelulares IL-2, IL-10, IL-12, IL-17, IFN-γ e TNF-α produzidas por células NK, nos esplenócitos e infiltrados tumorais dos camundongos do grupo controle, tratados com as vacinas de células dendríticas e células NK, isoladamente;

Materiais & Métodos

3- Delineamento do Estudo:

O estudo foi composto por 30 camundongos fêmeas da linhagem Balb/C com idade entre 6-8 semanas, obtidos do biotério central, e acomodados no biotério setorial do Instituto de Pesquisa em Oncologia da Universidade Federal do Triângulo Mineiro (IPON-UFTM) por 15 dias antes de iniciar a experimentação. Os grupos experimentais foram divididos de 3 formas para avaliação, sendo: grupo controle com animais sem indução de tumor e sem tratamento, o grupo tumor sem tratamento, porém, com indução tumoral por células 4T1, o grupo tumor induzido tratado com vacina de células DCs e por fim o grupo tumor induzido tratado com vacina de células NK. Treze dias após a indução do tumor, os animais do grupo tratado com vacina de células dendríticas e células NK passaram a ser medido o volume tumoral e vacinados por via subcutânea com 3 doses de vacina de células DCs e NK isoladamente, durante 3 semanas consecutivas. A dose da vacina de células DCs foi administrada com volume de 50 µL a uma concentração de 5,0x106 células por camundongo, e a vacina de células NK com volume de 50 µL, porém a uma concentração de 8,0x105 células por camundongo. O protocolo de inoculação e tratamento compreendeu a um período total de 25 dias, ao final deste período, os camundongos foram eutanasiados e retirado o material biológico baço e infiltrado tumoral para preparação e realização da técnica de citometria de fluxo. Este estudo foi submetido ao Comitê de Ética em Pesquisa com Animais da UFTM (CEUA/UFTM), e foi aprovado com o número 378.

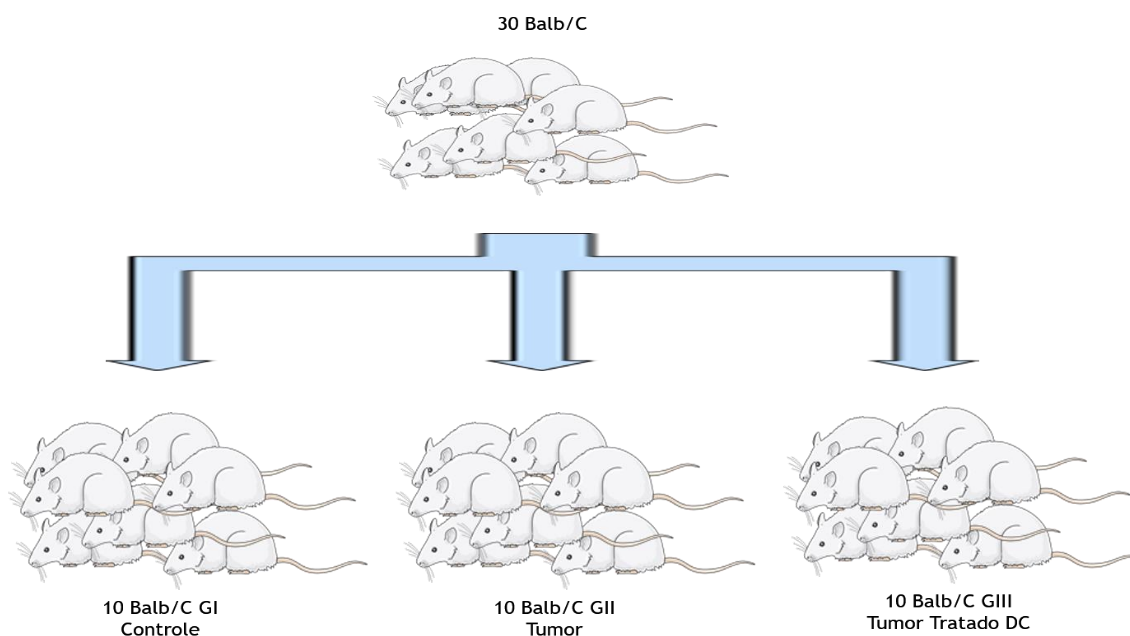


Figura 1: Esquema de separação dos grupos experimentais.

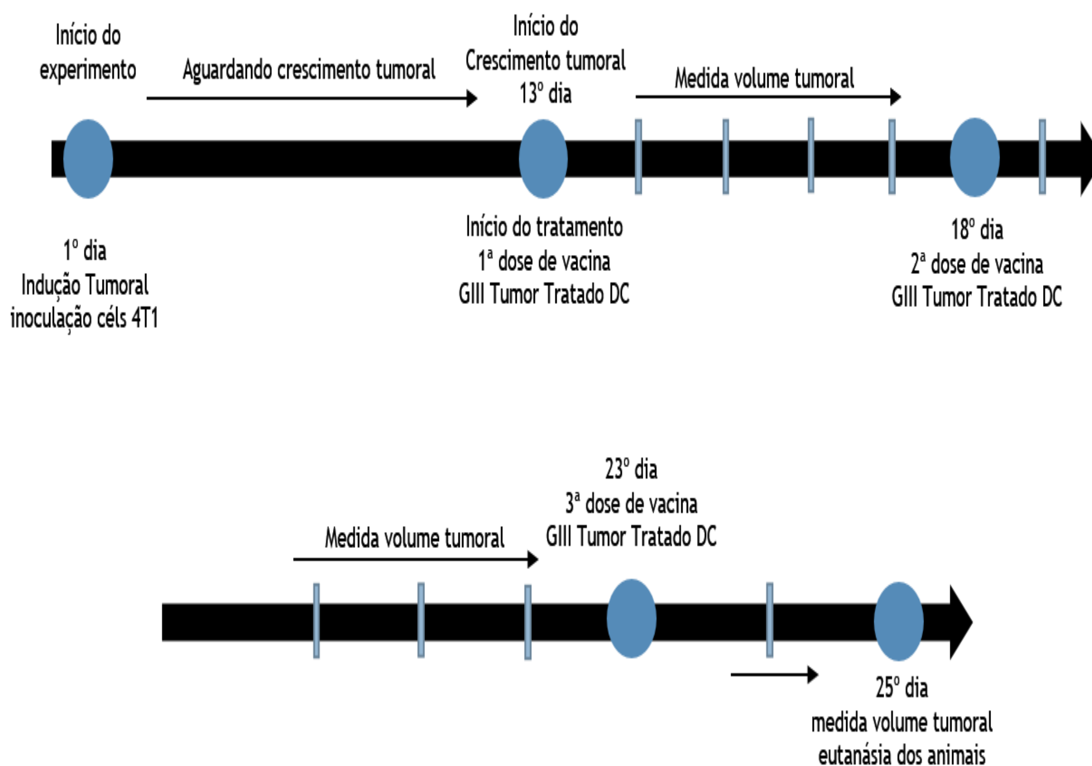


Figura 2: *Desenho experimental realizado com os grupos tumor e tratados com vacina.*

3.1- Carcinogênese induzida por Células 4T1:

Os animais dos grupos tumor induzido sem tratamento e do grupo tumor induzido tratados com as vacinas de células DCs e NK, foram submetidos a indução tumoral para o câncer de mama com células da linhagem 4T1, específicas para animais da linhagem Balb/C, capazes de gerar o crescimento tumoral semelhante ao câncer de mama em humanos compatível ao grau da doença em estágio IV (FERRARI-AMOROTTI et al., 2014). A inoculação foi feita no último par de mamas através da inoculação com seringa estéril a uma concentração de $2x10^5$ células para cada camundongo dos grupos experimentais descritos acima. Após o início da formação do tumor detectado por palpação iniciamos as medidas do tamanho tumoral semanalmente junto ao protocolo de tratamento com as vacinas de células DCs e células NK. As células 4T1 são previamente colocadas em cultura em meio específico para sua expansão, antes de serem inoculadas nos camundongos para o experimento utilizamos cepas ATCC® 4T1 CRL-2539TM, o período de expansão destas células é variável, dependendo de seu poder replicativo.

3.2- Citometria de fluxo:

O material biológico baço e infiltrado tumoral retirado dos animais que compuseram os grupos experimentais foram submetidos à realização da citometria de

fluxo, seguindo o protocolo preconizado pela BDbioscience®. As células foram colocadas em solução de lise BD FACSTM Lysing Solution® e submetidas as marcações extra e intracelulares do protocolo para células NK. Na execução as amostras foram incubadas com os anticorpos extracelulares por 30 minutos lavadas e incubadas com BD Cytofix/CytopermTM por 20 minutos e após a lavagem com BD Perm/Wash™ foram realizadas as marcações intracelulares. Para quantificarmos as células NK nos camundongos não tratados e tratados com vacina de células dendríticas e células NK, utilizamos os marcadores CD3 (linfócitos totais), NKp46 e NKG2D (receptores de ativação), Ly49G2 e NKG2A/C/E (receptores de inibição) e as interleucinas 2, 10, 12, 17, TNF-α e IFN-γ todos anticorpos da BD Bioscience®.

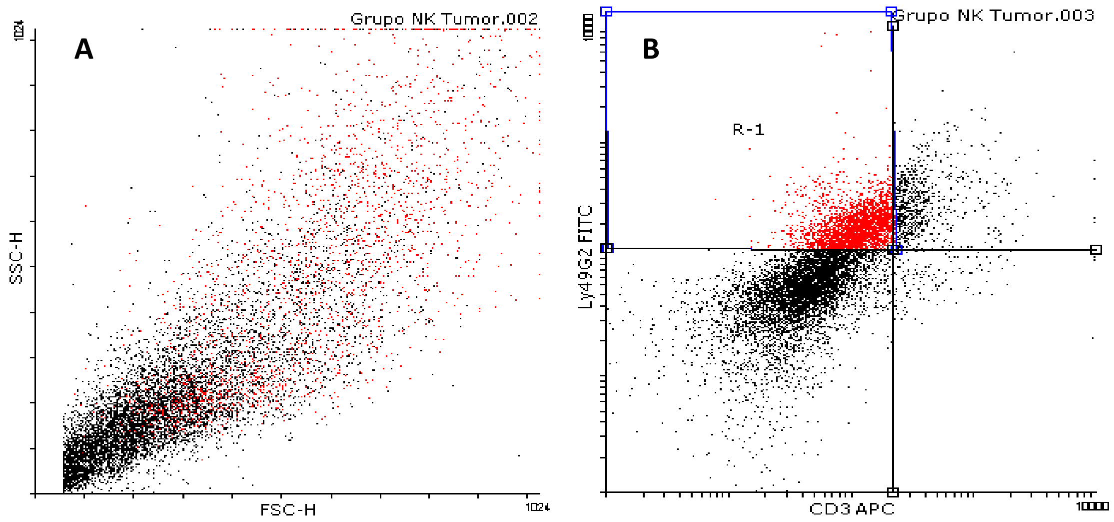
3.3- Painel de marcadores utilizados:

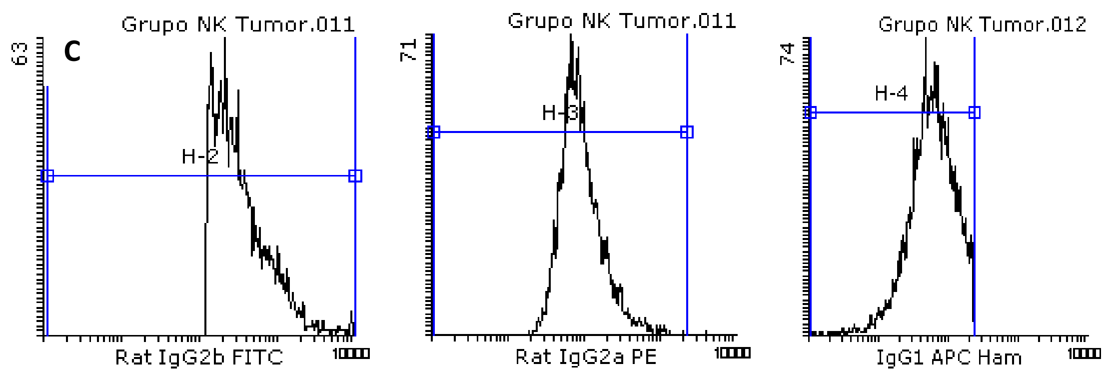
MARCAÇÃO CÉLULAS NK BAÇO/TUMOR		
Tubos	**Extracelular**	**Intracelular**
1	Controle	-----------------
2	Controle permeabilizado	-----------------
3	CD3e APC + Ly49G2 Fitc	-----------------
4	CD3e APC + NKG2A/C/E Fitc	-----------------
5	CD3e FITC + CD314 Pe (NKG2D)	-----------------
6	CD3e APC + CD335 Fitc (NKp46)	-----------------
7	NKG2A/C/E Fitc	IL-10 Pe/IL-2 Alexa Flúor
8	CD314 Pe (NKG2D)	Ly-6A/E PeCy7(IL-12)
9	CD335 Fitc (NKp46)	IL-17RB Alexa Flúor 647
10	Ly49G2 Fitc	IFN-γ PerCP-Cy5.5/MP6-XT22 APC (TNF-α)
11	Rat IgG2a Pe (ISO)	Rat IgG2b Fitc/Rat IgG1 APC (ISO)
12	Arm. Hamster IgG1 APC (ISO)	Rat IgG1 Fitc (ISO)

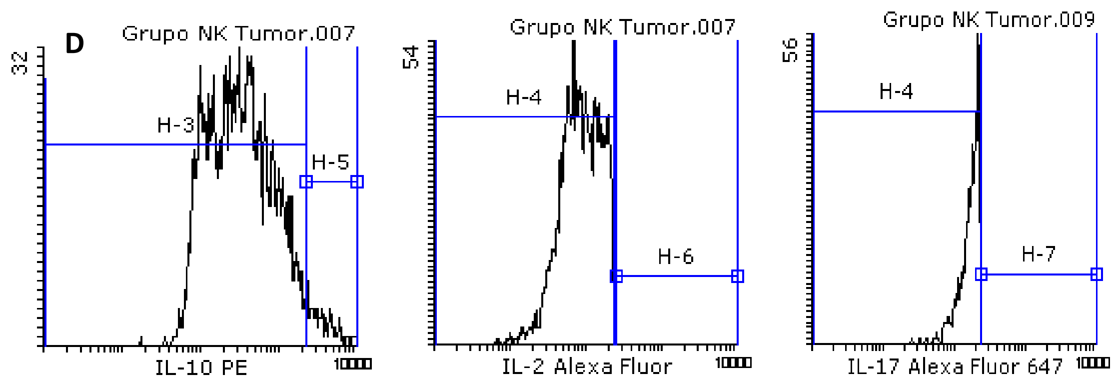
Tabela 1: *Marcadores utilizados nas células das amostras biológicas do baço e infiltrado tumoral dos camundongos do grupo controle, controle tumor, tratados com vacina de células dendríticas e tratados com vacina de células NK.*

3.4- Estratégia de seleção da Gate e Controles Isotipo:

Os resultados obtidos das células esplênicas e do infiltrado tumoral marcadas com os anticorpos citados passaram por uma estratégia de análise pelo Flowing Software 2.5.1 para obtenção do porcentual total de células NK no quadrante delimitado ao tamanho relativo (Forward Scatter, FSC) e granulosidade/complexidade (Side Scatter, SSC), como podemos observar na **figura (A)** e **(B)**. Os controles isotipos utilizados para exclusão das ligações inespecíficas são observados em **(C)**. Em **(D)** e **(E)** análises utilizadas para as marcações das citocinas IL-2, IL-10, IL-12 IL-17, IFN-γ e TNF-α e seus respectivos isotipos. Os dados obtidos das análises foram planilhados e submetidos a análise em software estatístico específico para realização dos cálculos.


