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JOYCE ALVES DOS SANTOS

# ANÁLISE DA TRANSFERÊNCIA CELULAR ADOTIVA DE MACRÓFAGOS ESTIMULADOS COM BCG NO CONTROLE DO CÂNCER DE MAMA TRIPLO-NEGATIVO EM MODELO MURINO

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas pela Universidade Federal de Alfenas. Área de concentração: Interação patógeno-hospedeiro.

Orientadora: Profa. Dra. Patrícia Paiva Corsetti de Almeida

Coorientador: Prof. Dr. Leonardo Augusto de Almeida

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A Banca examinadora abaixo-assinada aprova a Dissertação/Tese apresentada como parte dos requisitos para a obtenção do título de Mestra em Ciências Biológicas pela Universidade Federal de Alfenas. Área de concentração: Interação Patógeno-Hospedeiro.

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Profa. Dra Patricia Paiva Corsetti de Almeida
Instituição: Universidade Federal de Alfenas - UNIFAL-MG

Profa. Dra. Thaís Cristina Ferreira dos Santos
Instituição: Centro Nacional de Pesquisa em Energia e Materiais - CNPEM

Profa. Dra. Ana Carolina Silveira Rabelo
Instituição: Universidade Federal de Alfenas - UNIFAL-MG

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## RESUMO

O câncer de mama é uma das principais causas de morte entre mulheres no Brasil e no mundo. Entre seus subtipos, o câncer de mama triplo-negativo (TNBC) tem o pior prognóstico, por ser o mais agressivo e não apresentar três principais receptores (estrogênio, progesterona e HER2) que são os alvos de terapias específicas para o câncer de mama. No microambiente tumoral, há infiltração de células imunes, principalmente macrófagos que desempenham papéis importantes no combate tumoral. Os macrófagos podem ser induzidos a um estado anti-inflamatório pró-tumoral (M2), enquanto um fenótipo pró-inflamatório (M1) seria mais importante para o controle do tumor. A BCG (Bacilo Calmette-Guérin) é a única vacina que protege contra tuberculose em humanos e vem sendo estudada no tratamento de alguns tipos de cânceres. O objetivo desse trabalho foi avaliar o efeito do BCG na estimulação de macrófagos derivados da medula óssea (BMDMs) bem como o efeito *in vivo* utilizando BMDMs estimulados pelo BCG (BMDM+BCG) no tratamento do câncer de mama triplo negativo (células 4T1). Para isso, BMDMs derivados de camundongos fêmeas da linhagem BALB/c, foram estimulados com BCG ou seus controles e seus sobrenadantes foram coletados para análises de consumo de glicose, produção de lactato, NO e citocinas IL-10 e TNF-α. Ensaio de migração celular foi realizado para análise do efeito do meio condicionado sob as células 4T1. O estímulo com BCG em BMDMs levou ao aumento do consumo de glicose, produção de lactato, óxido nítrico e TNF-α além de impedir parcialmente a migração de células tumorais 4T1. Após análises, os BMDMs estimulados com BCG ou seus controles foram utilizados como tratamento do câncer de mama triplo negativo pela transferência celular adotiva *in vivo*. Foi realizada a indução do tumor de mama triplo negativo (células 4T1) em camundongos BALB/c e no dia 11 os animais foram tratados em dose única com injeção subcutânea no mesmo local da indução do tumor com: PBS, BMDMs+BCG, apenas BMDMs, BCG ou quimioterápico 5FU (5-Fluorouracil). O tratamento com BMDM+BCG diminuiu a perda de peso, melhorou o consumo de ração dos animais e diminuiu o volume tumoral. Além disso, a análise histopatológica da pele mamária, demonstrou que o tratamento BMDM+BCG levou a redução significativa no acúmulo de células tumorais, semelhante ao encontrado no grupo 5FU, porém, apresentou maior infiltração inflamatória no fígado e pulmão,

caracterizando uma resposta inflamatória mais robusta, que apesar de ser eficaz no combate as células cancerosas, pode gerar maior dano tecidual e comprometimento funcional dos órgãos. O tratamento apenas com BCG apresentou menor eficácia na diminuição do volume tumoral em relação ao tratamento com BMDM+BCG e demonstrou menor infiltração inflamatória nos tecidos. É possível concluir que o tratamento com BMDM estimulado com BCG apresenta eficácia no combate ao crescimento tumoral. Contudo, a resposta inflamatória robusta pode prejudicar a integridade dos órgãos, sendo necessários mais estudos para avaliar o tratamento a longo prazo, além de estratégias no controle da resposta inflamatória. Esta abordagem abre caminhos para terapias futuras contra o câncer de mama triplo-negativo.

**Palavras-chave**: BMDM; Células 4T1; Imunoterapia; Resposta imunológica.

## ABSTRACT

Breast cancer is one of the leading causes of death among women in Brazil and worldwide. Among its subtypes, triple-negative breast cancer (TNBC) has the worst prognosis, as it is the most aggressive and lacks the three main receptors (estrogen, progesterone, and HER2), which are the targets of specific breast cancer therapies. In the tumor microenvironment, there is an infiltration of immune cells, primarily macrophages, which play important roles in tumor combat. Macrophages can be induced into a pro-tumoral anti-inflammatory state (M2), whereas a pro-inflammatory phenotype (M1) would be more important for tumor control. BCG (Bacillus Calmette-Guérin) is the only vaccine that protects against tuberculosis in humans and has been studied for the treatment of certain types of cancer. The objective of this study was to evaluate the effect of BCG on the stimulation of bone marrow-derived macrophages (BMDMs) as well as the in vivo effect of using BCG-stimulated BMDMs (BMDM+BCG) in the treatment of triple-negative breast cancer (4T1 cells). For this purpose, BMDMs derived from female BALB/c mice were stimulated with BCG or their respective controls, and their supernatants were collected for analyses of glucose consumption, lactate production, NO, and cytokines IL-10 and TNF-α. A cell migration assay was performed to analyze the effect of the conditioned medium on 4T1 cells. BCG stimulation in BMDMs led to increased glucose consumption, lactate production, nitric oxide, and TNF-α, in addition to partially inhibiting the migration of 4T1 tumor cells. Following these analyses, BCG-stimulated BMDMs or their respective controls were used as a treatment for triple-negative breast cancer through in vivo adoptive cell transfer. TNBC (4T1) tumors were induced in BALB/c mice, and on day 11, the animals were treated with a single subcutaneous injection at the same tumor induction site with PBS, BMDMs+BCG, unstimulated BMDMs, BCG alone, or the chemotherapeutic agent 5FU (5-Fluorouracil). Treatment with BMDM+BCG reduced weight loss, improved food consumption in animals, and decreased tumor volume. Additionally, histopathological analysis of mammary skin tissue demonstrated that BMDM+BCG treatment significantly reduced the accumulation of tumor cells, similar to the findings in the 5FU group. However, it resulted in greater inflammatory infiltration in the liver and lungs, characterizing a more robust inflammatory response, which, despite being effective in combating cancer cells, may cause greater tissue damage and functional impairment of organs.

Treatment with BCG alone demonstred lower redution of tumor volume and lower inflammatory infiltration in tissues. It is possible to conclude that treatment with BCG-stimulated BMDMs is effective in combating tumor growth. However, due to the robust inflammatory response, it may compromise organ integrity, requiring further studies to evaluate long-term treatment and strategies to control the inflammatory response to maintain organ integrity. This approach paves the way for future therapies against triple-negative breast cancer.

**Keywords:** BMBM; 4T1 Cells; Immunotherapy; Immunology response.

## LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| 5FU | 5-Fluorouracil |
| ADC | Anticorpo monoclonal |
| ATP | Trifosfato de adenosina |
| BCG | Bacilo *Calmette-Guérin* |
| BMDM | Macrófagos derivados da medula óssea |
| CAR-T | Receptor de antígeno quimérico de células T |
| DC | Células dendríticas |
| IFN-ɣ | Interferon gama |
| IL-10 | Interleucina-10 |
| IL-1β | Interleucina-1beta |
| IL-6 | Interleucina-6 |
| iNOS | Óxido nítrico sintase induzível |
| MHC | Complexo principal de histocompatibilidade |
| NO | Óxido nítrico |
| PD-L1 | Ligante de morte programada 1 |
| RE | Receptor de estrogênio |
| RP | Receptor de progesterona |
| TAM | Macrófagos associados ao tumor |
| TME | Microambiente tumoral |
| TNBC | Câncer de mama Triplo negativo |
| TNF-α | Fator de necrose tumoral alfa |
| M1 | Macrófagos com fenótipo pró-inflamatório |
| M2 | Macrófagos com fenótipo anti-inflamatório |

**SUMÁRIO**

## 1 INTRODUÇÃO GERAL

O câncer de mama foi a segunda neoplasia com maior incidência em mulheres no Brasil em 2022, (atrás apenas do câncer de pele não melanoma) com 74 mil novos casos previstos por ano até 2025 (INCA, 2023). Dentre os seus subtipos, o câncer de mama triplo negativo (TNBC) está associado a um pior prognóstico sendo caracterizado pela ausência da expressão de receptores de estrogênio (RE), receptor de progesterona (RP) e receptor 2 do fator de crescimento epidérmico humano (HER2) (Yang *et al*., 2023).

Devido à ausência dos receptores que normalmente são utilizadas como parâmetro para definição do tipo de terapia para o câncer de mama, além da cirurgia e radioterapia, a quimioterapia segue como tratamento padrão para o TNBC. No entanto, o tratamento intravenoso pode levar a toxicidade medicamentosa apresentando limitação na solubilidade e absorção nas áreas cancerosas, afetando a taxa de sobrevivência após o tratamento (Wang *et al*.,2024).

Nos últimos anos, novas estratégias foram investigadas para o tratamento de pacientes com TNBC, entre eles a imunoterapia (Popovic *et al.,*2023). O objetivo das imunoterapias consiste na manipulação do sistema imune para a eliminação das células malignas, mantendo a integridade funcional das células normais (Zhang *et al.,* 2020). Dentre as imunoterapias aprovadas para o TNBC, existe a utilização de alguns tipos de anticorpos como os anticorpos monoclonais Pembrolizumabe (Keytruda®) e Atezolizumabe (Tecentriq®) ambos apresentam como alvo o receptor PD-L1 (ligante de morte programada 1) e o conjugado anticorpo-droga chamado sacituzumab govitecan (SG) direcionado ao Trop2 (antígeno de superfície celular de trofoblasto 2) (Trodelvy®) para pacientes com doença avançada submetidos a quimioterapia (Kaboli *et al*., 2024).

O maior desafio para o sucesso das imunoterapias é a capacidade de infiltrar no microambiente tumoral e superar a resistência precoce que, de acordo com os estudos, está relacionada a modulação desse ambiente promovendo a evasão das células tumorais a ação do sistema imune, facilitando a progressão tumoral (Mediratta *et al.,*2020).