A
Grupo NK Tumor.002
SSC-H
FSC-H
1024
B
Grupo NK Tumor.003
R-1
Ly49G2 FITC
CD3 APC
10000


C
Grupo NK Tumor.011
63
H-2
Rat IgG2b FITC
10000
Grupo NK Tumor.011
71
H-3
Rat IgG2a PE
10000
Grupo NK Tumor.012
74
H-4
IgG1 APC Ham
10000


D
Grupo NK Tumor.007
32
H-3
H-5
IL-10 PE
10000
Grupo NK Tumor.007
54
H-4
H-6
IL-2 Alexa Fluor
10000
Grupo NK Tumor.009
56
H-4
H-7
IL-17 Alexa Fluor 647
10000

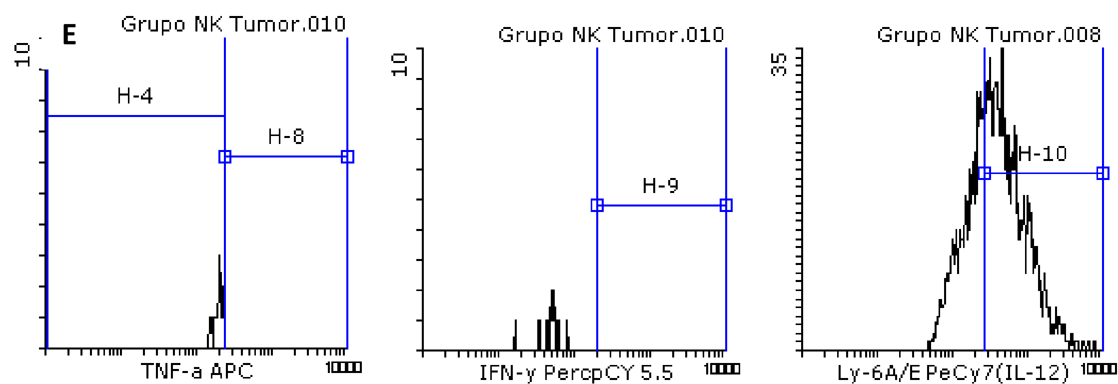


Figura 3: *Estratégia de análise realizada para as amostras do baço e infiltrado tumoral dos grupos controle, tumor induzido sem tratamento, tratados com vacina de células DC e tratados com vacina de células NK.*

3.5- Análise dos Dados:

Os valores das análises, percentual de células e intensidade média de fluorescência, foram utilizados para comparações entre os grupos e calculadas pelo software GraphPad Prism 5® realizando o teste de normalidade e para a comparação entre os três grupos utilizamos 1way ANOVA com post test de Kruskal-Wallis e Mann-Whitney t Test na comparação para dois grupos tanto dos resultados da citometria quanto do volume tumoral, considerando significativos resultados de p menores que 0,05 ($p<0{,}05$).

Resultados

4- Resultados:

4.1- Artigo 1

Targeted Oncology

Dendritic cell vaccination augments the profile of tumor infiltrating Natural Killer cells in tumor bearing mice.

--Manuscript Draft--

Manuscript Number:	
Full Title:	Dendritic cell vaccination augments the profile of tumor infiltrating Natural Killer cells in tumor bearing mice.
Article Type:	Original Research Article
Funding Information:	
Abstract:	We evaluated the Natural Killer (NK) cells in Dendritic Cell (DC) vaccine immunotherapy of 4T1 cell-induced breast cancer in Balb/C mice. We evaluated the expression of killer activation receptors NKp46, NKG2D, and inhibitory killer cell immunoglobulin-like receptor (KIR) Ly49G2, NKG2A/C/E and the interleukins (IL) 2, 10, 12, 17, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ). We used 30 Balb/C mice divided into untreated or control experimental group (GI), untreated induced tumor group (GII), and the DC vaccine-induced tumor group (GIII). For the vaccine we used bone marrow from donor mice. The 25-day experiment comprised tumor volume measurements and at the end of the treatment protocol mice were euthanized for removal of the spleen and evaluation of tumor infiltration by flow cytometry of the receptors NKp46, NKG2D, Ly49G2 and NKG2A/C/E and the cytokines IL-2, IL-10, IL-12, IL-17, TNF-α and IFN-γ. Our results showed that mice treated with the DC vaccine reduced the expression of inhibition receptors Ly49G2D and NKG2A/C/E (p=0.0079) in the spleen and tumor infiltrate, with increased fluorescence intensity of splenic IFN-γ-producing NKp46 cells, intratumoral TNF-α and IFN-γ-producing NKG2D cells (p=0.0019). We demonstrated that immunotherapy with DC can alter the profile of NK cells by directly influencing them into an antitumor response due to reduced expression of inhibition receptors and increased production of cytokines such as TNF-α and IFN-γ by cells. Also, the activation of receptors confers NK cells the ability to reverse tumor-induced immunosuppression, stimulating the immune system to eliminate tumor cells.
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Author Comments:	In this paper, we report on that dendritic cell vaccination can alter the immune profile by interacting with dendritic cells inducing vigorous immune responses against tumor cells. This is significant because stimulating the individual's own immune system to recognize these cells is a way to restore the immune system's functions without harming other healthy cells or organ dysfunction.
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Title

Dendritic cell vaccination augments the profile of tumor infiltrating Natural Killer cells in tumor bearing mice

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Uberaba December 05, 2019

Professor PhD **Martin Chopra**
Editor-in-chief
Targeted Oncology
Dear Prof. Chopra

We are enclosing the submission form of the manuscript **"Dendritic cell vaccination augments the profile of tumor infiltrating Natural Killer cells in tumor bearing mice"** by Eduardo Arthur Rodovalho Alves, Polyana Barbosa Silva, Eddie Fernando Candida Murta, and Márcia Antoniazi Michelin for consideration by the Editorial Board for publication in Targeted Oncology as original article.

We would like to say this paper is an original study and it has not been and will not be submitted to or accepted simultaneously to another journal, in whole or in part, it reports previously unpublished work and that ethics approval and written informed consent have been obtained, and explain whether any author has a conflict of interest.

We hope the paper will be acceptable for publication.

Sincerely,

Eduardo Arthur Rodovalho Alves

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Title

Dendritic cell vaccination augments the profile of tumor infiltrating Natural Killer cells in tumor bearing mice

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Highlights

Immunotherapy with DCs can modify the profile of NK cells

DCs and NKs cells can converge to profile immune system

Inhibition receptors of NK cells decrease in spleen and tumor infiltrated facilitates interactions between NK and DC cells

The increase activation receptors stimulate to clearance of tumor cells

Abstract

We evaluated the Natural Killer (NK) cells in Dendritic Cell (DC) vaccine immunotherapy of 4T1 cell-induced breast cancer in Balb/C mice. We evaluated the expression of killer activation receptors NKp46, NKG2D, and inhibitory killer cell immunoglobulin-like receptor (KIR) Ly49G2, NKG2A/C/E and the interleukins (IL) 2, 10, 12, 17, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ). We used 30 Balb/C mice divided into untreated or control experimental group (GI), untreated induced tumor group (GII), and the DC vaccine-induced tumor group (GIII). For the vaccine we used bone marrow from donor mice. The 25-day experiment comprised tumor volume measurements and at the end of the treatment protocol mice were euthanized for removal of the spleen and evaluation of tumor infiltration by flow cytometry of the receptors NKp46, NKG2D, Ly49G2 and NKG2A/C/E and the cytokines IL-2, IL-10, IL-12, IL-17, TNF-α and IFN-γ. Our results showed that mice treated with the DC vaccine reduced the expression of inhibition receptors Ly49G2D and NKG2A/C/E ($p=0.0079$) in the spleen and tumor infiltrate, with increased fluorescence intensity of splenic IFN-γ-producing NKp46 cells, intratumoral TNF-α and IFN-γ-producing NKG2D cells ($p=0.0019$). We demonstrated that immunotherapy with DC can alter the profile of NK cells by directly influencing them into an antitumor response due to reduced expression of inhibition receptors and increased production of cytokines such as TNF-α and IFN-γ by cells. Also, the activation of receptors confers NK cells the ability to reverse tumor-induced immunosuppression, stimulating the immune system to eliminate tumor cells.

Keywords: Natural Killer cells, Dendritic cells, Cellular Immunotherapy, NK cells activation.

1. Introduction

Once cancer is established, the tumor cells can be eliminated depending on proper immune responses. Immunotherapy as a tool has been widely used to stimulate the immune system by inducing an effective response against transformed cells without compromising neighboring cells, preventing tissue damage by limiting its functions [1].

However, the elimination of these cells depends on a variety of mechanisms and cells involving specific recognition of tumor cells [2]. Innate and adaptive immune cells actively prevent neoplastic development in a process called cancer immunosurveillance. Innate immune cells that include monocytes, macrophages, dendritic cells, and natural killer cells mediate immediate short-term responses by releasing cytokines or other mediators that directly lyse neoplastic or virus-infected cells [3].

Specifically, NK cells have a high capacity to lyse tumor or virus-infected cells, and their activation is due to the low expression of the major histocompatibility complex class I (MHC class I) [4]. The balance between NK cell membrane-expressed receptors confers inhibitory and activating stimuli, such as inhibitory KIRs (killer cell receptor-like receptor) inhibitors in humans and the Ly49 family of receptors in mice. MHC class I-binding maintains tolerance of NK cells to healthy host cells. Binding of these receptors gives NK cells recognition of modified cells due to the down-regulation of MHC I proteins characteristic of virus-infected or modified cells [5,6] and activation receptors recognize several ligands such as the FcγR receptor (CD16), which enables NK cell cytotoxicity (ADCC) to eliminate antibody opsonized cells [7].

The activation receptors are responsible for giving NK cells the signals responsible for the initiation of their specific functions, such as NCRs (natural cytotoxicity receptors), being NKp30, NKp44, NKp46, or NKG2D lectin family receptor the main ones in triggering targeted responses to neoplasic cells [8]. To perform their functions beyond activation receptors, NK cells also depend on the production of cytokines that are crucial for them to complete the activation cycle. After binding of activation receptors NKp46 or NKp30, NK cells produce IFN-γ. In contributing to destroying target cells, activated NK cells produce IFN-γ and TNF-α, and these last cytokines released by NK cells in the tumor microenvironment stimulates helper T cells to polarize to Th1 profile and accelerate macrophage and cytotoxic T lymphocytes activation [9].

In addition to these specific NK cell functions, their interaction with DC is of great importance in clinical practice and for the generation of vigorous immune responses. Several works demonstrated that the

effector functions of NK cells are stimulated by direct contact with activated DC, in a new concept for those cells calling them as dendritic-cell-activated killers (DAKs) [10–12].

However, the *in vivo* action of NK cells on DC-induced antitumor vaccine responses is not yet fully elucidated. Our objective in this study was to evaluate the role, systemic and intratumoral, of NK and their activator (NKp46 and NKG2D) and inhibitory (Ly49G2 and NKG2A/C/E) receptors, as well as the cytokines produced by those cells after immunotherapy with DC in breast tumor mice induced by 4T1 cells.

2. Materials and Methods

For this study, we used 30 female Balb/C mice from the sectoral bioterium of the Oncology Research Institute of the Federal University of Triângulo Mineiro (IPON-UFTM). The experimental groups were divided into three for evaluation: control group (GI) with animals without tumor induction; the untreated tumor group (GII) tumor-induced with 4T1 cells without any treatment; and finally, the tumor group treated with DC vaccine (GIII). Thirteen days after tumor induction, animals of group GIII were subcutaneously vaccinated receiving three doses of DC vaccines for three consecutive weeks, each dose with a volume of 50 µL and a concentration of $5.0x10^6$ cells per mouse. The inoculation and treatment protocol comprised a total of 25 days. In the end, mice were euthanized, and the biological material - spleen and tumor infiltrate - were removed to prepare and perform the flow cytometry technique.

2.1 4T1 cell tumor induction

Animals from experimental groups GII and GIII were inoculated with the 4T1 tumor line. The inoculation of the tumor cell line suspended in a physiological solution was administered in a single dose with a volume of 50 µL at a concentration of $2.0x10^5$ cells per animal in the last pair region of breasts. For tumor development, we waited 13 days for the confirmation of tumor formation being performed by palpation and inspection at the site of the breasts, thus starting the tumor measurement until the end of the experiment.

With a universal caliper rule, we performed weekly measurements of the tumor control group and the group on immunotherapeutic treatment with DC cells for each mouse until the day of final euthanasia. The measurements obtained were performed in the craniocaudal and anterolateral direction, considering the average values obtained after the calculations. Tumor values were calculated using the formula **$V=a.b^2/2$**, with (a) the largest diameter measurement and (b) the smallest diameter, where the median represented the values.

2.2 DCs cell vaccine production

To produce the DC vaccine (according to the protocol of Lutz et al., 1999) [13], we extracted bone marrow from the femur and tibia by washing the bones with 0.9% saline solution. These cells were then washed in incomplete IMDM medium and then cultured in IMDM medium containing 10% SBF and gentamicin. For differentiation, cultured cells were stimulated with GM-CSF (10 ng/µL) and IL-4 (10 ng/µL) on day 2, and with TNF-α (10 ng/µL) and tumor antigen on day 5, remaining in culture until day 7 to complete differentiation. After total differentiation, the DCs were washed and resuspended in 0.9% saline solution and finally used for inoculation in mice. Several research groups, including ours, use the dendritic cell vaccine to treat various types of cancer, aiming to induce immunological memory. However, the protocol employed in each group has specificities in cell differentiation and stimulation of these cells to be employed in the treatment.

2.3 Flow cytometry

On the 25th day of the protocol, the animals of each experimental group were euthanized, and splenic and intratumoral cells were obtained for flow cytometry analyses. Following the protocol recommended by BDbioscience®, the cells were placed in BD FACS™ Lysing Solution® and subjected to the protocol for extra and intracellular markings for NK cells.

Samples were incubated with extracellular antibodies for 30 minutes, washed and incubated with BD Cytofix/Cytoperm™ for 20 minutes, and after washing with BD Perm/Wash™, intracellular antibodies were used. To quantify NK cells in untreated and vaccine treated mice, we used CD3 (total lymphocytes), NKp46 and NKG2D (activation receptors), Ly49G2, and NKG2A/C/E markers (inhibitory receptors) and interleukins 2, 10, 12, 17, TNF-α, and IFN-γ (all BD Biosciences® antibodies). The data obtained from splenic cells and tumor infiltrate labeled with the antibodies underwent a strategy of analysis by obtaining the total percentage of cells with the selection of the amount of NK cells in the quadrant delimited by relative size (Forward Scatter, FSC) and granularity/complexity (Side Scatter, SSC).

2.4 Statistical analysis

Results were acquired by the BD FACSCalibur® instrument and analyzed using Flowing Software Version 2.5.1 software for the total percentage of NK cells for each specific marker, excluding other cell lines. The value of the analyses, cell percentage and mean fluorescence intensity were used for comparisons between groups and calculated by GraphPad Prism 5® software performing the normality test. For

comparison among the three groups we used 1way ANOVA test and Mann-Whitney t-Test comparing two groups by both cytometry and tumor volume results, considering significant p-values less than 0.05 ($p < 0.05$).