Entre as células do sistema imune inato infiltrantes no microambiente tumoral, os macrófagos são uma das populações que exercem importantes funções, gerando

formas distintas de ação. Os macrófagos chamados de “M1” ou pró-inflamatórios são caracterizados pela ativação clássica, já os macrófagos “M2” ou anti-inflamatórios, são ativados alternativamente. Estudos vem demostrando que macrófagos M1 apresentam uma ativação mais eficiente para combater as células tumorais comparado ao perfil M2 (Mantovani *et al.,*2017).

Considerando que a imunidade celular é crucial na erradicação das células tumorais, principalmente em tumores sólidos, estudos têm sido desenvolvidos na utilização de células imunes como tratamentos imunoterápicos. Como exemplo, as células modificadas através de técnicas de engenharia, entre elas o CAR-T (receptor de antígeno quimérico) que utiliza as células T, CAR-M utilizando macrófagos, além de manipulação de células NK e células dendríticas (DC) (Mishra; Malonia, 2023).

Um grande sucesso no campo da imunoterapia contra os tumores sólidos foi a introdução da administração de bactérias como *Mycobacterium bovis* (bacilo Calmette Guerín) - BCG de forma intravesical para o tratamento do câncer de bexiga em humanos (Morales *et al.,* 1976). Esta terapia é considerada até hoje como o tratamento de escolha para este tipo de câncer. A BCG permanece como uma das vacinas mais conhecidas que tem efeito e proteção benéfica associada a outras doenças, entre elas, o câncer (O’Neill; Netea, 2020). As micobactérias já são descritas por induzir a produção de citocinas pró-inflamatórias, pela ativação dos macrófagos, células dendríticas e células epiteliais via inflamassoma - NLRP3, levando a ativação das células da imunidade adaptativa (Jallad *et al*.,2014; Koti *et al*., 2017).

A proteção causada pela vacina BCG para aumentar a imunidade inata e unir respostas imunes adaptativas enfatiza a necessidade de mais estudos sobre o uso de terapias baseadas em microrganismos para o tratamento do câncer (Koti *et al*., 2020).

Muitos estudos têm focado na utilização dos anticorpos no tratamento do TNBC, porém pesquisas utilizando a bactéria BCG como uma forma de treinamento das células imunes inatas para o combate ao câncer de mama, ainda são limitadas. Pensando nesse contexto, o presente estudo objetivou utilizar a bactéria BCG para estimular macrófagos derivados da medula óssea murina a fim de verificar seu efeito em um modelo de terapia celular adotiva em tumores de mama triplo negativo.

# 2 REVISÃO DE LITERATURA

## 2.1 CÂNCER DE MAMA TRIPLO NEGATIVO E O MICROAMBIENTE TUMORAL

O câncer é definido como um conjunto de mais de 100 doenças que tem em comum o crescimento desordenado levando a formação de um aglomerado de células cancerosas que formam uma massa (tumor) que tem a capacidade de invadir tecidos e órgãos (INCA, 2022).

Entre os seus subtipos, o câncer de mama é a doença mais comum entre as mulheres em todo o mundo. Em 2020 ultrapassou o câncer de pulmão com a maior incidência global de câncer, apresentando uma estimativa de 2,3 milhões de novos casos (Sung *et al*.,2021). Para o Brasil, no ano de 2023, o risco estimado é de 66,54 casos a cada 100 mil mulheres, além de ocupar a primeira posição em mortalidade por câncer entre as mulheres (INCA, 2023).

Sendo uma doença heterogênea, o câncer de mama pode ser classificado em relação a presença ou ausência de três receptores: o receptor de estrogênio (ER), receptor de progesterona (PR) e receptor do fator de crescimento epidérmico humano-2+ (HER-2+). O câncer de mama triplo-negativo (TNBC) é assim classificado por não apresentar expressão de todos os receptores, ER, PR e HER-2, apresentando uma característica de desenvolvimento independente desses fatores (Yang P. *et al*., 2023).

O câncer de mama triplo negativo (TNBC) é uma doença frequentemente associada a um mau prognóstico em comparação com os outros subtipos, sendo responsável por aproximadamente 15% dos cânceres de mama diagnosticados, além de ser o subtipo com maior taxa de reincidência, menor sobrevida, maior taxa de crescimento e metástase (Howard; Olopade, 2021). Pacientes mais jovens ou que apresentam mutações da linhagem germinativa do câncer de mama são mais acometidas por esse subtipo (Kumar *et al*., 2023).

A progressão do câncer normalmente é relacionada a alterações genéticas e epigenéticas nas células cancerosas, porém estudos recentes têm demonstrado que o microambiente tumoral também é importante para determinar o comportamento do tumor (Kim; Bae, 2016).

O microambiente tumoral (TME) se refere ao ambiente onde ocorre a

iniciação, progressão e invasão celular. É constituído por células não imunes, como fibroblastos, adipócitos, células musculares, endoteliais e vasculares, além de células imunes como macrófagos, células NKs (assassinas naturais) e linfócitos T, sendo regulados por múltiplas vias de sinalização (Baghban *et al*.,2020).

O metabolismo celular no TME também influencia na formação do tumor. A primeira alteração específica do câncer identificada foi o fenômeno Warburg que consiste no aumento da glicólise anaeróbica mesmo na presença de oxigênio, resultando na produção aumentada de lactato (Beckers *et al*., 2007). O lactato produzido pelas células cancerosas condiciona seu ambiente reduzindo o pH e favorecendo a evasão na atuação do sistema imune (Koukourakis *et al.,* 2006). Além disso, os intermediários que são produzidos por essa via, são utilizados como vias secundárias para fornecer aminoácidos necessários para sustentar a proliferação celular (Ghergurovic *et al.,* 2021).

De acordo com a modulação da resposta imune, o microambiente tumoral pode ser classificado como imunossupressor ou imunorreativo, por meio da proporção de células imunes, liberação de citocinas e composição estromal. Estes fatores contribuem para a classificação de diferentes subtipos do TNBC (Gruosso *et al.,*2019).

Um grupo de estudos conseguiu identificar três subtipos de TNBC, sendo eles denominados de imunidade alta (H), imunidade média (M) e imunidade baixa (L) com base no perfil imunogenômico. O subtipo imunidade alta (H) apresentou maior infiltração de células imunológicas associado a um prognóstico mais favorável em relação aos outros grupos, o que demonstrou a importante relação entre as células imunes do microambiente tumoral e a classificação de TNBC (He *et al*., 2018).

O tratamento atual padrão do TNBC é a quimioterapia não específica, devido ao fato desse subtipo não apresentar receptores que normalmente são alvos de terapias específicas. Apesar da quimioterapia apresentar progressos, poucos pacientes atingem uma resposta completa, constituindo menos de 30%, com remissão de apenas 2 a 3 meses. Além disso, observa-se alta frequência de quimioresistência que limita a eficácia da quimioterapia convencional, particularmente em estágios avançados de TNBC (Li *et al*.,2022). Portanto, é necessário o desenvolvimento de novas terapias para os pacientes que não respondem aos tratamentos tradicionais.

Para estudar o câncer de mama, é possível utilizar linhagens em modelos murinos como a linhagem triplo-negativa 4T1 que são originárias do tumor de glândulas mamárias de camundongos da linhagem BALB/c. O carcinoma mamário murino 4T1 é uma linhagem celular tumoral muito utilizada em estudos por serem semelhantes ao câncer de mama triplo negativo estágio IV em humanos, apresentando alto potencial metastático além de serem uma linhagem não imunogênica (Tao *et al*.; 2008).

## 2.2 IMUNOTERAPIAS APLICADAS AO CÂNCER DE MAMA TRIPLO NEGATIVO

Por muito tempo, a imunoterapia não foi considerada adequada para o TNBC, até que recentemente alguns estudos encontraram resultados promissores (Li Z *et al.*, 2018). De forma geral, a imunoterapia contra o câncer inclui inibidores de "checkpoint" imunológico, terapia celular adotiva e vacinas.

O tratamento único com inibidores de "checkpoint", normalmente não tem apresentado efeitos suficientes para eliminar o TNBC avançado, mas a associação de alguns quimioterápicos junto a imunoterapia tem demonstrado resultados positivos. Atualmente, um estudo demonstrou que a porcentagem de resposta positiva ao tratamento foi maior entre pacientes com TNBC que receberam pembrolizumabe (anticorpo monoclonal) associado a quimioterapia neoadjuvante, do que aqueles que receberam placebo junto a quimioterapia. Outro estudo, demonstrou que atezolizumabe (anticorpo monoclonal) junto a nab-paclitaxel (Abraxane) prolongou a sobrevida livre de progressão em pacientes com TNBC metastático (Altundag K. 2019; Cetin; Gumusay, 2020). Esses achados demonstram resultados positivos com a utilização da imunoterapia associada a quimioterapia no tratamento do TNBC.

A utilização de células T do sangue periférico geneticamente modificadas, geralmente conhecidas como receptores de antígenos quiméricos (CARs) são introduzidos nas células T para que estas atuem como medicamentos capazes de interagir dinamicamente com o ambiente biológico do paciente, sendo capazes de reconhecer, ativar, proliferar e exercer função citotóxica específica, independente da apresentação do complexo principal de histocompatibilidade (MHC), tem ganhado atenção no tratamento de muitos tipos de câncer, incluindo o TNBC (Kumar *et al*.,

2023).

Para desenvolver terapêuticas CARs eficazes para TNBCs é necessário encontrar antígenos apropriados e que sejam compartilhados entre os pacientes, porém, por se tratar de um subtipo de câncer heterogêneo, existe a dificuldade de identificar esses antígenos em comum (Yuetao *et al.,* 2020).

Os anticorpos monoclonais (ADCs) também tem apresentado grande destaque como imunoterapia. Atualmente, o anticorpo Sacituzumab govitecan-hziy (SG) direcionado ao antígeno de superfície do trofoblasto 2 (Trop2) (Trodelvy®), foi aprovado como tratamento no TNBC e apresentou maior sobrevida dos pacientes em relação a quimioterapia (Hu *et al*., 2024).

Outros estudos utilizando células imunes, além das células T, tem apresentado resultados promissores. Bernál-Estevez e colaboradores (2021) em um estudo clínico com alguns pacientes portadores de TNBC, utilizaram células dendríticas autólogas em combinação com a quimioterapia neoadjuvante (Doxorrubicina e Ciclofosfamida) e não apresentaram efeitos adversos como reações locais, além de favorecer a recuperação da capacidade funcional das células T, após estímulo *in vitro*. Esses resultados mostram que a utilização de células imunes autólogas podem ser promissoras e devem ser mais estudadas para utilização em tratamento do TNBC.