3. Results

Evaluation of tumor volume from animals during the 25-day experimental period showed that the untreated group had a larger tumor volume than the dendritic cell vaccine treated group. (*p=0,00120/**p=0,0098) (Fig. 1).

Cytometry performed on the spleen and tumor infiltrate samples for NK cells showed an increased density of these splenic cells for the untreated tumor group GII (p=0.0079). In the tumor, higher density infiltrates for mice treated with DC cells GIII (p=0.0019) data not shown.

Figure 2 shows the percentage of splenic cells and tumor infiltrate based on the activation and inhibition markers studied. (Fig. 2) (A) shows a more significant number of cells expressing the activation receptors NKp46 and NKG2D, respectively, for the untreated control group GI (p=0,0019). In (B), the groups with a higher number of cells expressing inhibition receptors Ly49G2 in group GI compared to GIII (p=0,0019). Graphs (C) and (D) show a higher percentage of cells expressing receptors, respectively, NKp46, NKG2D, and Ly49G2, NKG2A/C/E in the tumor infiltrate in group GII (p=0,0079).

In (Figure 3), the mean fluorescence intensity (MFI) for activation receptors and splenic cell interleukin production are represented for groups GI, GII, and GIII. In graph (A) and (B), we have the positive cells respectively for NKp46 and NKG2D along with the cytokines produced by them. Regarding the IL-10, IFN-γ, and IL-12 producing NKp46 cells, we obtained significance respectively, in the comparisons between the GIvsGII, GIIvsGIII, and GIvsGIII groups (p=0,0019) and for the NKG2D cells with their cytokines. Significant findings were in all parameters for IL-10 between GIvsGII, IL-2 GIIvsGIII, IL17 GIvsGII, TNF-αGIvsGII, IFN-γGIvsGIII and IL-12 GIvsGII all with value p=0,0019. In (C) and (D) MFI values for mice tumor cells for receptors and cytokine production; we observed in (C) for NKp46 receptor significance for IL-10, IFN-γ, and IL-12, respectively, in comparison for GIIvsGIII groups (p=0,0019).

The results in (Figure 4) demonstrate comparisons between tumor infiltrate and splenic cell groups for inhibition receptors and cytokine production. In graph 4(A), the results show significantly higher MFI values of IL10-producing Ly49G2 cells (p=0,0019) and for the IL-1 producing cells in the comparison between GIvsGII. For IFN-γ (p=0,0019), comparing GIIvsGIII, the highest intensity is for the latter group,

and for IL-12 producing cells (p=0,0019), the same result is observed in the comparison. In (B) for IL-10 producing IFN-γ NKG2A/C/E cells and IL-12, respectively, the significant values are between GIvsGIII (p=0,0019), GIvsGIII/GIIvsGIII (p=0,0088) and GIvsGIII (p=0,0019) all above results for splenic cells of the experimental groups. In (C), the results for IL-10, TNF-α, IFN-γ, and IL-12 producing tumor infiltrate Ly49G2 cells are significant between groups (p=0,0079) with the predominance of higher fluorescence intensity, respectively, for these cytokines in group GII. In (D) the results show significant values for NKG2A/C/E cells producing interleukins 10, 2 and IFN-γ where only for interleukin 2 the GIII group has higher fluorescence intensity than the GII group (p=0,0022). For the other cytokines group GII presented higher fluorescence intensity for IL-10 (p=0,0022) and IFN-γ (p=0,0079).

4. Discussion

In the present study, we used the immunotherapy with DC cell vaccine to treat the induced breast tumors in mice and evaluate the profile of NK cells in the control, tumor and tumor treated with DC cell vaccine groups.

NK cells and all cells of the immune system migrate throughout the body via the circulatory system, continuously examining for invading microorganisms or tumor cells, responding to them in secondary lymphoid organs such as lymph node or spleen. [14]. However, to increase their cytolytic functions in the interaction with DC, they need to produce IL-2 and IL-12 by increasing the cytolytic function of mature NK cells [15].

In the tumor microenvironment stimulatory cytokines that attenuate NK cell responses are IL2, IL-15, IL-18, IL-21, and IFN-γ, in contrast to immunosuppressive cytokines such as transforming growth factor β (TGF-β), IL-10 and IL-6, directly or indirectly inhibit antigen presentation by presenting cells (APCs) or stimulate regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) to produce additional immunosuppressive factors [16].

Considering cytokine synergism between NK cells and DC, in studies, Ferlazzo et al. verified the binding potential between the maturation of DC cells and activation of NK cells, concluding that isolated resting NK cells are directly activated by interaction with DC cells. Moreover, results show that they recruit NK cells to infected tissues creating positive feedback that drives cells to Th1 cell-mediated immunity [7].

Activation receptors such as NKp46/NCR1 (CD335) in mice and NKp46 in humans are recognized as natural cytotoxicity receptors (NCRs) that recognize viral proteins, bacteria, and tumor cells efficiently triggering the release of cytotoxins from apoptotic granules, cytokines, and chemokines of the target cell

[17]. Another activating receptor expressed on NK cells is NKG2D, which interacts with MHC I molecules in stress-induced cells via MICA and MICB proteins and members of the UL16 binding family of ULBP protein family [18].

Deng et al. in their studies proved the importance of testing these receptors in hypothetical models since the results demonstrated that even with the release of soluble NKG2D-L by B16 tumor cells it did not affect NK cell functions at all but resulted in a higher reactivity of the NKG2D-L NK and faster tumor rejection [19].

In due course, we observed the critical role of NK cells on DC immunotherapy by assessing the percentage of total cells in the spleen and tumor infiltrate, knowing that the presence of these cells in tissues is crucial for the elimination of neoplastic cells and stimulating adaptive immunity [20]. Our results demonstrate a higher density of $NKp46^{+}$ cells in the tumor infiltrate of non-vaccine treated groups.

According to Wendel and colleagues 2008, the migration of NK cells to the tumor infiltrate occurs spontaneously in response to tumor cells, because these cells express CXCR3 chemokine, which is essential for NK cell infiltration into CXCL10-producing solid tumors [21,22].

Oppenhein et al. 2005 found that the immune response against overexpressed stress antigens in tumors and absence of stimulation by microorganisms is reminiscent of tumor surveillance make NK cells unviable from activating, so chronic activation of these cells leads to CD8 T cell-like exhaustion reflecting in an adaptation of NK cells to the tumor microenvironment [23,24].

On the other hand, studies have reported that sustaining NKp46 expression in tumors, for example, in breast cancer, is associated with low regulation of other activation receptors such as NKG2D, NKp30, and NKp44, characterizing NKp46 as a promising target in directing NK cell activation against cancer [25].

Although not significant, our results show an inversion of fluorescence intensity for positive NKp46 and NKG2D cells in the spleen of untreated mice concerning the tumor infiltrate of this same group for both markers, as in treated mice these cells remain at constant levels. In these two tissues, these findings may be correlated due to the exhaustion of NK cells present in the tumor infiltrate, this could be one of the factors for tumor cell evasion against effector cell attack [26,27].

Studies involving the lectin-like activation receptor type C NKG2D, expressed in both NK cells, demonstrate that systemic and intratumor NK cells with low expression of this receptor are correlated with NK cell functions and tumor cells immune-evasion [28]. However, the interaction of this receptor with

NKp46 can activate NK cells by stimulating the production of type I interferons through cellular contact with DC cells suggesting another cooperation option between these cells [29].

In our study involving NKG2D, we found high expression of these receptors in the group of mice treated with the DC vaccine, suggesting that the treatment increased the expression of these receptors generating NK cells capable of exerting their cytolytic function.

As it is yet not clear how NK cells are released into the circulation [30], in our results we observed an increased density of splenic NK cells in the control group expressing the NKp46 receptor, meeting the findings of Sojka and colleagues who distinguished these cells as resident NK tissue cells (trNK), which are similar to conventional NK cells (cNK), and exhibit high levels of TNF-related apoptosis-inducing ligand (TRAIL) [31].

When activated, NK cells need to cease their response at any time, given that all target cells have been deleted. For this, we have inhibition receptors like the NKG2A, alongside with helping in homeostasis, can suppress the activation of NK cells and prevent the recognition of our cells. That receptor interacts via NKG2A/HLA-E binding, decreasing response effectiveness against tumor cells [32], and KIR Ly49G2 binding MHC I prevent NK cells from exerting their cytotoxic function [33].

Our results showed a higher expression of Ly49G2 and NKG2A/C/E receptors in both splenic cells and tumor infiltrate for untreated groups, and a decrease in cells expressing these receptors for DC treated group, demonstrating the influence of the dendritic cells on NK cells in the effectiveness of DC vaccine treatment.

Zhang et al. 2019 found that virus-infected cells with low NKG2A receptor expression were prevented from replicating and evading immunosurveillance [34]. When dealing with dendritic cells in solid tumors, NK cells that have low regulation of these receptors assist in the elimination and combat of neoplastic cells.

In antiviral responses and against neoplastic cells, NK cells produce large-scale IFN-γ, thereby exerting their effector function, stimulating other immune response cells [35]. As well as interferon acting on NK, other cytokines are essential for the maturation, activation and survival of these cells, such as interleukins (IL). IL-2, IL-12, IL-15, IL-18 and IL-21 positively regulate their functions, independently or in cooperation with other cytokines such as IL-23 and IL-27, which may enhance or suppress NK cell function, depending on the context in which they are found [16,36].

In the tumor microenvironment, TGF-β, IL-10, and IL-6 suppress NK cell activity not only directly but also indirectly, affecting immunosuppressive cells and antagonizing the effect of stimulating cytokines. Such events suppress the antitumor response of NK cells and promotes tumor evasion and progression [37].

Evaluating our study, we observed that NK cells were stimulated in the case of activation receptors and cytokine production in splenic cells and tumor infiltrate. IFN-γ and IL-12 production by NKp46 cells is seen in the spleen and TNF-α and IFN-γ by NKG2D cells in the tumor infiltrate for the group treated with DC cell. The production of these cytokinesis essential for up-regulation of NK cells. Although we observed the production of IFN-γ and IL-12 by infiltrating cells expressing NKp46 receptors in the untreated tumor group, these cells were presented in a smaller amount because this same group presented IL-10 producing NKp46 cells, indicating that treatment potentiates the production of TNF-α, IFN-γ, and IL-12 in both tissues.

Finally, for inhibition receptors and cytokine production, our results show that IFN-γ and IL-12 producing Ly49G2 cells were found significantly more in the spleen of the group treated with DCs, and for this same group, we found non-significant results for IL-10. However, in the tumor infiltrate for the NKG2A/C/E-treated group of DC cells we found only significant values for IL-2 production. The study of these receptors is currently widely used in immunotherapies using monoclonal antibodies (mAbs) blocking receptor inhibition in target cells for greater effectiveness in cancer vaccine treatment [38].

Given the findings for activation receptors and cytokine production by NK cells, it is observed that cytokines responsible for stimulating and regulating responses mediated by these cells were found to be significant since untreated mice showed inefficient responses in tumor regression. In this scenario, we can infer that DC vaccination and NK cell cooperation are necessary for successful treatments. Our work is the first to correlate NK cell activation and inhibition receptors and cytokine production against dendritic cell autologous cell vaccine treatment.

Concluding our findings, we demonstrated that immunotherapy with DC is able to alter the profile of NK cell by directly influencing them in the antitumor response due to reduced inhibition receptor expression and increased production of cytokines such as TNF-α and IFN-γ by cells with activation receptors, conferring NK cells the ability to reverse tumor-induced immunosuppression, stimulating the immune system to eliminate tumor cells.

Conflicts of interest

The authors report no conflicts of interest.

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Declaration of competing interest

The authors declare that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

Author Contributions

Eduardo Arthur Rodovalho Alves designed, performed and wrote most experiments, Polyana Barbosa Silva designed and performed the experiments, Márcia Antoniazi Michelin and Eddie Fernando Cândido Murta planned the experiments and contributed to the analysis/interpretation of the data and writing of the manuscript.

Ethical approval

All procedures performed in this study involving mice where in accordance with the ethical standards of the institutional research committee, flowing national standards (Animal Use Ethics Committee – CEUA- Federal University of Triangulo Mineiro- UFTM, protocol number 378-2016) according CONCEA – 2016 Brazil.

References:

[1] F. Bellati, C. Napoletano, I. Ruscito, V. Visconti, M. Antonilli, M.L. Gasparri, I.G. Zizzari, H. Rahimi, I. Palaia, A. Rughetti, P. Benedetti Panici, M. Nuti, Past, Present and Future Strategies of Immunotherapy in Gynecological Malignancies, Curr. Mol. Med. 13 (2013) 648–669. doi:10.2174/1566524011313040014.

[2] J.D. Clough, Immune recognition, Suppressor Cells and Their Factors. (2018) 3–20. doi:10.1201/9781351077026.

[3] I. Waldhauer, A. Steinle, NK cells and cancer immunosurveillance, Oncogene. 27 (2008) 5932–5943. doi:10.1038/onc.2008.267.

[4] M. Sternberg-Simon, P. Brodin, Y. Pickman, B. Önfelt, K. Kärre, K.J. Malmberg, P. Höglund, R. Mehr, Natural killer cell inhibitory receptor expression in humans and mice: A closer look, Front. Immunol. 4 (2013) 1–12. doi:10.3389/fimmu.2013.00065.

[5] M. Cheng, Y. Chen, W. Xiao, R. Sun, Z. Tian, NK cell-based immunotherapy for malignant diseases, Cell. Mol. Immunol. 10 (2013) 230–252. doi:10.1038/cmi.2013.10.

[6] L.L. Lanier, Natural killer cell receptor signaling, Curr. Opin. Immunol. 15 (2003) 308–314. doi:10.1016/S0952-7915(03)00039-6.

[7] G. Ferlazzo, M.L. Tsang, L. Moretta, G. Melioli, R.M. Steinman, C. Münz, Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells, J. Exp. Med. 195 (2002) 343–351. doi:10.1084/jem.20011149.

[8] A.M. James, A.D. Cohen, K.S. Campbell, Combination immune therapies to enhance anti-tumor responses by NK cells, Front. Immunol. 4 (2013) 1–12. doi:10.3389/fimmu.2013.00481.

[9] C. Chester, K. Fritsch, H.E. Kohrt, Natural killer cell immunomodulation: Targeting activating, inhibitory, and co-stimulatory receptor signaling for cancer immunotherapy, Front. Immunol. 6 (2015) 1–9. doi:10.3389/fimmu.2015.00601.

[10] N.C. Fernandez, A. Lozier, C. Flament, P. Ricciardi-Castagnoli, D. Bellet, M. Suter, M. Perricaudet, T. Tursz, E. Maraskovsky, L. Zitvogel, Dendritic cells directly trigger NK cell functions: Cross-talk relevant in innate anti-tumor immune responses in vivo, Nat. Med. 5 (1999) 405–411. doi:10.1038/7403.

[11] M. Cooper, NK cell and DC interactions, Trends Immunol. 25 (2004) 47–52. doi:10.1016/j.it.2003.10.012.

[12] T. Walzer, M. Dalod, S.H. Robbins, L. Zitvogel, E. Vivier, Review article Natural-killer cells and dendritic cells : “ l ’ union fait la force ”, Blood. 106 (2005) 2252–2258. doi:10.1182/blood-2005-03-1154.Supported.