## 2.3 MACRÓFAGOS ASSOCIADOS AO TUMOR NA RESPOSTA IMUNE ANTITUMORAL

Os macrófagos associados ao tumor (TAM) representam 50 a 80 % das células mesenquimais do microambiente tumoral e participam ativamente do processo do desenvolvimento do tumor. Os TAMs se originam de monócitos circulantes no sangue que são recrutados pela produção de citocinas e quimiocinas secretadas pelas células tumorais e quando atingem o tecido alvo se diferenciam em macrófagos (Santoni *et al.,* 2018).

Os TAMs são compostos por dois subtipos: macrófagos M1, classicamente ativados e macrófagos M2 alternativamente ativados. No início da formação do câncer, os macrófagos são ativados com um perfil semelhante a M1 pró-inflamatórios, ativando uma resposta imune contra a formação do tumor (Noy;

Pollard, 2014). Após o estabelecimento do câncer, esses macrófagos são induzidos a se tornarem pró-tumorais (M2) promovendo angiogênese, remodelação tecidual, supressão da imunidade antitumoral, favorecendo a progressão do tumor e metástase (Basak *et al.,* 2023).

Os macrófagos são tradicionalmente considerados antitumorigênicos pela expressão de níveis elevados de fator de necrose tumoral (TNF), óxido nítrico sintase induzível (iNOS) ou moléculas MHC classe II, e considerados pró-tumorigênicos pela expressão de arginase-1 (ARG1) e IL-10 (DeNardo; Rufell, 2019).

Os macrófagos M2 são os mais abundantes no microambiente tumoral e normalmente são associados a um pior prognóstico e baixa sobrevida não apenas no câncer de mama, mas em vários outros tipos de canceres (Boutilier; Elsawa, 2021). De forma indireta, as citocinas produzidas pelos macrófagos M2 podem induzir células T regulatórias, inibir células dendríticas (DCs) e células T CD8+, levando a imunossupressão no microambiente tumoral. Alguns estudos demonstraram que inibir o recrutamento de macrófagos com esse fenótipo, aumenta a infiltração de células T, melhorando a resposta ao tratamento quimioterápico (Klug *et al*., 2013). Por outro lado, a apresentação de antígenos e a fagocitose podem ser características antitumorigênicas importantes de macrófagos, embora sejam funções pouco observadas em TAMs com fenótipo M2 (DeNardo; Ruffell, 2019)

Os macrófagos têm sido usados em terapias desde 1970, onde Dr. Fidler da Universidade da Pensilvânia, usou macrófagos para intervir na metástase tumoral. Os macrófagos foram isolados da cavidade peritoneal de camundongos C57B16 com tumores subcutâneos B16 e estimulados com linfócitos de camundongos com tumores, sendo estes injetados intravenosamente em camundongos portadores do tumor. Os resultados mostraram uma redução significativa nas metástases pulmonares (Filder IJ., 1974). Desde então, os macrófagos têm sido explorados em ensaios clínicos para tratar uma variedade de tumores humanos. Vários ensaios clínicos têm utilizado macrófagos como agentes terapêuticos, mas os resultados desses ensaios ainda não foram demonstrados (Guo; Qian, 2024).

Alguns estudos recentes também demonstraram que o Paclitaxel®, agente antineoplásico amplamente usado para tratar vários tipos de tumores sólidos, promoveu a imunidade antitumoral reprogramando macrófagos M2, para o fenótipo

M1 em modelos murinos de tumores de mama e melanoma (Wanderley *et al*.,2018), demonstrando a grande participação dos macrófagos no tratamento do câncer.

## 2.4 USO DO BCG COMO MODULADOR DA RESPOSTA IMUNE E UTILIZAÇÃO NO CÂNCER

A bactéria *Mycobacterium bovis* (Bacilo Calmette-Guérin) - BCG é uma cepa atenuada da vacina viva da *Mycobacterium bovis* desenvolvida há mais de 100 anos que protege contra infecções micobacterianas, responsáveis pela doença chamada tuberculose (Zumla *et al.*, 2013). Administrada em dose única, a vacina BCG também tem efeitos protetores heterólogos contra outras infecções, diminuindo a mortalidade principalmente em crianças (Biering-Sørensen *et al.,* 2017).

A explicação para esse efeito protetor não específico deve-se a imunidade treinada. Esse termo consiste em alterações metabólicas e epigenéticas que são adquiridas após um segundo estímulo por células da imunidade inata, incluindo macrófagos, células assassinas naturais (NK- *natural killer)*, células dendríticas, e células epiteliais, levando a intensificação ou à tolerância de respostas imunes (Netea et al, 2020).

Uma das primeiras estratégias imunoterapêuticas no câncer, foi desenvolvida por William Coley (Levine DB; 2008) no final do século XIX utilizando a injeção de toxinas de *Streptococcus* diretamente em tumores ósseos, sendo associada a indução de um fenótipo de imunidade treinada (Colley WB; 1891). Apesar do tratamento bem-sucedido a abordagem recebeu muitas críticas, por ser uma terapia arriscada, podendo gerar complicações. Além disso, outros métodos estavam em desenvolvimento como a radioterapia, que ofuscaram seu trabalho. Em 1930, seguindo a abordagem de Colley, a vacina BCG começou a ser estudada no tratamento do câncer e em 1950 foi desenvolvida como uma imunoterapia para tratar o câncer de bexiga (Old *et al*.,2008).

A administração intravesical da BCG foi uma das primeiras imunoterapias aprovadas pela FDA (Agência de Alimentos e medicamentos dos Estados Unidos) e continua sendo o tratamento padrão de escolha para câncer de bexiga em humanos (Moreo *et al*.,2023). Estudos recentes, demonstraram que a vacinação com BCG poderia induzir alterações epigenéticas no compartimento de células-tronco

hematopoiéticas gerando macrófagos de memória protetores (Kaufmann *et al*., 2018).

Apesar da vacina da BCG ser utilizada como padrão ouro apenas no câncer de bexiga, muitos estudos têm sido desenvolvidos em outros tipos de cânceres (Antonelli *et al.,* 2020). Estudos clínicos demonstraram efeitos positivos com a BCG no tratamento de pacientes com melanoma (Stewart; Levine, 2011). Também já foi estudado o uso da BCG para o tratamento em casos de leucemia e sarcoma (Li *et al*., 2006; Frampton JE, 2010). No câncer de mama, estudos demonstraram que o BCG recombinante expressando MUC1 e outros fatores de crescimento tiveram resultados positivos em modelos animais e humanos (Yuan *et al*., 2009; Convit *et al*.,2015).

A utilização clínica da BCG, apesar de alguns estudos satisfatórios, ainda não se tem clareza dos mecanismos envolvidos na sua aplicação. Um estudo *in vitro* demonstrou que as células NK ativadas por BCG foram capazes de inibir a proliferação de células cancerosas do colo de útero por meio de apoptose (Lu X *et al.,* 2013). Outros trabalhos observaram a ativação da via STING e moléculas pró-inflamatórias levando ao recrutamento de macrófagos M1 e células T, levando a erradicação do tumor (Lombardo *et al.,* 2022).

Logo, promover o treinamento da imunidade utilizando estímulos bacterianos como BCG poderá ser uma relevante estratégia no combate aos canceres.

# 3 OBJETIVOS

## 3.1 OBJETIVO GERAL

Avaliar o efeito da utilização de macrófagos derivados da medula óssea (BMDMs) estimulados com BCG, na migração de células tumorais mamárias 4T1 e no controle do crescimento do câncer de mama triplo negativo em modelo murino.

## 3.2 OBJETIVOS ESPECÍFICOS

a) Estimular BMDMs com BCG *in vitro* e analisar o perfil metabólico, produção de óxido nítrico e citocinas;
b) Avaliar o efeito do meio condicionado de BMDMs com seus estímulos na migração de células tumorais 4T1 *in vitro;*
c) Induzir o câncer de mama triplo negativo em camundongos da linhagem BALB/c e analisar o efeito dos tratamentos com BMDMs, BMDMs+BCG, BCG ou 5FU;
d) Quantificar a produção de citocinas provenientes da cultura de esplenócitos dos animais em diferentes tratamentos;
e) Analisar as alterações leucocitárias do sangue periférico dos animais após os tratamentos;
f) Identificar as alterações histopatológicas dos tecidos pulmonar, hepáticos e mamário após os tratamentos.

## 4 ARTIGO

# Analysis of adoptive cell transfer of BCG-stimulated macrophages in the control of triple-negative breast cancer in a murine model

# Analysis of adoptive cell transfer of BCG-stimulated macrophages in the control of triple-negative breast cancer in a murine model

Joyce Alves dos Santos[1], Evandro Neves da Silva[1], Thiago Caetano Andrade Belo[1], Ana Clara Moreira[1], Yasmin Vieira Braida[1], Leonardo Augusto de Almeida [1], Patrícia Paiva Corsetti [1]*

[1] Laboratory of Molecular Biology of Microorganisms, Federal University of Alfenas (UNIFAL), Alfenas, 37130-001, Minas Gerais, Brasil.

*Corresponding author: Patrícia Paiva Corsetti

Laboratório de Biologia Molecular de Microrganismos, Universidade Federal de Alfenas, Rua Gabriel Monteiro da Silva, 700, Alfenas, 37130-001, Minas Gerais, Brasil.
E-mail: patricia.corsetti@unifal-mg.edu.br).

## Abstract

Breast cancer is one of the leading causes of death among women in Brazil and worldwide. Among its subtypes, triple-negative breast cancer (TNBC) has the poorest prognosis. Macrophages infiltrated in the tumor microenvironment play important roles, as they can be induced into either a pro-tumoral M2 state or an anti-tumoral M1 phenotype. Currently, BCG (Bacillus Calmette-Guérin) has shown promise in cancer treatment, as seen with bladder cancer. This study aimed to evaluate the effect of BCG in stimulating bone marrow-derived macrophages (BMDMs) in vitro, as well as in vivo effect in treating murine mammary carcinoma induced by 4T1 mammary carcinoma cells. For this purpose, BMDMs were stimulated with BCG for 6 and 12 hours, and the effect of their supernatant on 4T1 tumor cells was analyzed. BCG stimulation led to increased nitric oxide and TNF-α release, partially inhibiting 4T1 cell migration in a wound healing assay. In the murine model, female BALB/c mice with induced cancer (4T1 cells) received a single dose of PBS, BCG-stimulated BMDMs, unstimulated BMDMs, BCG, or the chemotherapeutic agent 5FU (5-Fluorouracil). Treatment with BMDM+BCG reduced weight loss, improved feed intake and decreased tumor volume. Histologically, it led to a significant reduction in tumor cells in mammary tissue, similar to 5FU, but presented greater inflammatory infiltration in the liver and lung, which may compromise the integrity of the organs. BCG alone showed smaller reduction of tumor volume and presented a lower inflammatory response in the tissues. It can be concluded that treatment with BCG-stimulated BMDM is effective in combating tumor growth. However, the robust inflammatory response may harm organ integrity; therefore, further studies on the long-term effects of this treatment are needed, in addition to exploring approaches to control the excessive inflammatory response. This approach opens new avenues for the development of effective immune therapies against triple-negative breast cancer.