[13] M.B. Lutz, N. Kukutsch, A.L.J. Ogilvie, S. Rößner, F. Koch, N. Romani, G. Schuler, An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow, J. Immunol. Methods. 223 (1999) 77–92. doi:10.1016/S0022-1759(98)00204-X.

[14] D.K. Sojka, Z. Tian, W.M. Yokoyama, Tissue-resident natural killer cells and their potential

diversity, Semin. Immunol. 26 (2014) 127–131. doi:10.1016/j.smim.2014.01.010.

[15] G. Trinchieri, Natural killer cells wear different hats: effector cells of innate resistance and regulatory cells of adaptive immunity and of hematopoiesis, Semin. Immunol. 7 (1995) 83–88. doi:10.1006/smim.1995.0012.

[16] G.M. Konjević, A.M. Vuletić, K.M. Mirjačić Martinović, A.K. Larsen, V.B. Jurišić, The role of cytokines in the regulation of NK cells in the tumor environment, Cytokine. 117 (2019) 30–40. doi:10.1016/j.cyto.2019.02.001.

[17] S. Sheppard, I.S. Schuster, C.E. Andoniou, C. Cocita, T. Adejumo, S.K.P. Kung, J.C. Sun, M.A. Degli-Esposti, N. Guerra, The Murine Natural Cytotoxic Receptor NKp46/NCR1 Controls TRAIL Protein Expression in NK Cells and ILC1s, Cell Rep. 22 (2018) 3385–3392. doi:10.1016/j.celrep.2018.03.023.

[18] J. Hilpert, L. Grosse-Hovest, F. Grünebach, C. Buechele, T. Nuebling, T. Raum, A. Steinle, H.R. Salih, Comprehensive Analysis of NKG2D Ligand Expression and Release in Leukemia: Implications for NKG2D-Mediated NK Cell Responses, J. Immunol. 189 (2012) 1360–1371. doi:10.4049/jimmunol.1200796.

[19] W. Deng, B.G. Gowen, L. Zhang, L. Wang, S. Lau, A. Iannello, J. Xu, T.L. Rovis, N. Xiong, D.H. Raulet, A shed NKG2D ligand that promotes natural killer cell activation and tumor rejection, Science (80-.). 348 (2015) 136–139. doi:10.1126/science.1258867.

[20] T.K. Erick, L. Brossay, Phenotype and functions of conventional and non-conventional NK cells, Curr. Opin. Immunol. 38 (2016) 67–74. doi:10.1016/j.coi.2015.11.007.

[21] M. Wendel, I.E. Galani, E. Suri-Payer, A. Cerwenka, Natural killer cell accumulation in tumors is dependent on IFN-γ and CXCR3 ligands, Cancer Res. 68 (2008) 8437–8445. doi:10.1158/0008-5472.CAN-08-1440.

[22] S.K. Tripathy, P.A. Keyel, L. Yang, J.T. Pingel, T.P. Cheng, A. Schneeberger, W.M. Yokoyama, Continuous engagement of a self-specific activation receptor induces NK cell tolerance, J. Exp. Med. 205 (2008) 1829–1841. doi:10.1084/jem.20072446.

[23] D.E. Oppenheim, S.J. Roberts, S.L. Clarke, R. Filler, J.M. Lewis, R.E. Tigelaar, M. Girardi, A.C. Hayday, Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance, Nat. Immunol. 6 (2005) 928–937. doi:10.1038/ni1239.

[24] S. Bessoles, C. Grandclément, E. Alari-Pahissa, J. Gehrig, B. Jeevan-Raj, W. Held, Adaptations of natural killer cells to self-MHC class I, Front. Immunol. 5 (2014) 1–6. doi:10.3389/fimmu.2014.00349.

[25] L. Gauthier, A. Morel, N. Anceriz, B. Rossi, A. Blanchard-Alvarez, G. Grondin, S. Trichard, C. Cesari, M. Sapet, F. Bosco, H. Rispaud-Blanc, F. Guillot, S. Cornen, A. Roussel, B. Amigues, G. Habif, F. Caraguel, S. Arrufat, R. Remark, F. Romagné, Y. Morel, E. Narni-Mancinelli, E. Vivier, Multifunctional Natural Killer Cell Engagers Targeting NKp46 Trigger Protective Tumor Immunity, Cell. 177 (2019) 1701-1713.e16. doi:10.1016/j.cell.2019.04.041.

[26] S. Jonjić, M. Babić, B. Polić, A. Krmpotić, Immune evasion of natural killer cells by viruses, Curr. Opin. Immunol. 20 (2008) 30–38. doi:10.1016/j.coi.2007.11.002.

[27] Q. Yang, S.R. Goding, M.E. Hokland, P.H. Basse, Antitumor activity of NK cells, Immunol. Res. 36 (2006) 13–25.

[28] H. Saito, T. Osaki, M. Ikeguchi, Decreased NKG2D expression on NK cells correlates with impaired NK cell function in patients with gastric cancer, Gastric Cancer. 15 (2012) 27–33. doi:10.1007/s10120-011-0059-8.

[29] M. Draghi, A. Pashine, B. Sanjanwala, K. Gendzekhadze, C. Cantoni, D. Cosman, A. Moretta, N.M. Valiante, P. Parham, NKp46 and NKG2D Recognition of Infected Dendritic Cells Is Necessary for NK Cell Activation in the Human Response to Influenza Infection, J. Immunol. 178 (2007) 2688–2698. doi:10.4049/jimmunol.178.5.2688.

[30] R.J. Benschop, M. Schedlowski, H. Wienecke, R. Jacobs, R.E. Schmidt, Adrenergic control of natural killer cell circulation and adhesion, Brain. Behav. Immun. 11 (1997) 321–332. doi:10.1006/brbi.1997.0499.

[31] D.K. Sojka, B. Plougastel-Douglas, L. Yang, M.A. Pak-Wittel, M.N. Artyomov, Y. Ivanova, C. Zhong, J.M. Chase, P.B. Rothman, J. Yu, J.K. Riley, J. Zhu, Z. Tian, W.M. Yokoyama, Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells, Elife. 2014 (2014) 1–21. doi:10.7554/eLife.01659.

[32] P. André, C. Denis, C. Soulas, C. Bourbon-Caillet, J. Lopez, T. Arnoux, M. Bléry, C. Bonnafous, L. Gauthier, A. Morel, B. Rossi, R. Remark, V. Breso, E. Bonnet, G. Habif, S. Guia, A.I. Lalanne, C. Hoffmann, O. Lantz, J. Fayette, A. Boyer-Chammard, R. Zerbib, P. Dodion, H. Ghadially, M. Jure-Kunkel, Y. Morel, R. Herbst, E. Narni-Mancinelli, R.B. Cohen, E. Vivier, Anti-NKG2A mAb

Is a Checkpoint Inhibitor that Promotes Anti-tumor Immunity by Unleashing Both T and NK Cells, Cell. 175 (2018) 1731-1743.e13. doi:10.1016/j.cell.2018.10.014.

[33] I. Barao, M. Alvarez, E. Ames, M.T. Orr, H.E. Stefanski, B.R. Blazar, L.L. Lanier, S.K. Anderson, D. Redelman, W.J. Murphy, Mouse Ly49G2+ NK cells dominate early responses during both immune reconstitution and activation independently of MHC, Blood. 117 (2011) 7032–7041. doi:10.1182/blood-2010-11-316653.

[34] C. Zhang, X. mei Wang, S. ran Li, T. Twelkmeyer, W. hong Wang, S. yuan Zhang, S. feng Wang, J. zheng Chen, X. Jin, Y. zhang Wu, X. wen Chen, S. dian Wang, J. qi Niu, H. rong Chen, H. Tang, NKG2A is a NK cell exhaustion checkpoint for HCV persistence, Nat. Commun. 10 (2019). doi:10.1038/s41467-019-09212-y.

[35] A. Aquino-López, V. V. Senyukov, Z. Vlasic, E.S. Kleinerman, D.A. Lee, Interferon gamma induces changes in Natural Killer (NK) cell ligand expression and alters NK cell-mediated lysis of pediatric cancer cell lines, Front. Immunol. 8 (2017). doi:10.3389/fimmu.2017.00391.

[36] J.R. Ortaldo, R. Winkler-pickett, J. Wigginton, M. Horner, E.W. Bere, A.T. Mason, N. Bhat, J. Cherry, M. Sanford, D.L. Hodge, H.A. Young, Regulation of ITAM-positive receptors : role of IL-12 and IL-18, 107 (2019) 1468–1476. doi:10.1182/blood-2005-04-1579.Supported.

[37] N.W. Zwirner, C.I. Domaica, Cytokine regulation of natural killer cell effector functions, BioFactors. 36 (2010) 274–288. doi:10.1002/biof.107.

[38] N. van Montfoort, L. Borst, M.J. Korrer, M. Sluijter, K.A. Marijt, S.J. Santegoets, V.J. van Ham, I. Ehsan, P. Charoentong, P. André, N. Wagtmann, M.J.P. Welters, Y.J. Kim, S.J. Piersma, S.H. van der Burg, T. van Hall, NKG2A Blockade Potentiates CD8 T Cell Immunity Induced by Cancer Vaccines, Cell. 175 (2018) 1744-1755.e15. doi:10.1016/j.cell.2018.10.028.

Legends

Fig. 1. Tumor measurements in mice submitted to Dendritic cells Immunotherapy. Measures from the 13th day, when the tumor nodule formation was detected by palpation. We performed the tumor measurements, and on the 25th day, at the end of the treatment, the animals were euthanized. Comparisons were performed by Mann Whitney t-Test, and significant values of $p < 0.05$ were considered. For the median tumor group (Min.- Max.) 48,19 (0,1250-186,60) and group DCs 0,1714 (0,0-2,686).

Fig. 2. Activation and inhibition receptors in NK cells present on spleen or tumor obtained from tumor-induced mice treated with Dendritic cells. Comparisons between groups using 1way ANOVA. **(A)** Percent of cells for NKp46 and NKG2D receptors obtained from mice spleen. In (**B**), percentage of cells expressing Ly49G2 and NKG2A/C/E receptors obtained from the spleen. On (**C**) and (**D**) cell percentage for activation and inhibition receptors, respectively, NKp46, NKG2D and Ly49G2, NKG2A/C/E in the tumor infiltrate of mice used in the study.

Fig. 3. Cytokine synthesis by NK cells positive for activators receptors NKp46 and NKG2D from spleen and tumor infiltrate obtained from tumor-induced mice submitted to Immunotherapy with DCs. In (**A**) MFI values obtained for NKp46 cells producing IL-10, IL-2, IL-17, TNF-α, and IFN-γ in mice spleen, significant values are observed for IL-10, IFN- γ and IL-12. **(B)** Results for IL-10, IL-2, IL-17, TNF-α, and IFN-γ and IL-12 producing NKG2D cells in the comparisons show significant values for all interleukins. In **(C)** and (**D**) the same conditions as described above, but in the tumor infiltrate samples from the GII and GIII mice.

Fig. 4. Cytokine synthesis by NK cells positive for inhibitors receptors Ly49G2 and NKG2A/C/E from spleen and tumor infiltrate obtained from tumor-induced mice submitted to Immunotherapy with DCs. In (**A**) MFI values obtained for the IL-10, IL-2, IL-17, TNF-α, IFN-γ, and IL-12 producer Ly49G2 cells in the spleen of mice, significant values are observed for IL-10. 10, IFN-γ and IL-12 in the GIvsGII groups for IL-10 and GIIvsGIII for IFN-γ and IL-12. (**B**) Results obtained for IL-10, IL-2, IL-17, TNF-α, IFN-γ, and IL-12 producing NKG2A/C/E cells in the comparisons, IFN-γ, and IL-12 for the GIvsGIII IL-

10, GIvsGIII and GIIvsGIII groups for IFN-γ and GIvsGIII IL-12. In (C) and (D) in the same way, but with the tumor infiltrate samples from the GII and GIII mice.

Figures

Figure 1

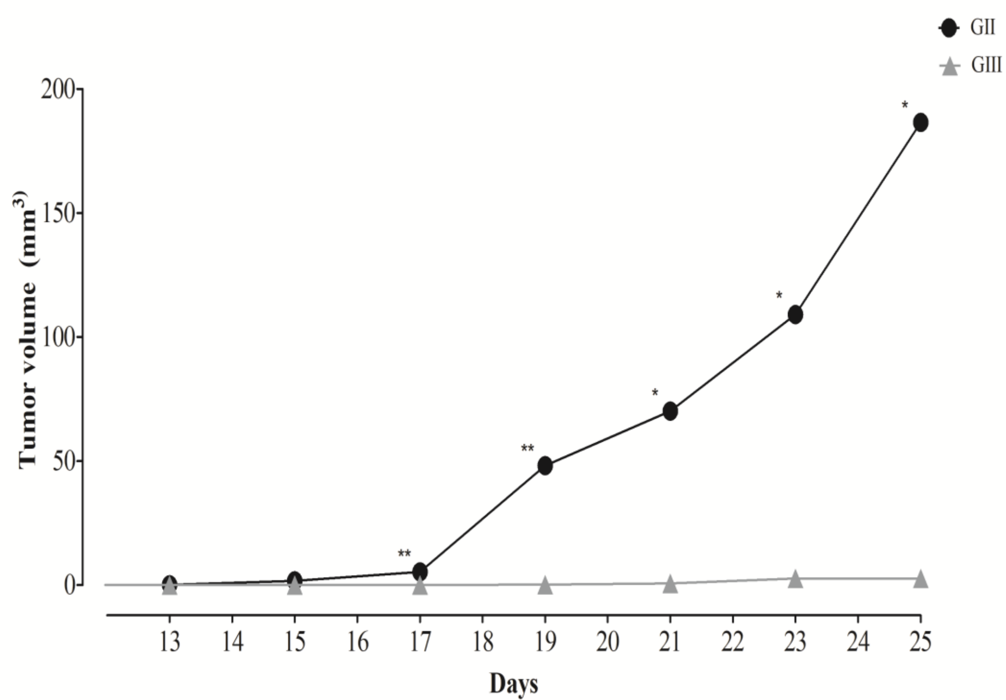


Figure 2

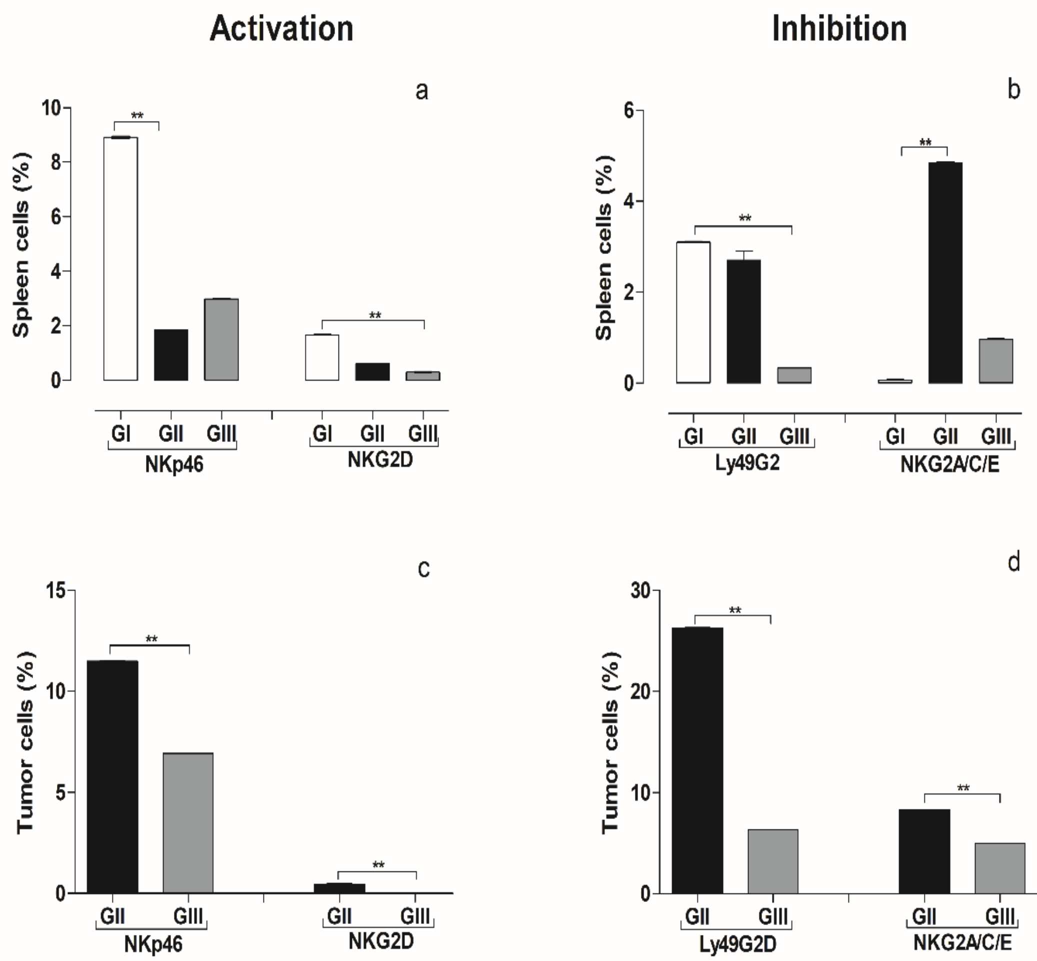


Figure 3

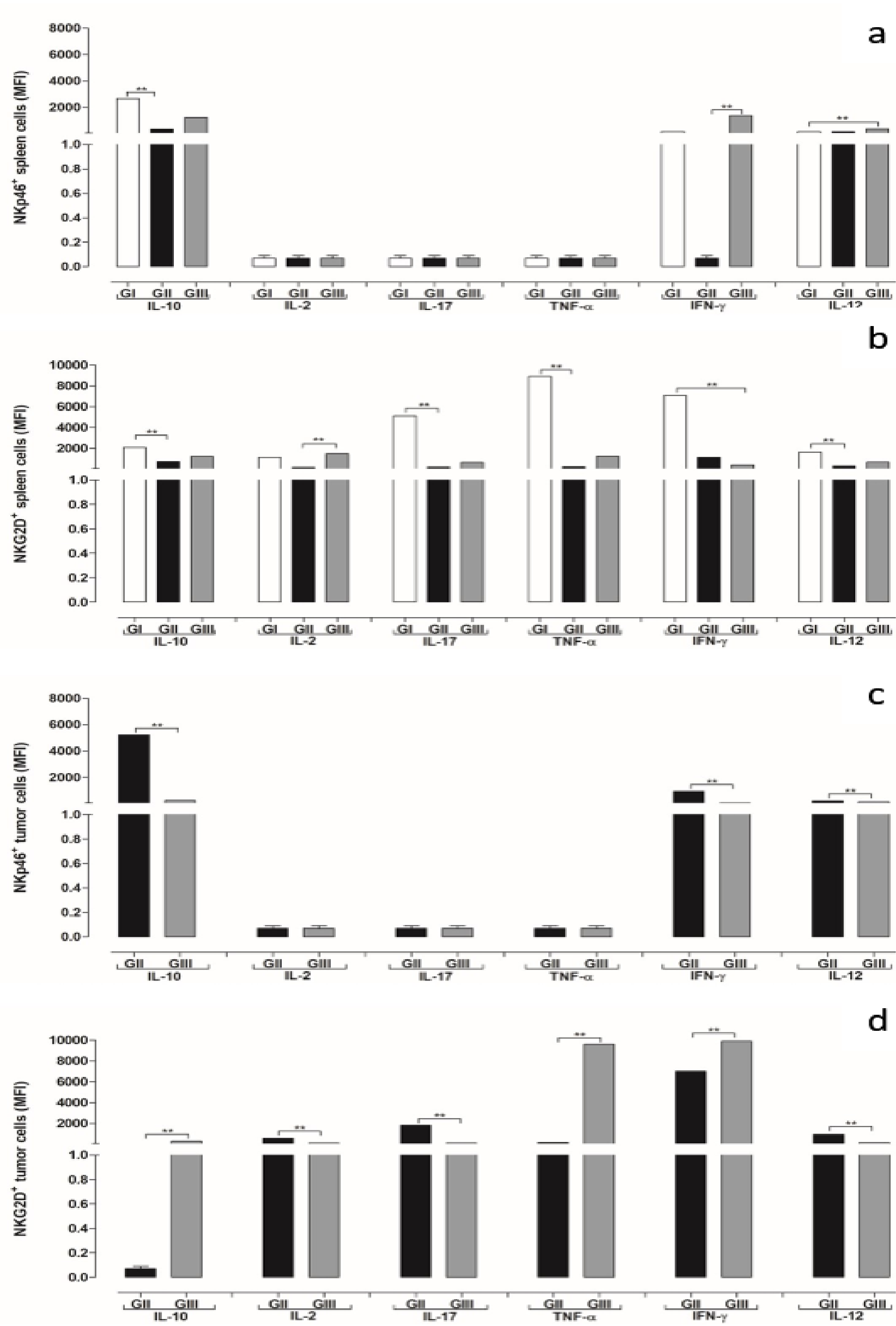
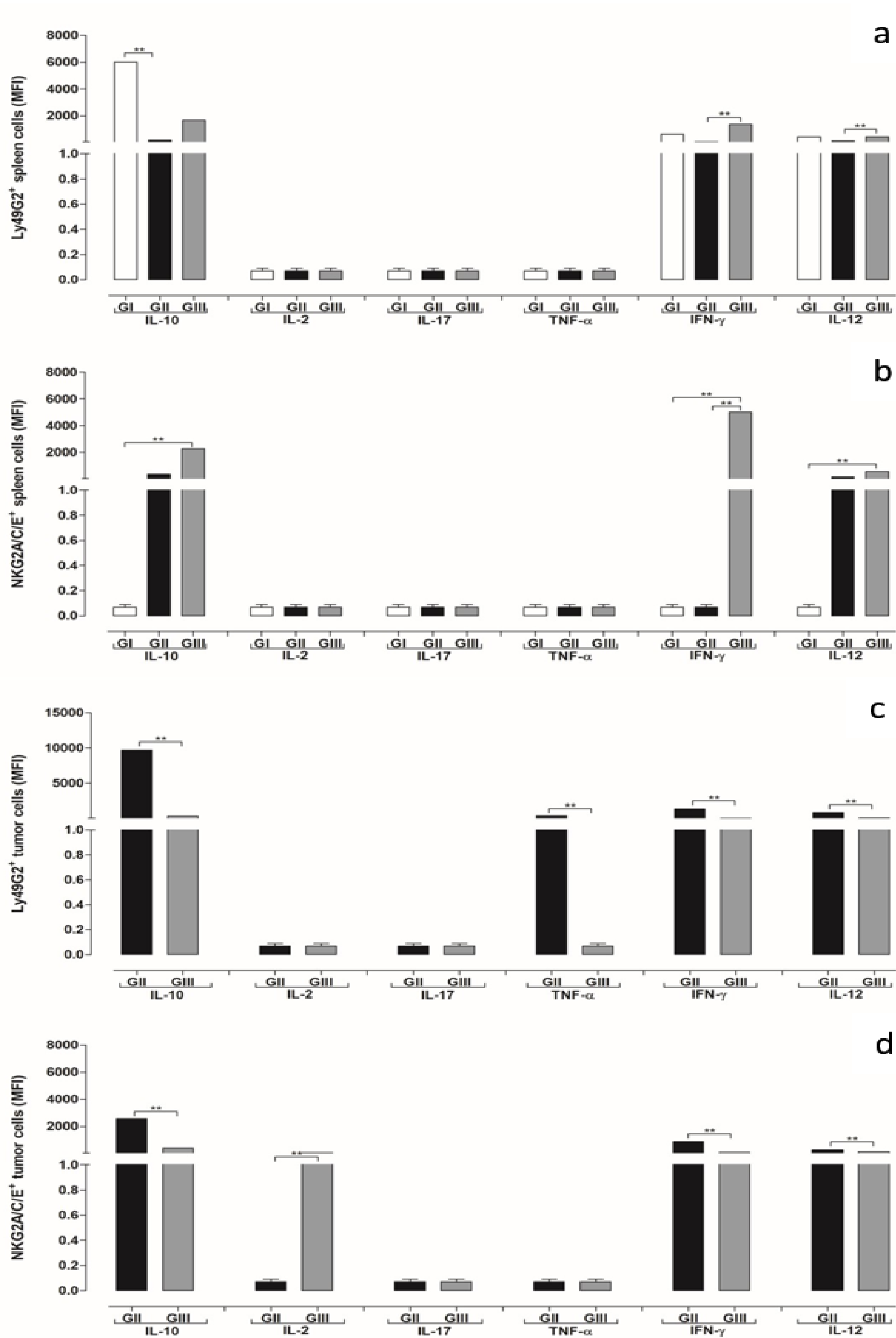


Figure 4



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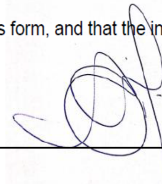

Date: December, 05th 2019.

4.2- Artigo 2

Immune profile of treatment with natural killer (NK) cells vaccine in mice induced breast tumor with 4T1 cells.

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Objective: In this study we evaluated the profile of the Natural Killer (NK) cell immune response in NK cell vaccine immunotherapeutic treatment in mice induced by breast cancer through NKp46, NKG2D activation, Ly49G2, NKG2A/C/E inhibition and production of interleukins (IL) 2, 10, 12, 17, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ). **Materials and methods:** Thirty Balb/C mice were used to compose the experimental groups: Control (C), untreated tumor (T) and tumor treated with NK cell vaccine (TNK). In the preparation of the vaccine we used bone marrow donor mice and the experimental period was 25 days, performing measurements of tumor volume and immunotherapeutic treatment for 3 weeks ending with the euthanasia of mice to remove the spleen and tumor infiltrate to perform flow cytometry for NKp46, NKG2D, Ly49G2 and NKG2A/C/E receptors; IL-2, IL-10, IL-12, IL-17, TNF-α and IFN-γ cytokines. **Results:** Our results show that mice treated with NK cell vaccine have a higher density of splenic and intratumor cells expressing NKp46 (p=0.0019), Ly49G2 (p=0.0079) and NKG2A/C/E receptors (p=0.0079) and higher fluorescence intensity for cytokine production by splenic cells NKp46, NKG2D and NKG2A/C/E producing IFN-γ (p=0.0019/0.0019 and 0.0088), NKG2D producing of TNF-α and IFN-γ (p=0.0019) and NKG2A/C/E-producing intratumoral tumors of IL-10 and IL-12 (p=0.0022/0.0079), TNF-α-producing NKG2D (p=0.0079) develop immunosuppression preventing NK cells from performing their effector functions. **Conclusion:** In NK cell immunotherapy the proper balance between activation and inhibition receptors along with cytokine production is required for proper activation of these cells to exert their effector functions, increased expression of Ly49G2 cells prevents NK cells from performing their functions. effectors stimulating systemic expansion and migration of this phenotypic profile to TME preventing its activation.

Keywords: Natural Killer cells, cellular immunotherapy, NK cell activation.

1- Introduction

Immunotherapies are very promising in the treatment of cancer, based on the essence of using the patient's own immune system to effect specific antitumor responses. New therapies are based on the use of checkpoint inhibitors, immunomodulators, and genetically engineered cells to block signaling pathways or receptors that act by generating an immunosuppressive state in the microenvironment or systemically affecting host cell responses to dissipate neoplastic cells. (JESPERSEN et al., 2017).

Recent approaches that use in vivo cells to attack tumor cells reinforce issues involving limitations in treatment for patients treated with chemotherapy or radiotherapy, leading to immunodepression, such as the elimination of healthy T cells that may change or be stimulated to contribute in tumor regression (POPP; MAQUAT, 2015).

In cancer treatments, understanding natural killer cell (NK) cell therapy by advancing these studies contributes to improved cancer treatment of various types of cancer.

NK cells are part of the innate immune system and similarly to cytotoxic T lymphocytes perform their specific functions and are categorized by their great potential in eliminating tumor cells. Its activation is driven by a balance between activating and inhibitory signals, without prior sensitization to exert its antitumor function (DI VITO et al., 2019).

These cells have demonstrated their potential in the treatment of various types of cancer, but the efficacy in treating solid tumors is still unsatisfactory, and one of the main reasons for this limitation is the immunosuppressive effect of the tumor microenvironment (TME) (HABIF et al., 2019).

In TME tumor-associated cells and tumor cells produce various cytokines and other factors that directly or indirectly affect NK cell activation, such as interleukin (IL) 10 and 6 among others, and also have a restriction on modulation of NKp30 activation receptors, NKp44 or NKG2D and receive signals from inhibitory receptors increasing local immunosuppression (BÖTTCHER et al., 2018).

In addition to the issues surrounding the tumor microenvironment, recent discoveries have revealed that impaired metabolism is associated with progressive NK cell dysfunction, understanding these mechanisms would be a better way to understand their contribution to current therapies. (CONG et al., 2018).

Foster cell therapies bring benefits that allow ex vivo manipulation with approaches to enabling NK cells to survive by persistently performing their functions in vivo. (KIESSLING, 1976; SUEN et al., 2018).

For this, the expression of NK cell surface receptors responsible for their mediated recognition, and cytokine-mediated cytotoxic functions, such as the production of TGF-β produced by regulatory T lymphocytes (Treg) among other cells, inhibits the expression of NKp30 and NKG2D which are crucial for NK cell-mediated recognition and cell death, as well as IL-4 which may reduce the ability of NKs to produce cytokines, preventing interaction with dendritic cells (DCs), and subsequently, the NKG2D itself hypoxia that is associated with the tumor microenvironment may lead to decreased expression or function of NK receptors (CASTRICONI et al., 2003; MARCENARO et al., 2005).

In tumor biology the number of infiltrating cells is extremely relevant, relevant considerations such as limiting factors for the effectiveness of the anti-tumor immune response include the relative amount of intratumor cells and functional capacity, and the increased relationship between effector cells and target cell is key to eliminating tumor cells in vitro and in vivo (MELERO et al., 2014).

Focusing on cellular immunotherapy, our study aims to show how NK cells behave systemically and in the tumor microenvironment to treatment with the autologous NK cells vaccine, bringing a better understanding of their role in antitumor immune responses adding the ability to interact with other cells stimulating effector responses by the immune system, but we were unsuccessful in treatment due to the high expression of the Ly49G2 receptor in cell differentiation and IL-10 production in the spleen and tumor infiltrate of the treated mice.