**Keywords**: BCG; Tumor microenvironment; Inflammatory response; Adoptive cell transfer.

## Introduction

Breast cancer is the most common cancer type among women and one of the leading causes of mortality. According to the World Cancer Research Fund (1), approximately 2.2 million cases were reported globally in 2022, making it the second-highest incidence of malignancies. Among its subtypes, triple-negative breast cancer (TNBC) is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression, and is associated with a poorer prognosis (2). Due to the absence of these receptors, which are typically targeted by specific therapies, TNBC treatment is limited to surgery and chemotherapy, highlighting the need for new therapeutic approaches (3).

In recent years, new strategies have been investigated for TNBC treatment, including immunotherapy, such as checkpoint inhibitors and antibody-drug conjugates. However, early resistance remains a major challenge, related to the modulation of the tumor microenvironment, which promotes immune evasion and tumor progression (4).

In the tumor microenvironment, macrophages are one of the main cell populations with important functions, capable of being classically activated to a pro-inflammatory "M1" profile or alternatively to an anti-inflammatory "M2" profile, with M1 being more efficient in tumor combat (5). Given the crucial role of cellular immunity in eradicating tumor cells, the use of certain cell types in immunotherapy treatments has increased (6).

A pioneering success in immunotherapy for solid tumors was the intravesical use of *Mycobacterium bovis* - BCG (Bacillus Calmette-Guérin) for bladder cancer treatment (7), which remains known for its broad protective effect associated with other diseases, including cancer (8). *Mycobacteria* induce the production of pro-inflammatory cytokines through the activation of macrophages, dendritic cells, and epithelial cells via the NLRP3 inflammasome, activating adaptive immunity (9). This response, integrating innate and adaptive immunity, underscores the need to explore microorganism-based therapies in cancer treatment (10). This study aimed to use BCG to stimulate bone marrow-derived macrophages *in vitro* and assess its effect as adoptive cell therapy in controlling TNBC induced by 4T1 cells.

## Materials and Methods

### Animal model and cell line

This study was conducted following a protocol approved by the Ethics Committee on Animal Experimentation at the Federal University of Alfenas-UNIFAL/MG (ANNEX A - CEUA 39/2022). Female BALB/c isogenic mice, aged between 7 and 9 weeks, from the Central Animal Facility of the Federal University of Minas Gerais (UFMG), were used (n=53). They were maintained under controlled temperature conditions (12-hour light/dark cycle), regular cleaning, and provided with filtered water and food ad libitum. The 4T1 cell line, used as a breast cancer model (murine triple-negative mammary carcinoma), was donated by the Department of Parasitology, Microbiology, and Immunology of the Federal University of Juiz de Fora (UFJF). Cells were thawed and placed in cell culture flasks with RPMI medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and amphotericin B (2 mg/mL) in a $CO_2$ incubator at 37ºC. BCG (*Mycobacterium bovis*) Moreau Rio de Janeiro strain was obtained from the Microorganism Molecular Biology Laboratory stock at the Federal University of Alfenas.

### Bone marrow-derived macrophages and cell stimulation

Bone marrow-derived cells were obtained from the femurs and tibias of BALB/c mice (n=3) and differentiated into macrophages (BMDM) as previously described by our group (11). Murine BMDMs (5 x $10^5$ cells per well) were cultured in two culture plates, followed by stimulation with medium (negative control), BCG at a multiplicity of infection (MOI) of 1:0.1, proteins extracted from 4T1 tumor cells (PTN 4T1) at a concentration of 2 µg/mL, and a combination of BCG and proteins extracted from 4T1 tumor cells (BCG+PTN 4T1). Additionally, 1 µg/mL LPS was used as a positive control. The BMDMs were maintained in a $CO_2$ incubator at 37ºC, with one plate collected at 6 hours and another at 12 hours to assess secreted molecules.

### Glucose and lactate quantification

Glucose quantification in the 6-hour and 12-hour BMDM supernatants was performed according to the GOD-POD enzymatic-colorimetric method described by Trinder (1969) (12). Lactate was measured using the enzymatic method (13), with the

final reaction read on a microplate reader (Bio-Tropsch Tek Instruments, Winooski, VT, USA) at 505 nm and 550 nm, respectively.

**Nitric oxide quantification**

The BMDM supernatants from each group post-stimulation were analyzed using the Griess method (14) to quantify nitric oxide. The final reaction was measured on a microplate reader at 405 nm, with wavelength correction set to 650 nm.

**Cytokine levels**

Cytokine levels in BMDM culture supernatants were quantified by ELISA for TNF-α and IL-10 cytokines using the Murine TNF-α / IL-10 / Mini ABTS ELISA development kit (PeproTech, Cranbury, NJ, USA). Final cytokine concentrations were calculated using the standard curve. The final reaction was read on a microplate reader (Bio-Tropsch Tek Instruments, Winooski, VT, USA) at 405 nm with wavelength correction set to 650 nm.

**Wound Healing assay**

A wound healing assay was performed as previously described (15). After scratching, the culture medium was replaced with 200 µL of the pre-collected BMDM supernatants. The chemotherapeutic 5FU was used as a positive control at 1.6 µL in 200 µL RPMI medium per well. Images were captured with an optical microscope (Zeiss Primo Vert, Oberkochen, Germany) equipped with a digital camera at time intervals, and the assay was completed after 72 hours. The wound area in each image was analyzed and quantified using Image J software (Image J National Institutes of Health, Bethesda, Maryland, USA). The percentage of closure was calculated at each time interval, using the following formula: % of closure= (initial area – final area/ final area final) ×100.

***In vivo* tumor model and adoptive cell transfer**

The 4T1 breast cancer cell line was used to induce triple-negative breast cancer, being pre-cultured in RPMI. After reaching 80% confluence, cells were detached with 0.25% trypsin-EDTA, washed twice in PBS, centrifuged at 1200 rpm at

4ºC for 10 minutes, and resuspended in sterile saline. For the breast cancer model, BALB/c mice were divided into two groups (n=25 each); the first group received a subcutaneous injection in the last left mammary gland with $1x10^4$ 4T1 cells in 50 µL of saline per animal. The group that did not receive tumor cells served as the control. Mice were monitored daily for body weight and food intake. Additionally, feces were collected on days 0, 7, 13, 15, and 17, and fecal consistency was scored based on the index by Sherman et al. (1983) (16): 0, normal (firm and well-formed stools); 1, soft consistency (soft and formed stools); 2, mild diarrhea (fluid stools, often yellowish); and 3, severe diarrhea (watery stools).

After 10 days of tumor induction, BMDMs were derived and cultured as described for treatment purposes. One group of BMDMs remained unstimulated, and another group was stimulated with BCG (MOI 1:0.1) 12 hours before animal treatment. BMDMs were detached with 0.25% trypsin-EDTA, washed twice in PBS, and resuspended in sterile saline. On day 11, tumor-bearing mice were randomly divided into 5 groups (n=5) and treated with saline, unstimulated BMDMs ($1 \times 10^6$ cells per animal), BCG-stimulated BMDMs ($1 \times 10^6$ cells per animal) for 12 hours (MOI 1:0.1), BCG ($1 \times 10^5$ CFU per animal), or the chemotherapeutic 5FU (50 mg/kg) for subcutaneous injection in the last left mammary gland. No-tumor-bearing animals were also subdivided into 5 groups and received the same treatments. After treatment, mice were monitored for 7 days and euthanized at the end of this period. Tumors and other organs were collected, weighed, and analyzed. Tumor volumes were calculated using the formula: tumor volume ($mm^3$) = $L\ W^2\ /2$, where L is the longest diameter of the tumor, and W is the shortest.

**Splenocyte culture and cytokine measurement**

Spleens were collected, and splenocytes were cultured ($1 \times 10^6$ cells per well) as previously described by our group (17). Cells were stimulated with BCG (MOI:1:0.1), proteins extracted from 4T1 tumor cells (2 µg/mL), or medium (negative control) and maintained in a $CO_2$ incubator at 37ºC. Supernatants were collected after 48 hours, and IFN-γ and IL-10 levels were quantified by ELISA.

**Differential blood leukocyte count**

Cardiac blood from animals was obtained after euthanasia using a sterile

syringe. Fresh blood was used to prepare a blood smear for each animal. After drying, slides were stained with the rapid Panoptic method based on Romanowsky's principle (18). The procedure involves sequential submersion in three solutions containing Triarylmethane, Xanthene, and Thiazines for 15 seconds. Each submersion lasts 5 seconds, followed by a water rinse and air drying in an upright position. Slides were analyzed by light microscopy, and at least 100 leukocytes were counted.

**Histological processing and analysis**

Skin (of the last left breast), liver (median lobe) and left lung samples were fixed in 10% formaldehyde solution (0.1M, pH 7.2) for 24h, dehydrated in increasing ethanol series (70-99.8%), clarified in xylene and embedded in histological grade paraffin. The skin was embedded glycol methacrylate, while liver and lung samples were embedded in histological grade paraffin (19). Histological sections with 4-µm thick were cut in a rotary microtome (Leica Multicut, Wetzlar, Germany), stained with hematoxylin and eosin, and mounted on histological slides with Entelan (Merk, Darmstadt, Germany). Both organs were observed and digital images captured by using a bright field photomicroscope (Axio Scope A1, Carl Zeiss, Oberkochen, Germany). For each organ and animal, 20 histological fields randomly sampled were analyzed using x40 objective lens and x10 ocular lens (x400 magnification) (20).

Histopathological analysis was performed in a semi-quantitative manner based on the observation and characterization of classical alterations in each of the organs investigated as follows: (i) Skin: epidermal and dermal thickness, dermal cellularity, distribution of connective tissue and epidermal annexes (sebaceous glands and hair follicles), presence and distribution of tumoral cells (ii) Liver: hepatocyte cords organization, interstitial cellularity, distribution of sinusoidal capillaries, congestion or vascular collapse, inflammatory infiltrate, cellular degeneration, morphology of hepatocyte nucleus, cellular hypertrophy or atrophy; (iii) Lung: Alveolar hyperinflation or collapse, interstitial cellularity, alveolar exudate, congestion or vascular collapse (20). A pathologist blinded to the treatment groups performed all histopathological analyses. Healthy animals were used as controls for lung and liver microstructural normality.