2. Materials and Methods

The study brings together the making of the NK cell vaccine from the bone marrow taken from Balb/C mice on what we verified the expression of differentiated cell receptors in culture before starting immunization and "*in vivo*" testing with mice from

experimental groups. Once differentiated, we initiated the treatment protocol and receptor and cytokine production evaluations by flow cytometry of the spleen and tumor infiltrate samples from each group.

2.1- **Experimental groups and research design**

Thirty Balb/C mice were used in the study and distributed in 3 experimental groups for evaluation, control group (C) with animals without tumor induction and without treatment, the tumor group (T) without treatment that received only the inoculation of 4T1 cells and finally the tumor group induced with 4T1 cells and treated with NK cell vaccine (TNK). Thirteen days after tumor induction and the tumor formation began, animals from the NK cell-treated tumor group received the vaccine subcutaneously in 3 doses of vaccine for 3 consecutive weeks, each dose with a volume of 50 µL and a concentration of $8.0x10^5$ cells per mouse. The inoculation and treatment protocol comprised a total of 25 days and at the end, the mice were euthanized for biological material removal and preparation for the flow cytometry technique.

2.2- **4T1 cell tumor induction**

Animals in the experimental untreated tumor and NK cell treated tumor were inoculated with the 4T1 tumor line suspended in physiological solution and administered in a single dose at 50 µL volume at a concentration of $2.0x10^5$ cells per animal in the last pair region. of breasts. For tumor development we waited 13 days, once tumor formation was confirmed by palpation and inspection at the breast site, we began tumor measurement until the last day of the experiment.

With a universal caliper we performed weekly measurements of the untreated tumor group and the NK cell treated group in each mouse until the day of final euthanasia. The measurements obtained were performed in the craniocaudal and later lateral direction considering the average values obtained after the calculations. The calculations of the tumor values were obtained through the formula $\mathbf{V=a.b^2/2}$ being (a) the largest diameter measurement and (b) the smallest diameter, where the values were represented by the median.

2.3- **Production NK Cell Vaccine**

The euthanized animals belonging to the cell donor group for differentiation underwent femoral and tibial extraction and bone marrow cells are removed using a 13x4.5mm needle syringe and washed with saline, depositing the Petri dish solution.

To production NK cell vaccine according to the protocol of Luevano et al, 2012 (LUEVANO; MADRIGAL; SAUDEMONT, 2012), bone marrow cells were washed with incomplete RPMI 1640 medium and cultured after counting at a concentration of $32.5x10^6$ cells / mL in complete RPMI medium. To promote terminal proliferation/differentiation of NK cells, culture was stimulated with IL-2 (10 IU/mL), M-CSF (10 ng/mL), IL-15 (10 ng/mL) and IL-18 (0.33 ng/mL) on day 1 and thereafter with IL-15 (100 ng/mL) and IL-18 (0.33 ng/mL) on days 3, 5 and 7 and on day 20 with cytokine IL-2 (10 IU/mL) and maintained in culture until day 21 for complete differentiation.

2.4- **Flow Cytometry**

On the 25th day the animals that composed the experimental groups were euthanized and the splenic and intratumoral cells were obtained by dissemination for flow cytometry. Following the protocol recommended by BDbioscience®, the cells were placed in BD FACSTM Lysing Solution® lysis solution and subjected to the protocol extra and intracellular markings for NK cells.

In the run the samples were incubated with the extracellular antibodies for 30 minutes washed and incubated with BD Cytofix/Cytoperm™ for 20 minutes and after washing with BD Perm/Wash™ the intracellular markings were performed. To quantify NK cells in untreated and NK cell vaccine treated mice, we used the CD3 (total lymphocyte), NKp46 and NKG2D (activation receptor), Ly49G2 and NKG2A/C/E (inhibitory receptor) markers and interleukins. 2, 10, 12, 17, TNF-α and IFN-γ all BD Bioscience® antibodies. The data obtained from splenic cells and tumor infiltrate labeled with the aforementioned antibodies underwent a strategy of analysis by obtaining the total percentage of cells with selection of the amount of NK cells in the quadrant delimited to relative size (Forward Scatter, FSC) and granularity/complexity (Side Scatter, SSC).

2.5- **Statistical analysis**

By the BD FACSCalibur® instrument we obtained the results and analyzed by the Flowing Software Version 2.5.1 software through the total percentage of NK cells for each specific marker excluding other cell lines. The analysis values, cell percentage and mean fluorescence intensity were used for comparisons between groups and calculated by the GraphPad Prism 5® software performing the normality test and for comparison between the three groups we used One-way ANOVA test and Mann-Whitney t Test

comparing for two groups both cytometry and tumor volume results, considering significant p-values less than 0.05 ($p < 0.05$).

3. Results

In the analysis of the differentiated cells in the cell cultures we verified the expression of the expressed receptors, for that, we used CD3 and CD4 and CD8 T cells total lymphocyte markers, together with the activation and inhibition receptors of NK NKG2A/C/E, Ly49G2 and NKp46. In **Figure 1 (A)** we see the percentage of differentiated cells for each of the above receptors, in **1 (B)** the mean fluorescence intensity (MFI) emitted by all labeled cells. Evaluating the graphs, we observed a percentage of cells expressing the Ly49G2 and NKp46 receptors and despite a higher number for Ly49G2 the activity represented by fluorescence intensity is low, but for NKp46 this activity is representatively increased. No statistical tests were performed for these evaluations, because the observation of receptor expression was observed without distinction of comparative groups.

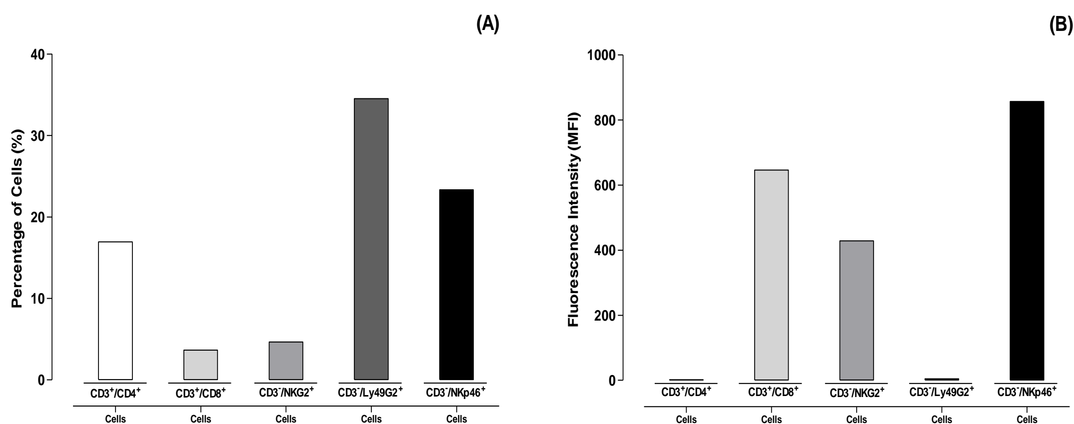


Figure 1: Cell percentage and mean fluorescence intensity (MFI) of cell differentiation in culture: In (A) the percentage cell number for NKG2A / C / E, Ly49G2 and NKp46 receptors of differentiated bone marrow cells , and (B) the mean fluorescence intensity for cells labeled with the receptors used in (A).

In the evaluation of the animals during the experimental period of 25 days, we demonstrated that the T and TNK groups presented similar tumor volumes and there was no significant tumor regression in the comparison between the two groups **(Figure 2)**.

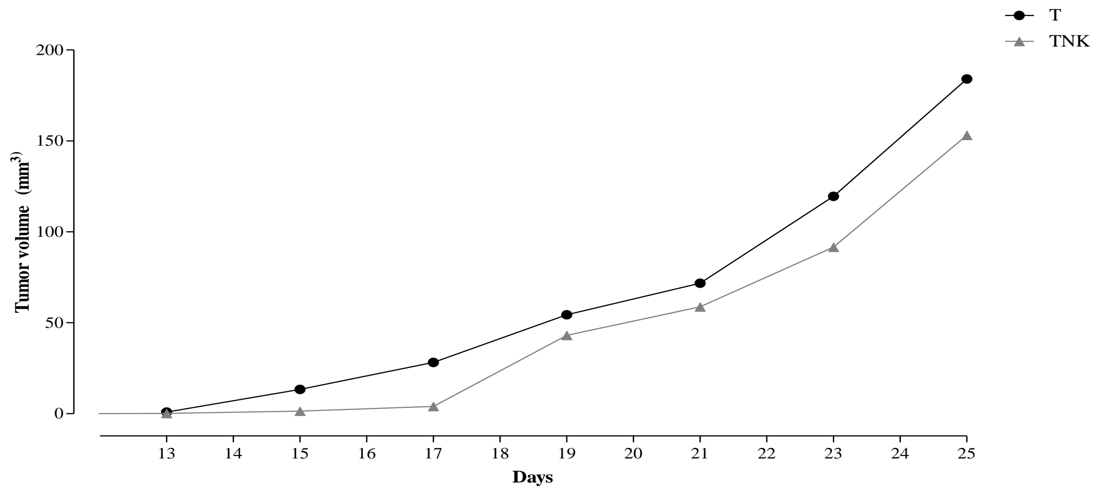


Figure 2: Tumor measurements of untreated tumor and NK cell treated mice: tumor size measurements from the 13th to 25th final day of treatment where the animals were euthanized. Comparisons were performed by Mann Whitney t Test and significant values of p <0.05 were considered**.**

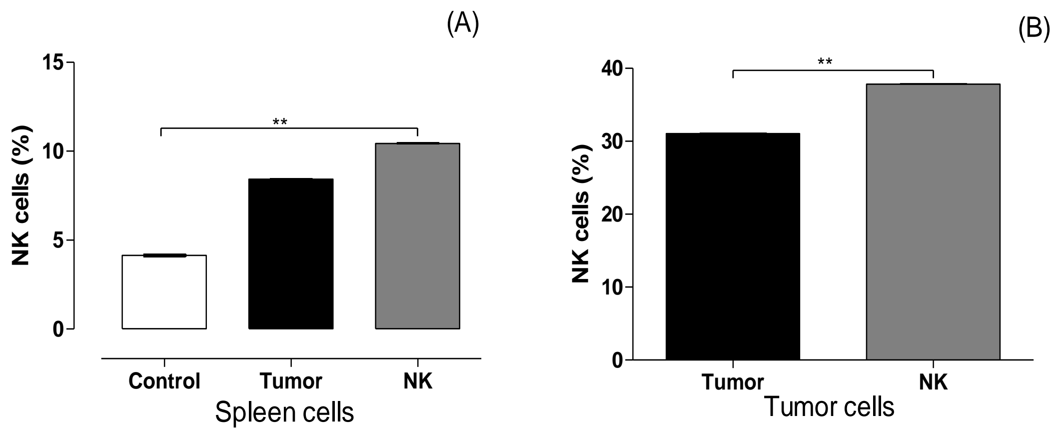


Figure 3: Values obtained for the total percentage of splenic cells and tumor infiltrate of mice from the control group, untreated tumor group and tumor group treated with DC cells. Comparison between 2 groups Mann Whitney t Test. In (A) in the total cell percentage, the NK-treated group has higher NK cell percentage values than the control group. (B) the higher percentage number of cells is observed in the NK cell treated group compared to the untreated tumor group.

The flow cytometry performed for the spleen and tumor infiltrate in **figure 3** shows the percentage of NK cells obtained in each of the samples in the studied groups. An increased splenic NK cell percentage is noted for the TNK group **(fig. 3 (A))**; (p=0.0019) compared to group C, and an increase of these cells in the tumor infiltrate for group TNK compared to group T **(fig. 2 (B))** (p = 0.0079).

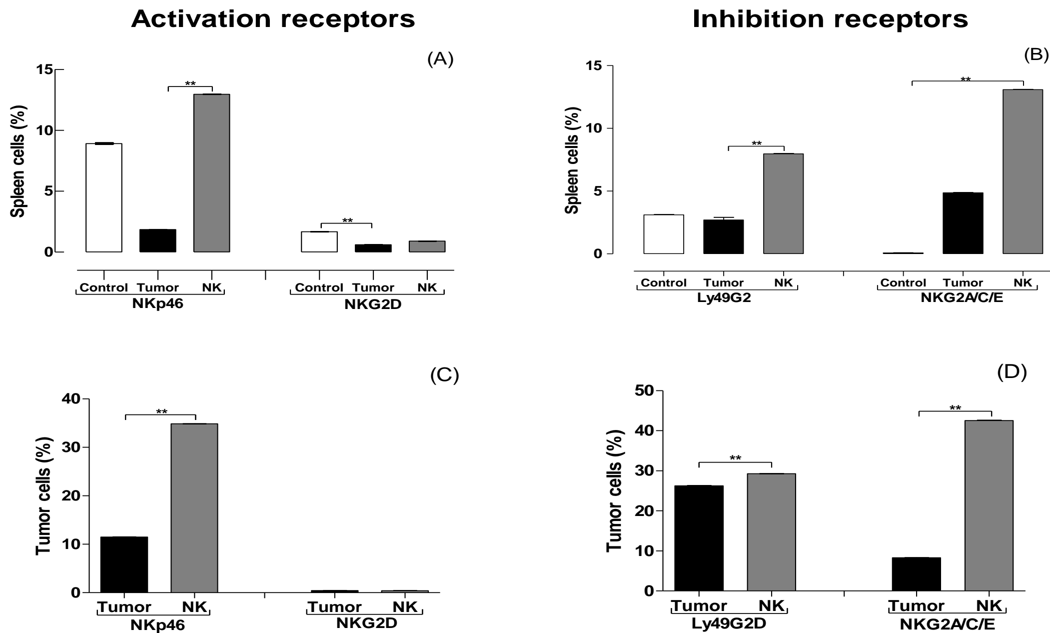


Figure 4: Values obtained for the activation and inhibition receptors present in the splenic cells and tumor infiltrate of the untreated and uninduced control, untreated and NK cell treated mice: For comparisons between groups 1way ANOVA and Mann Whitney t-Test were used. **(A)** Percentage of NK cells for NKp46 and NKG2D receptors obtained from spleen. In **(B)** percentage of NK cells expressing Ly49G2 and NKG2A/C/E receptors obtained from the mouse spleen. **(C)** and **(D)** NK cell percentage for activation and inhibition receptors, respectively, NKp46, NKG2D and Ly49G2, NKG2A/C/E in mouse tumor infiltrate**.**

In **figure 4** the percentage of splenic cells for the activation and inhibition markers studied shows in **(A)** a larger number of cells for the NKp46 activation receptors for the TNK group (p = 0.0019) and for the NKG2D receptor (p = 0.0019) higher cell density for group C. In (B) the groups expressing the largest number of splenic cells for inhibition receptors Ly49G2 and NKG2A/C/E (p = 0.0019) is for those in the TNK group in compared to group C and group T. In graph **(C)** and **(D)** we have a higher percentage of NK cells in the tumor infiltrate expressing the receptors, respectively, NKp46, NKG2D and Ly49G2, NKG2A/C/E (p = 0,0079 and 0,0079) for the TNK group compared to the T group.

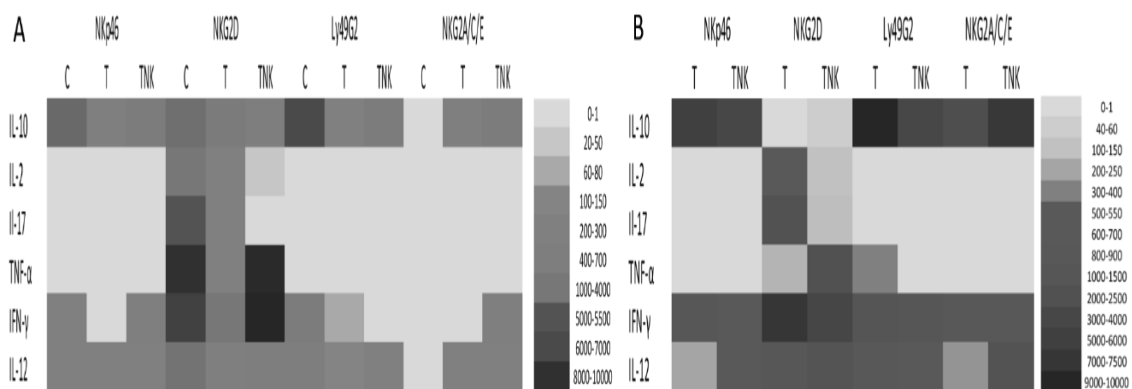


Figure 5: Heat map graph depicting median values for activation and inhibition receptors and cytokine production from spleen and tumor infiltrate samples from untreated control, untreated tumor, and tumor treated with NK: Em **(A)** cells.) MFI values obtained for NKp46, NKG2D, Ly49G2 and NKG2A/C/E receptors producing splenic cell IL-10, IL-2, IL-17, TNF-α, IFN-γ and IL-12) **(B)** MFI values obtained for receptors and cytokine production of tumor infiltrate cells.

In **Figure 5 (A)** the mean fluorescence intensity (MFI) for activation and inhibition receptors interleukin production of splenic and intratumor cells are represented for the groups. In the heat map graph, we have respectively positive cells for activation receptors NKp46 and NKG2D along with the produced cytokines. Regarding the IL-10, IFN-γ and IL-12 producing NKp46 cells, we obtained significance respectively, in the comparisons between the C *vs* T, TNK *vs* T and C *vs* T groups (p=0.0019/0.0019 and 0.0090) and for the cells NKG2D and their respective cytokines, the significant values observed, were for all parameters, IL-10 among the C *vs* TNK, IL-2 C *vs* TNK, IL17 C *vs* TNK, TNF-α C *vs* TNK, IFN-γ T *vs* TNK and IL-12 C *vs* T all p=0.0019. In inhibition receptors and cytokine production we observed for the Ly49G2 receptor significance for IL-10, IFN-γ and IL-12 producing cells respectively in the comparison between the CvsT, C *vs* TNK and C *vs* T groups (p=0.0019). For the IL-10, IFN-γ and IL-12 producing NKG2A/C/E cells, the comparisons, respectively, between the C *vs* TNK, C *vs* TNK / T *vs* TNK and C *vs* TNK groups (p=0.0019/0.0088 and 0.0019).

Next, comparisons between tumor infiltrate cell groups for inhibition receptors and cytokine production, in graph **5 (B)** we show the MFI values for IL-10 producing NKp46 cells (p=0.0079) for group T and IFN-γ producing cells (p=0.0079) the highest intensity was also observed for this same group, as for IL-12 producing NKp46 cells (p=0.0079) higher density for TNK group. For NKG2D IL-10, TNF-α and IL-12 producing NKG2D intratumoral cells (p=0.0079), greater intensity was observed for the TNK group and to produce IL-2, IL-17 and IFN-γ cytokines. γ (p=0.0079) the highest intensity is observed for group T. Results for IL-10, TNF-α, IFN-γ and IL-12 producing tumor infiltrate Ly49G2 cells are significant between groups (p=0.0079) with predominance of higher fluorescence intensity respectively for these cytokines in group T. In the NKG2A/C/E cells producing interleukins 10, IFN-γ and IL-12 results show significant values for interleukins 10 and 12 in the TNK group (p=0.0022 / 0.0079) having higher fluorescence intensity than the T group, and for IFN-γ the T group showed higher fluorescence intensity compared to the TNK group.

4. Discussion

Immunotherapies are very promising in the treatment of cancer, but some limitations are observed when tumor cells exhibit low immunogenicity impairing antigen processing and presentation associated with low expression of distinct tumor-specific antigens, hindering successful immunotherapy.

Our study shows some of the limitations that may help elucidate and understand how NK cell vaccine treatment may not be as effective in eradicating neoplastic cells, given the type of microenvironment involved in immunotherapy.

In order to contribute to a better breast cancer treatment protocol, in this study we have developed the NK cell vaccine that has now become promising for treatment, but major challenges persist, especially regarding triple negative breast cancer (TNBC) (AMES; MURPHY, 2014; PARK et al., 2012).

At "*in vitro*" and "*in vivo*" studies by Shenouda and colleagues found that differentiation of peripheral blood NK cells from healthy, co-cultured breast cancer donors has the ability to expand similarly, but also demonstrated that NK cells of breast cancer donors showed greater cytotoxicity against allogeneic and autologous derived tumor cells in vitro (SHENOUDA et al., 2017).

Our cell differentiation results for making the NK cell vaccine show clear expression of the higher cell density specific receptors for the Ly49G2 and NKp46 receptors and despite a lower density for NKG2A/C/E activity demonstrated by fluorescence intensity is larger for the NKp46 receptor, this condition confers the vaccine produced a potential to recognize tumor cells and eliminate them.

In order to increase the cytotoxicity of cancer-derived NK cells and generate cellular products that can overcome the tumor suppressor microenvironment Shenouda and colleagues also compared the functionality and phenotype of expanded breast cancer donor NK cells to those of donor found that expanded NK cells demonstrate increased expression of CD69 and NKp44 activation receptors compared to peripheral blood cells (PBMCs), with decreased expression of CD11c and CD27 cell maturation markers (SHENOUDA et al., 2017).

As we did not verify the conditions of Shenouda and collaborators we can infer that the cells of cell differentiation in our studies did not show a significant decrease in inhibition receptors, especially Ly49G2, which may suggest the development of checkpoint therapies.

As already described NK cells migrate spontaneously to the tumor microenvironment through chemokine expression, and when infiltrated due to stress generated by the microenvironment these cells may be down-regulated activation receptors and not respond to stimuli generated by the target cell increasing so local immunosuppression (BÖTTCHER et al., 2018).

The results we obtained for spleen and intratumor NK cell density show that NK-treated mice have increased cell accumulation in both tissues, elucidating that the vaccine stimulated the expansion of these cells and greater migration to tumor tissue confirming migratory stimuli by chemokine expression, moreover, these cells may be suffering from down-regulation of activation receptors leading to immunosuppression and non-responsiveness to target cells.

Activation receptors responsible for conferring NK cell responsiveness against target cells may be poorly expressed by NK by poor regulation of expression, for example, of NKp46 and NKG2D, Romero et al. reported in their postulates that patients with acute myeloid leukemia (AML) suffer from interference of mono- and polymorphonuclear phagocytes by negatively regulating the expression of NKp46 and NKG2D cell surface receptor density in NK cells, a phenomenon that occurs by a mechanism. dependent on the availability of reactive oxygen species (ROS) from phagocytes (ROMERO et al., 2006).

However as much we haven't evaluated the functionality of NK cells, in our results, the expression of NKp46 and NKG2D receptors in the spleen and tumor infiltrate was higher in the NK-treated group, however this result did not affirm the effectiveness of NK cells in eliminating tumor cells, since we did not see significant results for tumor regression, suggesting that the influence of other cells on the microenvironment, such as mono- or polymorphonuclear cells, affected responsiveness to target cells.

In addition to activation receptors, which are key in stimulating effector responses in NK cells, we have inhibition receptors, which perform indispensable functions in the performance of their function in immune responses. NK cell activity is mediated by activation receptors such as NKG2D CD94/NKG2 complex receptors (NKG2A, NKG2C, NKHG2E) form heterodimers with CD94 binding to HLA-E and NKG2D forming MHC class I homodimers generating potent cytotoxic response, even in the presence of MHC class I molecules inhibitory (KAISER et al., 2008).

Expecting to achieve a vigorous immune response by NK cells against tumor cells in our findings these receptors were expressed in the spleen and tumor infiltrate of NK-treated mice suggesting, however, that these cells were not activated due to exhaustion generated in the tumor microenvironment, causing the dysfunction of these cells.

This statement meets the findings of Cong and collaborators (CONG et al., 2018) who reported impaired NK cell viability in lung cancer patients during cancer promotion or progression, concluding that NK cells prevent tumor initiation but gradually lose their antitumor function.

Following NK cells to exert their lytic activity, they need to produce cytokines that make them release the contents of their perforin granules and granzymes, or they can alternatively utilize apoptosis mechanisms through TNF, FAS and TRAIL, the major cytokines involved. in NK activity are IL-2, IL-12, IL-15 and IL-18 which induce proliferation and stimulate activity (LIEBERMAN, 2010).

Our results involving the cytokines tested in this study we saw that there was a relatively increased IL-10 production in both spleen and tumor infiltrate tissues. This cytokine when present in immune responses involving NK cells affects the cytotoxicity of these cells, generating an immunosuppressive environment (SZKARADKIEWICZ et al., 2010).

Many treatments using NK cells are studied through previous IL-2 infusion to facilitate the stimulation of these cells. Our study aimed to verify the potential of NK cells alone by generating autologous NKs from pluripotent cells (HPSCs). differentiated in culture and directly reinfused in the treated mice.

Because we failed in tumor regression, and in NK cell culture inhibition receptor expression prevailed in higher density than activation receptors, even though NKp46 showed higher activity.

We observed that the cells, when infused in the treated mice, increased the number of systemic as well as intratumoral NK cells, with a relevant expression for all activation and inhibition receptors researched, may have generated an imbalance involving effector responses by NK cells, thus obtaining A balance in the expression of these receptors in the production of the NK cell vaccine may be an alternative in improving its effectiveness by aiding tumor regression and effectively activating NK cells.

Concluding our findings, we demonstrate with our results an increased expression of the inhibition receptor Ly49G2 on cell differentiation, this seems to stimulate the systemic expansion of these cells and a mass migration of this phenotypic profile to TME, preventing effective NK cells expressing activation receptors from remaining. dormancy contributing to tumor progression, and interleukin 10 production in both tissues contribute to a systemic and intratumoral immunosuppressive profile.

References:

ALEIXO, A. A R.; MICHELIN, M. A; MURTA, E. F. C. Dendritic cell vaccine and cancer treatment: new patents. **Recent patents on endocrine, metabolic & immune drug discovery**, v. 8, n. 1, p. 26–9, 2014.

AMES, E.; MURPHY, W. J. Advantages and clinical applications of natural killer cells in cancer immunotherapy. **Cancer Immunology, Immunotherapy**, v. 63, n. 1, p. 21–28, 2014.

BANCHEREAU, J. et al. Mmunobiology of. **Immunology**, n. 18, p. 767–811, 2000.

BELLATI, F. et al. Past, Present and Future Strategies of Immunotherapy in Gynecological Malignancies. **Current Molecular Medicine**, v. 13, n. 4, p. 648–669, 1 abr. 2013.

BÖTTCHER, J. P. et al. NK Cells Stimulate Recruitment of cDC1 into the Tumor Microenvironment Promoting Cancer Immune Control. **Cell**, v. 172, n. 5, p. 1022-1037.e14, 2018.

CASTRICONI, R. et al. Transforming growth factor β1 inhibits expression of NKP30 and NKG2d receptors: Consequences for the NK-mediated killing of dendritic cells. **Proceedings of the National Academy of Sciences of the United States of America**, v. 100, n. 7, p. 4120–4125, 2003.

CHENG, M. et al. NK cell-based immunotherapy for malignant diseases. **Cellular and Molecular Immunology**, v. 10, n. 3, p. 230–252, 2013.

CHESTER, C.; FRITSCH, K.; KOHRT, H. E. Natural killer cell immunomodulation: Targeting activating, inhibitory, and co-stimulatory receptor signaling for cancer immunotherapy. **Frontiers in Immunology**, v. 6, n. DEC, p. 1–9, 2015.

CLOUGH, J. D. Immune recognition. **Suppressor Cells and Their Factors**, p. 3–20, 2018.

CONG, J. et al. Dysfunction of Natural Killer Cells by FBP1-Induced Inhibition of Glycolysis during Lung Cancer Progression. **Cell Metabolism**, v. 28, n. 2, p. 243- 255.e5,

2018.
COOPER, M. NK cell and DC interactions. **Trends in Immunology**, v. 25, n. 1, p. 47–52, 2004.
DA CUNHA, A.; ANTONIAZI MICHELIN, M.; CÂNDIDO MURTA, E. F. Phenotypic profile of dendritic and T cells in the lymph node of Balb/C mice with breast cancer submitted to dendritic cells immunotherapy. **Immunology Letters**, v. 177, p. 25–37, 2016.
DI VITO, C. et al. NK cells to cure cancer. **Seminars in Immunology**, v. 41, n. March, p. 101272, 2019.
GAO, J. et al. Mechanism of Action of IL-7 and Its Potential Applications and Limitations in Cancer Immunotherapy. **International Journal of Molecular Sciences**, v. 16, n. 5, p. 10267–10280, 2015.
GARCÍA-HERNÁNDEZ, M. L.; TING KOH, Y.; KAST, W. M. Tumor Immune Escape Mechanisms. **Cancer Drug Resistance SE - 31**, p. 577–602, 2006.
GUILLEREY, C.; HUNTINGTON, N. D.; SMYTH, M. J. Targeting natural killer cells in cancer immunotherapy. **Nature Immunology**, v. 17, n. 9, p. 1025–1036, 2016.
HABIF, G. et al. Targeting natural killer cells in solid tumors. **Cellular and Molecular Immunology**, v. 16, n. 5, p. 415–422, 2019.
HANAHAN, D.; WEINBERG, R. A. The Hallmarks of Cancer. **Cell**, v. 100, n. 3, p. 57–70, 2000.
HU, W. et al. Cancer immunotherapy based on natural killer cells: Current progress and new opportunities. **Frontiers in Immunology**, v. 10, n. MAY, p. 1–16, 2019.
INCA. **Estimativa 2018, Incidência de câncer no Brasil**. [s.l: s.n.].
INTERNATIONAL AGENCY FOR RESEARCH ON CANCER (IARC). **Latest global cancer data, 2018World Health Organization**. [s.l: s.n.]. Disponível em: <http://www.who.int/cancer/PRGlobocanFinal.pdf>.
JAMES, A. M.; COHEN, A. D.; CAMPBELL, K. S. Combination immune therapies to enhance anti-tumor responses by NK cells. **Frontiers in Immunology**, v. 4, n. DEC, p. 1–12, 2013.
JESPERSEN, H. et al. Clinical responses to adoptive T-cell transfer can be modeled in an autologous immune-humanized mouse model. **Nature Communications**, v. 8, n. 1, 2017.
KAISER, B. K. et al. Structural basis for NKG2A/CD94 recognition of HLA-E. **Proceedings of the National Academy of Sciences of the United States of America**, v.