**Stereological and histomorphometric analysis**

Quantitative microstructural analysis of both organs was performed using the stereological method and computational planimetry. Thus, the volume density (Vv, %) of hepatocytes, sinusoidal capillaries, alveoli and alveolar septum was estimated using the relationship Vv = ΣP/PT, where ΣP represents the number of points that fall on the structure of interest, and PT is the total number of points used in the counting system (here= 100) (19-20). Furthermore, the number density (Nv) of interstitial cells and alveoli was determined according to the following formula: Nv= QA/TA, where QA represents the number of structures of interest observed in the test area, and TA represents the dimension of the test area. In the lung, Nv results were normalized according to the histological area occupied by alveolar septum. Epidermis thickness was assessed by computational planimetry using the linear measurement tool of the software the image analysis software Image Pro-Plus 4.5 (Media Cybernetics Inc., Silver Spring, Maryland, USA). This parameter was measured in the center of the skin image and in 2 different equidistant lateral points to the right and left. The mean thickness was then calculated. The Image Pro-Plus 4.5 software was also used to estimate all stereological parameters (21).

**Statistical Analyses**

Data are expressed as mean ± SEM. All in vitro experiments were conducted in triplicate. Different treatments were compared using the ANOVA. Statistical significance was accepted at $P<0,05$. All statistical analyses were performed using GraphPad Prism version 7.0c (GraphPad Software).

**Results**

**BCG induces greater activation of the anaerobic glycolytic pathway, NO production, and TNF-α release compared to stimulation with 4T1 tumor proteins in BMDMs**

To investigate the effect of BCG on macrophage metabolism, BMDMs were stimulated with BCG, tumor proteins (PTN 4T1), and PTN 4T1+BCG as described in figure 1A. It was observed that stimulation with BCG resulted in higher glucose consumption compared to the group stimulated with PTN 4T1 at 12 hours (Fig. 1B),

accompanied by greater lactate production from 6 to 12 hours (Fig. 1C). Additionally, the group stimulated with PTN 4T1+BCG showed glucose consumption and lactate production like the BCG group. During the initial 6-hour period, a trend of higher glucose consumption was observed in the group of BMDMs stimulated with PTN 4T1 compared to the other groups. However, this consumption did not sustain at 12 hours. In contrast, the groups stimulated with BCG and PTN4T1+BCG maintained high consumption levels.

Regarding NO production (Fig. 1D), a byproduct of macrophage activation in immune responses, it was observed that during the first 6 hours of stimulation, all groups exhibited similar production levels with no statistical differences. At 12 hours, the PTN 4T1 group showed a decrease in production, like the unstimulated group (medium). On the other hand, the BCG-stimulated group showed increased production at 12 hours. Interestingly, the PTN 4T1+BCG combination exhibited lower NO production compared to BCG alone.

BCG and PTN 4T1+BCG stimulation led to low TNF-α production at 6 hours (Fig. 1E), while the medium and PTN 4T1 groups showed no detectable production. At 12 hours, both BCG and PTN 4T1+BCG showed higher TNF-α production, with no statistical difference between them, and both were higher than the PTN 4T1 and medium groups. Regarding IL-10, similar production levels were observed in the BCG and PTN 4T1+BCG groups (Fig. 1F) at 6 hours, while the PTN 4T1-stimulated group showed lower production, like the unstimulated group. However, at 12 hours, there was a significant increase in IL-10 production in the PTN 4T1 group, surpassing all other groups, while the BCG and PTN 4T1+BCG groups maintained similar production, which was low and statistically comparable to the unstimulated group.

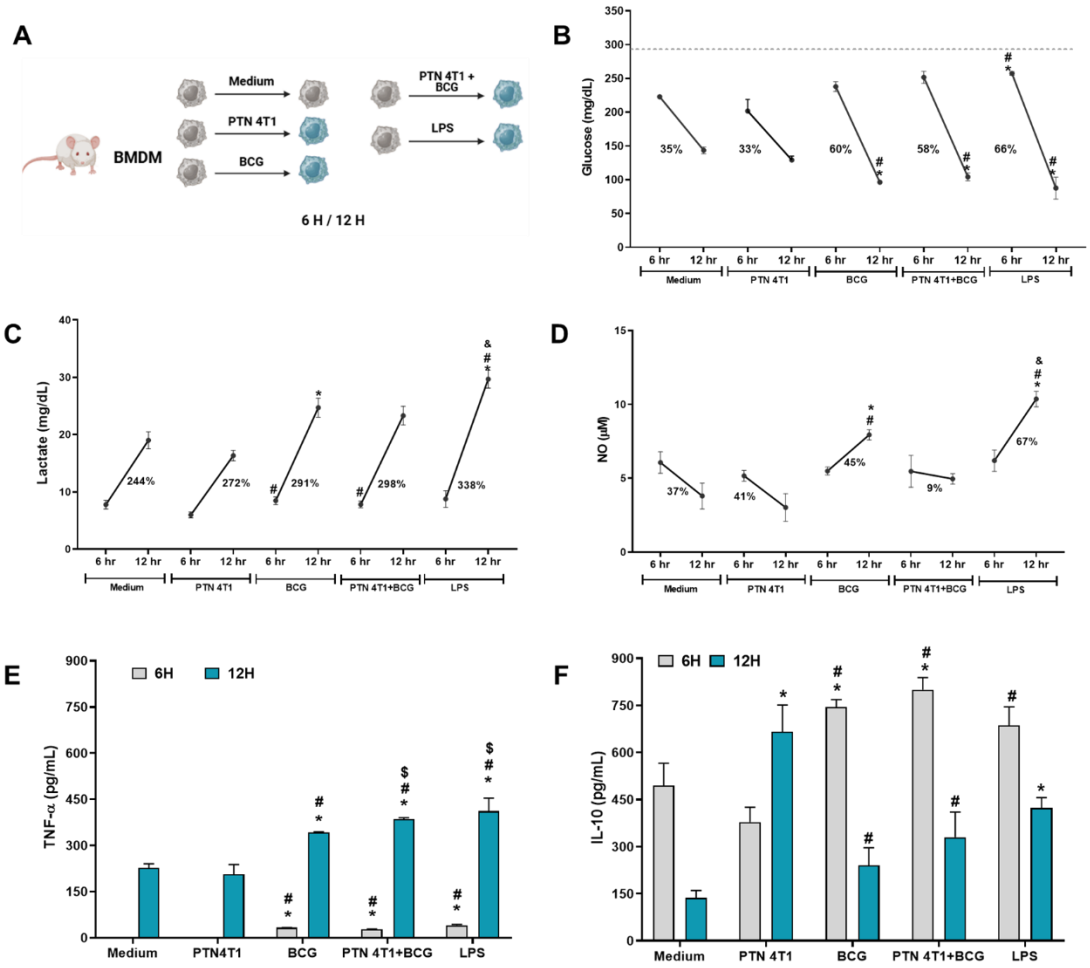


**Figure 1. BCG induces glycolytic activation, NO production, and TNF-α production in BMDMs.** BMDMs were stimulated with BCG, proteins extracted from 4T1 tumor cells (PTN 4T1), and a combination of both components. Schematic representation of the treatment model BMDM in vitro (A). Supernatants were collected and analyzed for glucose consumption (B), lactate production (C), NO production (D), TNF-α cytokine secretion (E), and IL-10 secretion (F). All values are means ± SD (n=3). * $p<0.05$ compared to the medium group; # $p<0.05$ compared to PTN 4T1; & $p<0.05$ compared to PTN 4T1+BCG; $ compared to BCG.

## Conditioned medium from BCG-stimulated BMDMs partially prevents migration of 4T1 breast cancer cells in vitro.

To analyze the effects of conditioned BMDM media on 4T1 tumor cell migration, a wound healing assay was performed by adding supernatants from the 6-hour (Fig. 2A) and 12-hour (Fig. 2B) time points into tumor cells. The supernatants of BCG-stimulated BMDMs significantly reduced tumor cell migration, resulting in 53% closure at 6 hours (Fig. 2C) and 67% at 12 hours (Fig. 2D). In contrast, the supernatants from PTN 4T1-stimulated BMDMs did not inhibit tumor cell migration, showing 90% closure at both 6 and 12 hours, similar to the unstimulated BMDM

group. For the PTN 4T1+BCG combination, a reduction in tumor cell migration was also observed, with 61% closure at 6 hours and 75% at 12 hours.

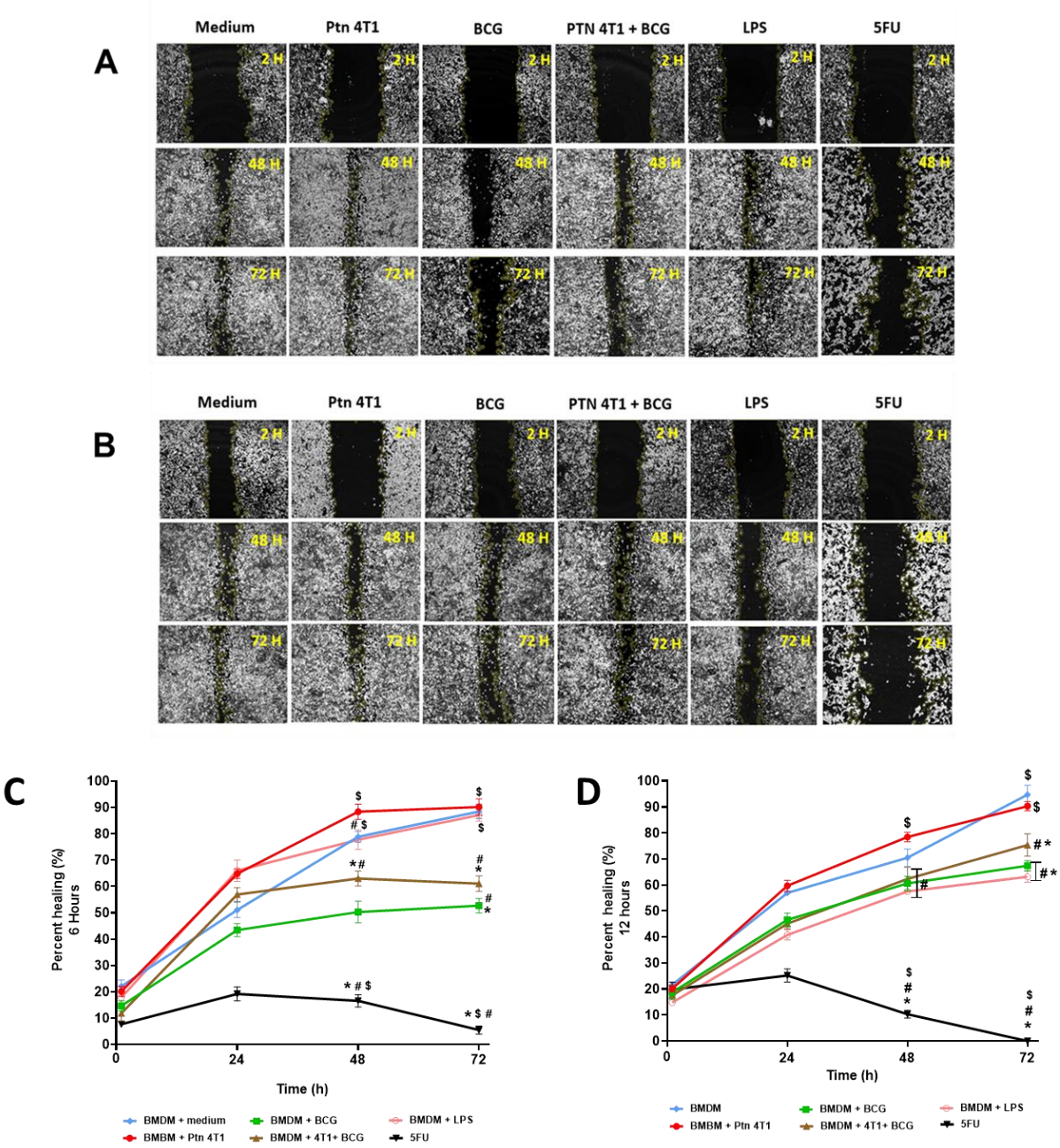


**Figure 2. Conditioned medium from BCG-stimulated BMDMs partially prevents migration of 4T1 tumor cells *in vitro*.** Supernatants from the 6-hour (A) and 12-hour (B) time points were added to pre-plated 4T1 tumor cells with a wound created in the center of each well. Images were taken at 2, 48, and 72 hours. Percentages of wound closure were calculated and are shown in the graphs with 6-hour (C) and 12-hour (D) supernatants. All values are means ± SD (n=3). * $p<0.05$ compared to the BMDM+medium group; # $p<0.05$ compared to BMDM+PTN 4T1; $ $p<0.05$ compared to BMDM+BCG.