105, n. 18, p. 6696–6701, 2008.
KIESSLING, R. Natural Killer Cells in the Mouse. **The Role of Products of the Histocompatibility Gene Complex in Immune Responses**, p. 77–85, 1976.
KIM, R. Cancer Immunoediting: From Immune Surveillance to Immune Escape. **Cancer Immunotherapy**, p. 9–27, 2007.
LANIER, L. L. Nk Cell Receptors. 1998.
LESTERHUIS, W. J.; HAANEN, J. B. A G.; PUNT, C. J. A. Cancer immunotherapy--revisited. **Nature reviews. Drug discovery**, v. 10, n. 8, p. 591–600, 2011.
LIEBERMAN, J. Anatomy of a murder: How cytotoxic T cells and NK cells are activated, develop, and eliminate their targets. **Immunological Reviews**, v. 235, n. 1, p. 5–9, 2010.
LUEVANO, M.; MADRIGAL, A.; SAUDEMONT, A. Generation of natural killer cells from hematopoietic stem cells in vitro for immunotherapy. **Cellular and Molecular Immunology**, v. 9, n. 4, p. 310–320, 2012.
MAILLIARD, R. B. et al. Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for the induction of NK cell helper function. **Journal of immunology (Baltimore, Md. : 1950)**, v. 171, n. 5, p. 2366–2373, 2003.
MARCENARO, E. et al. IL-12 or IL-4 Prime Human NK Cells to Mediate Functionally Divergent Interactions with Dendritic Cells or Tumors. **The Journal of Immunology**, v. 174, n. 7, p. 3992–3998, 2005.
MARTÍN-FONTECHA, A. et al. Induced recruitment of NK cells to lymph nodes provides IFN-γ for TH1 priming. **Nature Immunology**, v. 5, n. 12, p. 1260–1265, 2004.
MATIAS, B. F. et al. Influence of immunotherapy with autologous dendritic cells on innate and adaptive immune response in cancer. **Clinical Medicine Insights: Oncology**, v. 7, p. 165–172, 2013.
MELERO, I. et al. T-cell and NK-cell infiltration into solid tumors: A key limiting factor for efficacious cancer immunotherapy. **Cancer Discovery**, v. 4, n. 5, p. 522–526, 2014.
MULLER, N. et al. Engineering NK Cells Modified With an EGFRvIII-specific Chimeric Antigen Receptor to Overexpress CXCR4 Improves Immunotherapy of CXCL12/SDF-1alpha-secreting Glioblastoma. **J Immunother**, v. 38, n. 5, p. 197–210, 2015.
NELSON, R. P.; BALLOW, M. Immunomodulation and immunotherapy: drugs, cytokines, cytokine receptors, and antibodies. **The Journal of allergy and clinical immunology**, v. 111, n. 2 Suppl, p. S720–S743, 2003.
OISETH, S. J.; AZIZ, M. S. Cancer immunotherapy: a brief review of the history, possibilities, and challenges ahead. **Journal of Cancer Metastasis and Treatment**, v. 3,

n. 10, p. 250, 2017.

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

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

PARK, S. et al. Characteristics and outcomes according to molecular subtypes of breast cancer as classified by a panel of four biomarkers using immunohistochemistry. **Breast**, v. 21, n. 1, p. 50–57, 2012.

PAUL, S.; LAL, G. The molecular mechanism of natural killer cells function and its importance in cancer immunotherapy. **Frontiers in Immunology**, v. 8, n. SEP, 2017.

PEREZ-MARTINEZ, A. et al. Blood dendritic cells suppress NK cell function and increase the risk of leukemia relapse after hematopoietic cell transplantation. **Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation**, v. 17, n. 5, p. 598–607, 2011.

POPP, M. W.; MAQUAT, L. E. Attenuation of nonsense-mediated mRNA decay facilitates the response to chemotherapeutics. **Nature Communications**, v. 6, 2015.

QUAIL, D. F.; JOYCE, J. A. Microenvironmental regulation of tumor progression and metastasis. **Nature medicine**, v. 19, n. 11, p. 1423–37, 2013.

RODRIGUES, C. M. et al. The role of T lymphocytes in cancer patients undergoing immunotherapy with autologous dendritic cells. **Clinical Medicine Insights: Oncology**, v. 5, p. 107–115, 2011.

ROMERO, A. I. et al. NKp46 and NKG2D receptor expression in NK cells with CD56dim and CD56bright phenotype: Regulation by histamine and reactive oxygen species. **British Journal of Haematology**, v. 132, n. 1, p. 91–98, 2006.

ROSENBERG, S. A. Entering the mainstream of cancer treatment. **Nature Reviews Clinical Oncology**, v. 11, n. 11, p. 630–632, 2014.

SCOTT, A. M.; ALLISON, J. P.; WOLCHOK, J. D. **Monoclonal antibodies in cancer therapy.Cancer immunity**, 2012. Disponível em: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=6366145>

SEREN BERNARDONE, I. Role of NK cells and adaptive immunity in “immunoediting”: Recent developments. **Inmunología**, v. 27, n. 3, p. 141–146, 2008.

SHENOUDA, M. M. et al. Ex vivo expanded natural killer cells from breast cancer patients and healthy donors are highly cytotoxic against breast cancer cell lines and

patient-derived tumours. **Breast Cancer Research**, v. 19, n. 1, 2017.
SUEN, W. C. W. et al. Natural Killer Cell-Based Cancer Immunotherapy: A Review on 10 Years Completed Clinical Trials. **Cancer Investigation**, v. 36, n. 8, p. 431–457, 2018.
SZKARADKIEWICZ, A. et al. Natural killer cell cytotoxicity and immunosuppressive cytokines (IL-10, TGF-β1) in patients with gastric cancer. **Journal of Biomedicine and Biotechnology**, v. 2010, 2010.
WORLD HEALTH ORGANISATION. Global cancer data. **International Agency for Research on cancer**, n. September, p. 13–15, 2018.
ZHOU, J. Advances and Prospects in Cancer Immunotherapy. **New Journal of Science**, v. 2014, p. 1–13, 2014.

Comentários

5- Comentários:

Em busca de tratamentos para auxiliar o combate ao câncer nossos estudos visam estimular o sistema imune do indivíduo minimizando o desconforto ou efeitos colaterais causados pela quimioterapia ou radioterapia. Atualmente, estes esforços têm se tornado muito explorado em todo o mundo e a busca para atingir o padrão ideal no tratamento necessita de um conhecimento abrangente quanto aos mecanismos utilizados no estímulo das células imunes e vias de sinalização envolvidos. Na imunoterapia com a vacina de células DCs, as células NK junto a produção de citocinas, cooperam de forma satisfatória para rejeição e eliminação tumoral. Por outro lado, na imunoterapia com células NK a expressão de receptores de inibição em maior densidade na diferenciação celular sugere uma estimulação sistêmica deste perfil celular com migração evidente no infiltrado tumoral impedindo que células NK expressando os receptores de ativação reconheçam as células neoplásicas e produzam citocinas auxiliando outras células do sistema imune, para tanto, a contribuição deixada aqui traz abordagens ou formas de direcionar protocolos de tratamento eficazes contra o câncer. Aprofundar o estudo envolvendo a diferenciação de células NK a partir da medula no tratamento de tumores sólidos necessitam de maior atenção, uma vez que, o potencial destas células no combate às células neoplásicas tornara promissor, uma atenção especial para o desenvolvimento de células efetivas com baixa expressão dos receptores Ly49G2 seria um ponto chave a ser estudado.

Conclusões

6- Conclusões:

Concluindo os nossos estudos vimos que a diferenciação das células HPSCs em células NK é possível com a utilização de citocinas específicas como M-CSF, IL-2, IL-15 e IL-18 associado ao tempo de maturação de 21 dias. Na avaliação do tratamento com a vacina de células DCs, observamos a capacidade daquelas células alterar o perfil das células NK influenciando na resposta antitumoral, reduzindo a expressão dos receptores de inibição e aumentando a produção de TNF-α e IFN-γ ativando outras células do sistema imune caracterizando que há uma cooperação entre elas. O tratamento com a vacina de células NK em nossas avaliações observamos que existe uma deficiência em gerar respostas vigorosas, uma vez que obtivemos uma expressão evidente dos receptores de inibição, em específico Ly49G2, onde este pode ter contribuído para uma expansão destas células sistemicamente e maior migração para o TME impedindo a ativação efetiva das células NK.

7- Referências:

Banchereau J, Banchereau J, Briere F, et al (2000) Imunobiology of Immunology 767–811. doi: 10.1146/annurev.immunol.18.1.767

Bellati F, Napoletano C, Ruscito I, et al (2013) Past, Present and Future Strategies of Immunotherapy in Gynecological Malignancies. Curr Mol Med 13:648–669. doi: 10.2174/1566524011313040014

Cheng M, Chen Y, Xiao W, et al (2013) NK cell-based immunotherapy for malignant diseases. Cell Mol Immunol 10:230–252. doi: 10.1038/cmi.2013.10

Chester C, Fritsch K, Kohrt HE (2015) Natural killer cell immunomodulation: Targeting activating, inhibitory, and co-stimulatory receptor signaling for cancer immunotherapy. Front Immunol 6:1–9. doi: 10.3389/fimmu.2015.00601

Clough JD (2018) Immune recognition. Suppressor Cells and Their Factors 3–20. doi: 10.1201/9781351077026

Cooper M (2004) NK cell and DC interactions. Trends Immunol 25:47–52. doi: 10.1016/j.it.2003.10.012

da Cunha A, Antoniazi Michelin M, Cândido Murta EF (2016) Phenotypic profile of dendritic and T cells in the lymph node of Balb/C mice with breast cancer submitted to dendritic cells immunotherapy. Immunol Lett 177:25–37. doi: 10.1016/j.imlet.2016.07.009

Gao J, Zhao L, Wan Y, Zhu B (2015) Mechanism of Action of IL-7 and Its Potential Applications and Limitations in Cancer Immunotherapy. Int J Mol Sci 16:10267–10280. doi: 10.3390/ijms160510267

García-Hernández ML, Ting Koh Y, Kast WM (2006) Tumor Immune Escape Mechanisms. Cancer Drug Resist SE - 31 577–602. doi: 10.1007/978-1-59745-035-5_31

Guillerey C, Huntington ND, Smyth MJ (2016) Targeting natural killer cells in cancer immunotherapy. Nat Immunol 17:1025–1036. doi: 10.1038/ni.3518

Hanahan D, Weinberg RA (2000) The Hallmarks of Cancer. Cell 100:57–70. doi: 10.1016/S0092-8674(00)81683-9

Hu W, Wang G, Huang D, et al (2019) Cancer immunotherapy based on natural killer cells: Current progress and new opportunities. Front Immunol 10:1–16. doi: 10.3389/fimmu.2019.01205

INCA (2018) Estimativa 2018, Incidência de câncer no Brasil

International Agency for Research on Cancer (IARC) (2018) Latest global cancer data, 2018

James AM, Cohen AD, Campbell KS (2013) Combination immune therapies to enhance anti-tumor responses by NK cells. Front Immunol 4:1–12. doi: 10.3389/fimmu.2013.00481

Kim R (2007) Cancer Immunoediting: From Immune Surveillance to Immune Escape. Cancer Immunother 9–27. doi: 10.1016/B978-012372551-6/50066-3

Lanier LL (1998) Nk Cell Receptors

Lesterhuis WJ, Haanen JB a G, Punt CJ a (2011) Cancer immunotherapy--revisited. Nat Rev Drug Discov 10:591–600. doi: 10.1038/nrd3500

Mailliard RB, Son Y-I, Redlinger R, et al (2003) Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for the induction of NK cell helper function. J Immunol 171:2366–2373. doi: 10.4049/jimmunol.171.5.2366

Martín-Fontecha A, Thomsen LL, Brett S, et al (2004) Induced recruitment of NK cells to lymph nodes provides IFN-γ for TH1 priming. Nat Immunol 5:1260–1265. doi: 10.1038/ni1138

Muller N, Michen S, Tietze S, et al (2015) Engineering NK Cells Modified With an EGFRvIII-specific Chimeric Antigen Receptor to Overexpress CXCR4 Improves Immunotherapy of CXCL12/SDF-1alpha-secreting Glioblastoma. J Immunother 38:197–210. doi: 10.1097/CJI.0000000000000082

Nelson RP, Ballow M (2003) Immunomodulation and immunotherapy: drugs, cytokines, cytokine receptors, and antibodies. J Allergy Clin Immunol 111:S720–S743. doi: 10.1067/mai.2003.146

Oiseth SJ, Aziz MS (2017) Cancer immunotherapy: a brief review of the history, possibilities, and challenges ahead. J Cancer Metastasis Treat 3:250. doi: 10.20517/2394-4722.2017.41

Palucka K, Banchereau J (2012) Cancer immunotherapy via dendritic cells. Nat Rev Cancer 12:265–277. doi: 10.1038/nrc3258

Palucka K, Banchereau J (2013) Dendritic-Cell-Based Therapeutic Cancer Vaccines. Immunity 39:38–48. doi: 10.1016/j.immuni.2013.07.004

Paul S, Lal G (2017) The molecular mechanism of natural killer cells function and its importance in cancer immunotherapy. Front Immunol 8:. doi: 10.3389/fimmu.2017.01124

Perez-Martinez A, Iyengar R, Gan K, et al (2011) Blood dendritic cells suppress NK cell function and increase the risk of leukemia relapse after hematopoietic cell transplantation. Biol Blood Marrow Transplant 17:598–607. doi: 10.1016/j.bbmt.2010.10.019

Quail DF, Joyce J a (2013) Microenvironmental regulation of tumor progression and metastasis. Nat Med 19:1423–37. doi: 10.1038/nm.3394

Rodrigues CM, Matias BF, Murta EFC, Michelin MA (2011) The role of T lymphocytes in cancer patients undergoing immunotherapy with autologous dendritic cells. Clin Med Insights Oncol 5:107–115. doi: 10.4137/CMO.S6927

Rosenberg SA (2014) Entering the mainstream of cancer treatment. Nat Rev Clin Oncol 11:630–632. doi: 10.1038/nrclinonc.2014.174

Scott AM, Allison JP, Wolchok JD (2012) Monoclonal antibodies in cancer therapy. Cancer Immun. 12:8

Seren Bernardone I (2008) Role of NK cells and adaptive immunity in “immunoediting”: Recent developments. Inmunología 27:141–146. doi: 10.1016/S0213-9626(08)70062-3

World Health Organisation (2018) Global cancer data. Int Agency Res cancer 13–15

Zhou J (2014) Advances and Prospects in Cancer Immunotherapy. New J Sci 2014:1–13. doi: 10.1155/2014/745808

Aleixo A a R, Michelin M a, Murta EFC (2014) Dendritic cell vaccine and cancer treatment: new patents. Recent Pat Endocr Metab Immune Drug Discov 8:26–9

Matias BF, de Oliveira TM, Rodrigues CM, et al (2013) Influence of immunotherapy with autologous dendritic cells on innate and adaptive immune response in cancer. Clin Med Insights Oncol 7:165–172. doi: 10.4137/CMO.S12268

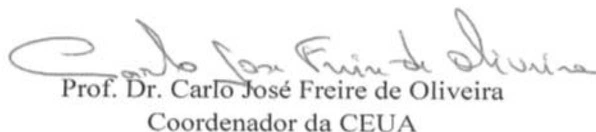
Anexo 1

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 das células natural killer (NK) na imunoterapia combinada entre as vacinas de células dendríticas e células NK em camundongos com tumor de mama experimental induzidos por 4T1", registrada com o nº **378**, 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	80 (30 machos e 50 fêmeas)
Peso/idade	20 à 30g/ 8 à 12 semanas (machos) 20 à 30g/ 8 semanas (fêmeas)
Gênero	Machos e fêmeas
Origem	Biotério Setorial do Instituto de Pesquisa em Oncologia - UFTM



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