**Treatment with BCG-stimulated BMDM reduces weight loss and improves food intake in mice with triple-negative breast cancer**

BCG is an attenuated bacterium that has been studied as a therapeutic option for several diseases, including bladder cancer, where it is used as an immunotherapy (Moreo et al., 2023). After observing the in vitro effect of BCG-stimulated macrophage supernatants on breast tumor cells, we sought to analyze it *in vivo* effect. Female BALB/c mice were subcutaneously inoculated with 4T1 tumor cells in the mammary glands. After 11 days, a single-dose treatment was administered using BMDMs without prior stimulation, BCG-stimulated BMDMs, BCG alone, and the chemotherapeutic agent 5FU. After a 7-day observation period, the animals were euthanized (Fig. 3A).

In terms of survival, there was an 80% reduction (1 death) in untreated tumor-bearing animals (PBS/4T1) and those treated with BCG (BCG/4T1), while the other treatment groups maintained 100% survival (Fig. 3B). Compared to control groups, all tumor-bearing animals showed a trend of weight loss (Fig. 3D), while the control groups showed a trend of weight maintenance (Fig. 3C) with no statistical difference. Animals treated with 5FU showed the greatest weight loss, whereas those treated with BCG showed a trend of weight maintenance, with these two groups being the only ones to show a statistically significant difference between each other on day 18 (Fig. 3D). To analyze which treatments showed the most weight loss, daily weight loss was assessed, and it was found that tumor-bearing groups overall experienced greater weight loss compared to non-tumor-bearing animals (Fig. 3E). Animals treated with BCG-stimulated BMDMs showed the lowest weight loss, followed by the BCG-treated group, in comparison to untreated tumor-bearing animals. The 5FU-treated group showed greater weight loss than the BMDM+BCG and BCG groups but did not show a statistically significant difference compared to untreated (PBS) and unstimulated BMDM-treated animals.

Food intake in non-tumor animals showed an increasing trend over time, with a slight reduction after treatment (Fig. 3F), while tumor-bearing animals showed a downward trend in food intake that remained since tumor induction, suggesting that tumor presence alone leads to reduced food intake (Fig. 3G). Additionally, total food intake was higher in tumor-bearing animals treated with BMDM+BCG compared to the PBS, BMDM, and 5FU groups, but interestingly, it did not exceed intake in

animals treated with BCG alone (Fig. 3H), which, despite higher food intake, showed greater weight loss. Thus, treatment with BMDM+BCG and BCG improved food intake compared to other treatments, although the BCG-treated group showed higher weight loss.

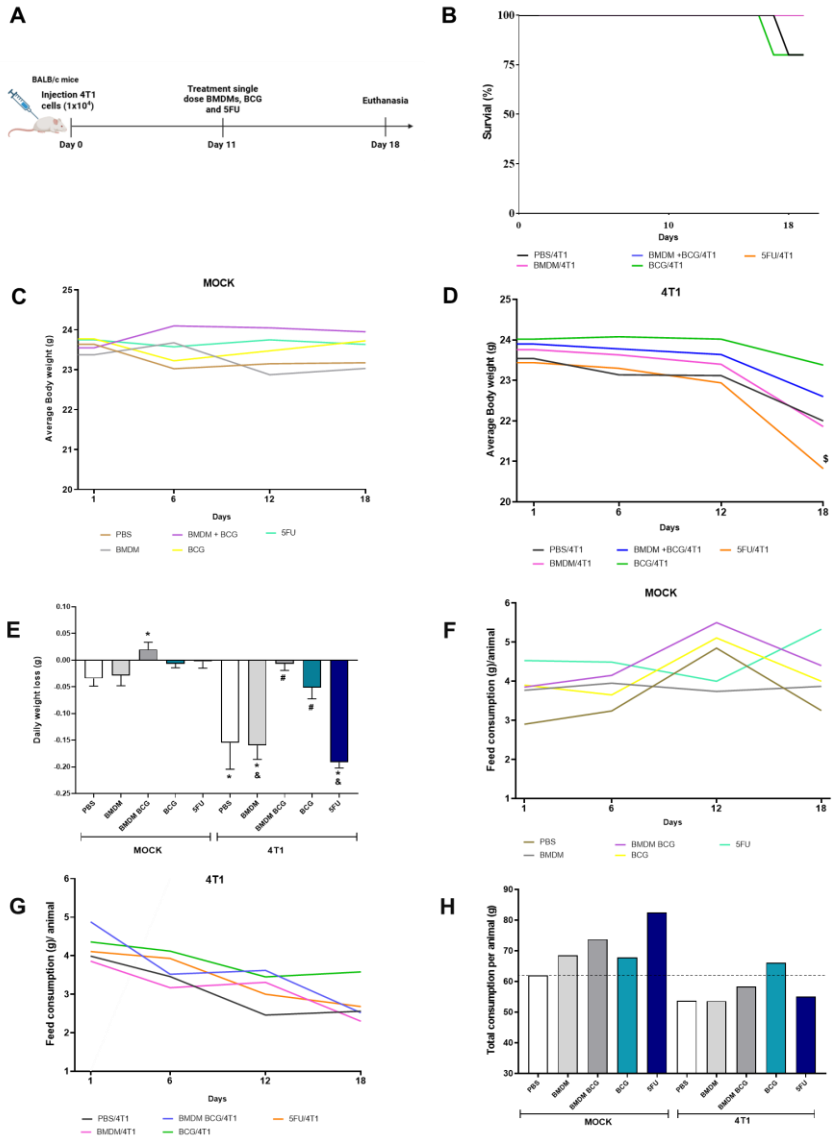


**Figure 3. Treatment with BCG-stimulated BMDMs reduces weight loss and improves food intake *in vivo*.** Schematic representation of the tumor induction model and in vivo treatment (A). 4T1 breast tumor cells (1x10⁴ per animal) were injected into the mammary gland of the mice. On day 11, a single dose treatment was administered using BMDMs, BCG-stimulated BMDMs, BCG, and 5FU. After 7 days, the animals were euthanized. Animal survival over the course of the study (A). Average body weight graph of control (B) and tumor-bearing animals (C) $ $p<0.05$ compared to BCG. Graph of average daily weight loss in control and tumor-bearing animals (D) *$p<0.05$ compared to PBS/control; # $p<0.05$ compared to PBS/4T1; & $p<0.05$ compared to BMDM+BCG/4T1. Food intake per animal in the control (E) and tumor-bearing (F) groups. Cumulative total food intake graph over the entire study (G). All values are means ± SD (n=5).

The effect of treatments on fecal patterns was analyzed using a fecal score (Supplementary Fig. S1). Tumor-bearing animals showed higher fecal scores compared to those without tumors. Among tumor-bearing groups, animals treated with 5FU had the highest scores on days 13, 15, and 17. Animals treated with BMDM+BCG and BCG showed lower scores.

**Treatment with BMDM+BCG reduces tumor weight and volume in mice with 4T1 cell-induced breast cancer.**

After treatments, tumors were collected and analyzed for weight and volume. Regarding tumor weight (Fig. 4A), the untreated group had the highest average compared to all treated groups. Among treated groups, mice receiving BMDMs and BMDM+BCG had the lowest average tumor weights, with no significant difference between them. The BCG-treated group had a higher mean with a statistically significant difference compared to PBS, BMDM, and BMDM+BCG groups. Tumor volume results followed a similar pattern (Fig. 4B).

The BMDM+BCG group showed the smallest tumor volume, similar to the 5FU-treated group, followed by the BMDM group, with slightly larger volume but no statistical difference among the three groups. In contrast, the BCG-treated group had a larger volume, with a statistically significant difference compared to all treated groups. A visual representation of the tumors can be seen in Fig. 4C. These results suggest that BMDM treatment may be more effective, both alone and when stimulated with BCG, compared to BCG treatment alone.

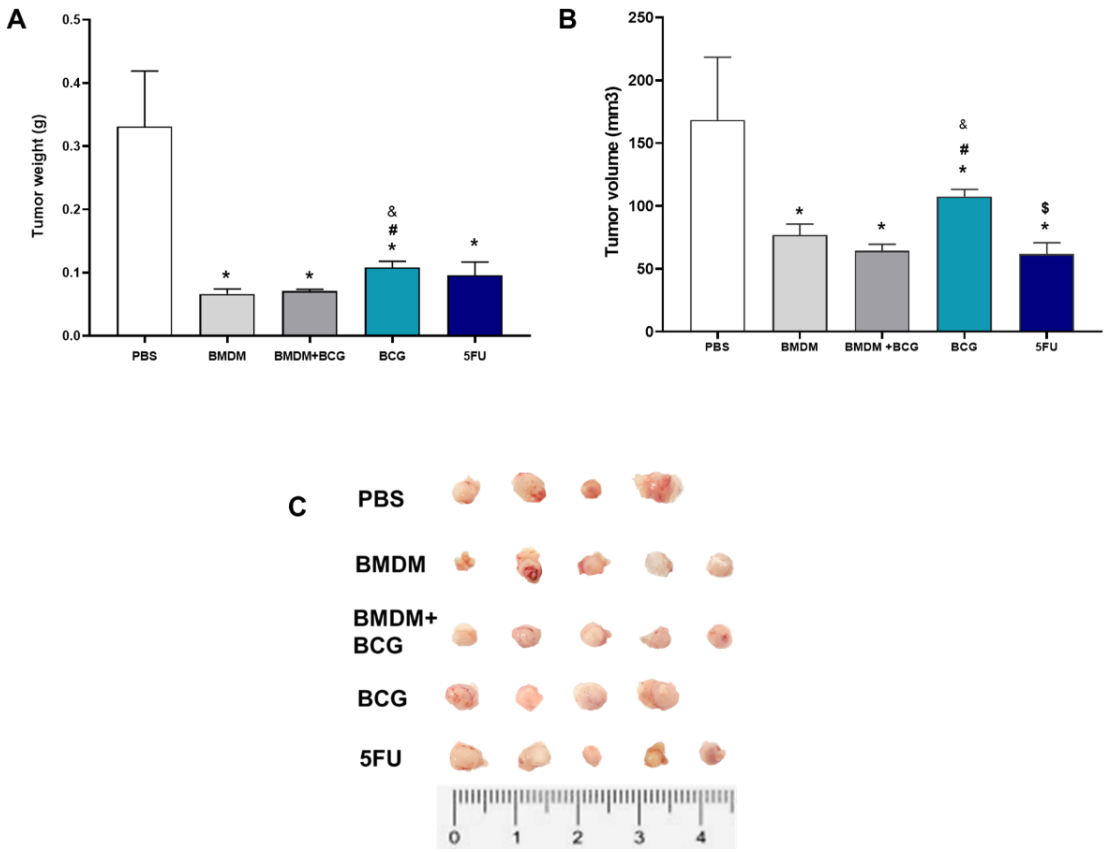


**Figure 4. Tumor weight and volume reduction in response to treatments.** Following treatments with PBS, BMDM, BMDM+BCG, BCG, and chemotherapeutic 5FU, tumors were collected and assessed for weight (A) and volume (B), using the tumor volume formula ($mm^3$) = L $W^2/2$. Tumor images are shown in (C). * $p<0.05$ vs PBS, # $p<0.05$ vs BMDM, & $p<0.05$ vs BMDM+BCG, $ $p<0.05$ vs BCG. Values are means ± SD (n=5).

## Effect of Treatments on Splenic Weight and Cytokine Production in Cultured Splenocytes of 4T1-Induced Mammary Cancer Mice

The spleens of the animals were collected and weighed, revealing an increase in spleen weight (splenomegaly) in the tumor-bearing groups, while control animals exhibited similar spleen weights (Fig. 5A). Among the tumor-bearing groups, the PBS group showed the lowest average spleen weight compared to all treatment groups.

To evaluate the impact of treatments on T cell responses, splenocytes from treated mice were stimulated with BCG and tumor proteins (PTN 4T1) for 48 hours, and the production of IFN-γ and IL-10 was analyzed in the supernatants. Tumor-bearing animals generally exhibited a tendency toward higher IFN-γ production (Fig. 5B). Splenocytes from tumor-bearing animals, except for the 5FU group, produced more IFN-γ without stimulation (medium) compared to control groups.

After stimulation with BCG, splenocytes from tumor-bearing animals treated

with BCG and 5FU produced more IFN-γ than those from tumor-free animals, while no significant differences were observed in the other groups. IL-10 production increased in all tumor-bearing groups, with lower levels observed in the BMDM+BCG and 5FU groups, though IL-10 levels remained predominant over IFN-γ (Figs. 5B-C).

After stimulation with PTN 4T1, splenocytes from the BMDM and BMDM+BCG groups produced higher levels of IFN-γ, indicating a pro-inflammatory response. In contrast, splenocytes from the BCG and 5FU groups exhibited dominant IL-10 production, suggesting an anti-inflammatory response. Stimulation with PTN 4T1 increased IL-10 production in the PBS and 5FU groups, while the other groups showed no significant changes compared to the controls (Figs. 5B-C).

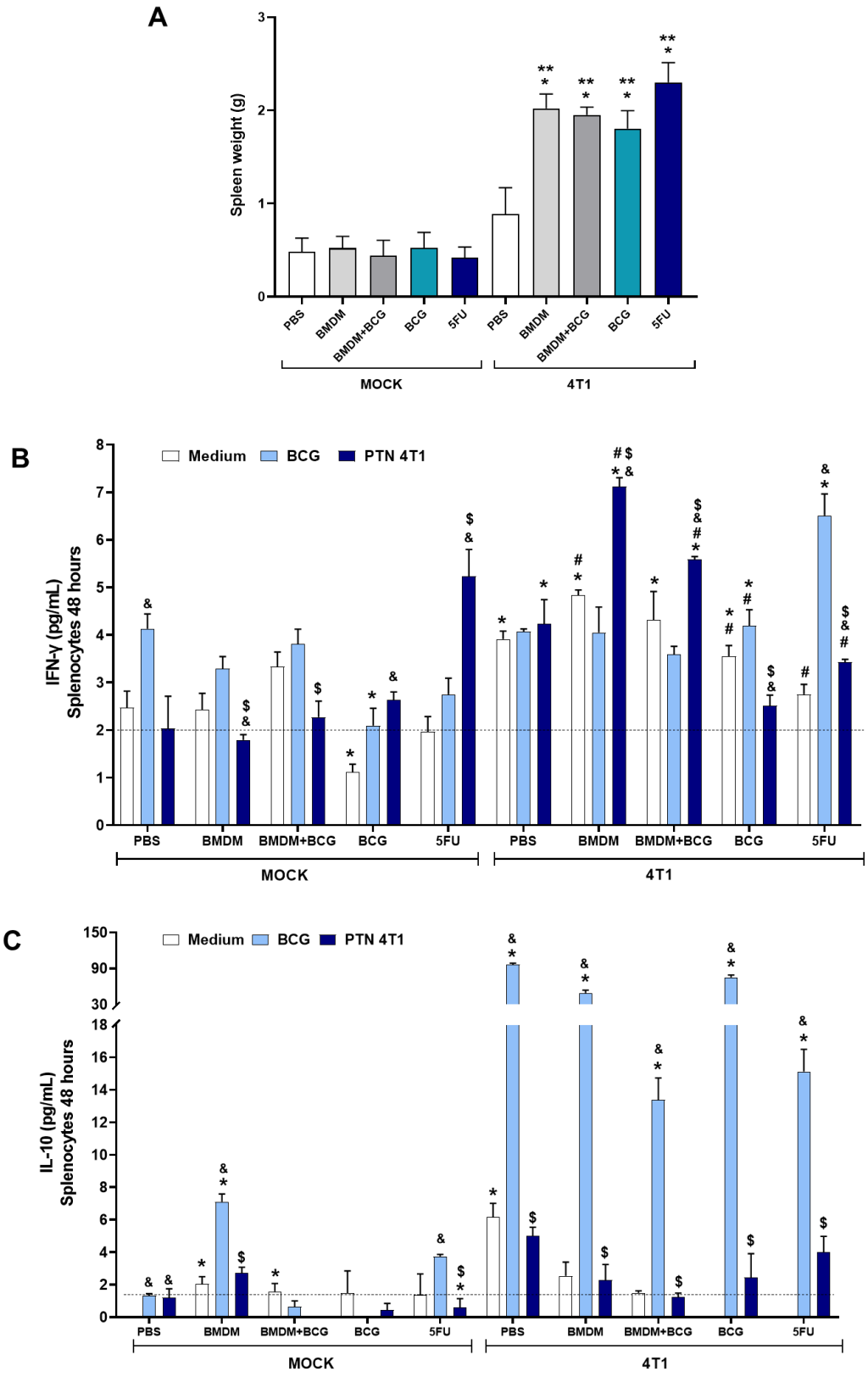


**Figure 5. Effect of treatments on spleen weight and cytokine production in splenocyte cultures.** Spleen weight (A). Production of IFN-γ (B) and IL-10 (C) by splenocytes stimulated with medium, BCG, and 4T1 tumor proteins. * p<0.05 compared to PBS; # p<0.05 compared to the corresponding control; & p<0.05 compared to medium; $ p<0.05 compared to BCG stimulation. ** p<0.05 compared PBS/4T1. All values are expressed as mean ± SD (n=5).

**Leukocyte alterations related to the presence of breast cancer in a murine model**

The analysis of blood smears revealed significant leukocyte alterations in tumor-bearing animals, particularly in the groups treated with BMDM, BMDM+BCG, and 5FU. A reduction in neutrophil count was observed in these groups, with the greatest decrease in the 5FU-treated group, while the PBS and BCG groups showed no significant differences (Supplementary Fig. S 2A). The relative number of lymphocytes increased in all tumor-bearing groups, with statistical significance in those treated with BMDM+BCG and 5FU (Fig. S 2B). Monocytes also increased in all tumor-bearing groups, with the most pronounced increase observed in the BMDM+BCG group (Fig. S 2C). Eosinophils increased in the PBS, BMDM, and 5FU tumor-bearing groups, while the BMDM+BCG and BCG groups showed only a trend toward an increase without statistical significance (Fig. S 2D). Notably, an increase in basophils was observed in animals treated with 5FU, whereas BCG treatment showed no significant increase. (Fig. S 2E).

**Histological alterations in the liver, lungs and mammary skin in response to treatments in a murine model**

The liver and lungs were evaluated for histological alterations resulting from tumor induction and the different treatments applied. As shown in Fig. 6A, histopathological analysis revealed that control animals presented typical hepatic microstructural organization, with evident hepatocyte cords, scarce connective tissue and low interstitial cellularity. In the groups with tumor induction, obliteration of the sinusoidal space and binucleated hepatocytes were more frequently observed in the groups PBS to BCG, as well as a marked increase in interstitial cellularity in the groups PBS and BMDM+BCG. Focal inflammatory infiltrate was also identified in PBS, with a predominance of polymorphonuclear cells with typical neutrophil morphology.

Quantitative microstructural analysis (Fig. 6B) indicated a significant reduction in the volumetric density of hepatocytes in the groups 5FU, which was accompanied by a larger histological area occupied by sinusoidal capillaries, as indicated by the results of vascular volumetric density in these groups compared to the other groups ($P<0.05$). In addition, a higher density of interstitial cells was identified in the groups

PBS and BMDM+BCG, indicating marked inflammatory infiltration in these groups compared to the other groups (P<0.05). In general, the group treated with BCG presented less inflammation and structural preservation of the liver tissue in relation to the other tumor groups, while the 5FU group presented changes such as vacuolization of hepatocytes and moderate dilation of sinusoidal capillaries.

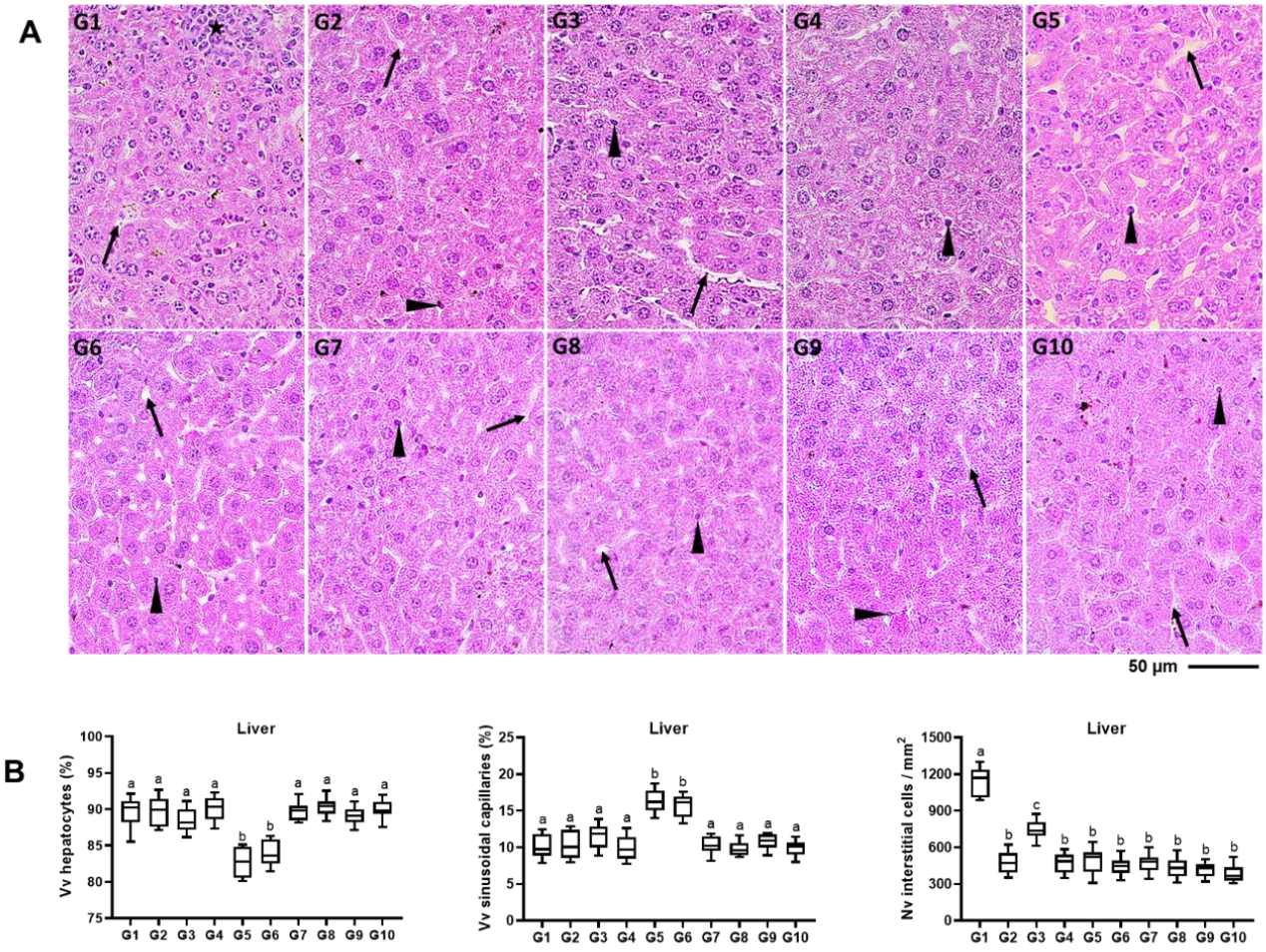


**Figure 6**. **Histological analysis of the liver of animals subjected to treatments.** Representative images (bright field microscopy, hematoxylin and eosin staining) (A). Groups= G1: Tumor + PBS, G2: Tumor + BMDM, G3: Tumor + BMDM+BCG, G4: Tumor + BCG, G5: Tumor + 5FU, G6: PBS, G7: BMDM, G8: BMDM+BCG, G9: BCG, G10: 5FU. Arrow: sinusoidal capillaries. Arrowhead: interstitial cell. Star: focal inflammatory infiltrate. Graphic of quantitative microstructural analysis (B). Data are expressed as median and interquartile interval. $^{a,b,c,d,e,f}$ Different letters indicate statistical differences among the groups (P<0.05), and groups with the same letter present similar statistical results (P>0.05).

Lung histoarchitecture can be observed in Fig. 7A. Control animals presented well-defined pulmonary alveoli with thin alveolar septa and reduced interstitial

cellularity mainly formed by mononuclear cells. Slight septal thickening and increased cellularity was identified in controls groups treaties with BMDM+BCG and 5FU. Septal thickening increased interstitial cellularity, and alveolar collapse were frequently observed in all animal groups subjected to tumor induction, especially in PBS, BMDM, BMDM+BCG and BCG. In all these groups, diffuse neutrophilic inflammatory infiltrate was evident, with a predominance of polymorphonuclear cells with typical neutrophil morphology.

Furthermore, all animals inoculated with tumor cells showed reduced volumetric density of pulmonary alveoli, greater septal thickening and distribution of interstitial/inflammatory cells (Fig. 7B) compared to control animals ($P<0.05$). Septal thickening and distribution of interstitial/inflammatory cells were greater in untreatment group and lower in treaties with 5FU animals compared to the other groups with tumor ($P<0.05$). The alveolar area was reduced in untreatment group and increased in treaties with 5FU animals compared to the other control groups ($P<0.05$).

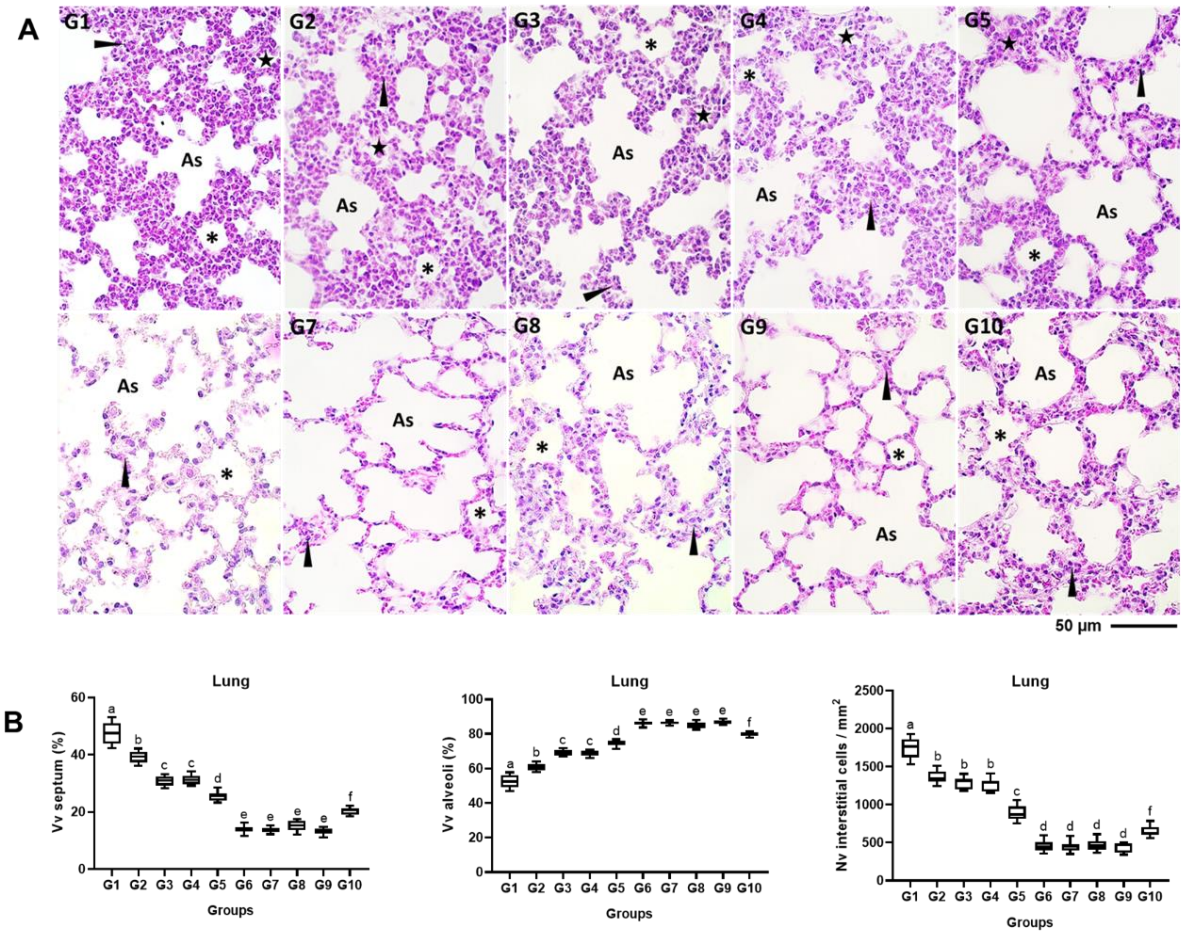

**Figure 7. Histological analysis of the lung of animals subjected to treatments.** Representative images (bright field microscopy, hematoxylin and eosin staining) (A). Groups= G1: Tumor + PBS, G2: Tumor + BMDM, G3: Tumor + BMDM+BCG, G4: Tumor + BCG, G5: Tumor + 5FU, G6: PBS, G7: BMDM, G8: BMDM+BCG, G9: BCG, G10: 5FU. Arrowhead: alveolar septum. Star: collapsed alveoli. Asterisk: Pulmonary alveoli. Graphic of quantitative microstructural analysis (B). Data are expressed as median and interquartile interval. $^{a,b,c,d,e,f}$ Different letters indicate statistical differences among the groups ($P<0.05$), and groups with the same letter present similar statistical results ($P>0.05$).

The histoarchitecture of mammary skin can be observed in Fig. 8A. In the control animals, the epidermis, dermis, and hypodermis were well-defined, with low cellularity, abundant connective tissue distribution, and clear profiles of sebaceous glands and hair follicles. In the tumor-induced groups, epidermal and dermal thickening was observed, accompanied by a marked increase in dermal cellularity composed of mononuclear and polymorphonuclear cells. A smaller accumulation of tumor cells was evident in the groups treated with BMDM+BCG and 5FU, as detailed in Fig. 8B. Tumor development was indicated by a large dermal accumulation of 4T1 cells, which exhibited enlarged, euchromatic nuclei with a round or ovoid morphology, along with numerous intensely basophilic nucleoli. At the tumor site, epidermal thickening was confirmed through histomorphometry in all inoculated groups compared to control animals ($P<0.05$).

Additionally, a pronounced dermal distribution of tumor cells was observed in the untreated, BMDM-treated, and BCG-treated groups, with a significant reduction in the numerical density of these cells in the BMDM+BCG and especially the 5FU groups ($P<0.05$). The ratio between the proportion of cellular nuclei and stromal tissue (Fig. 8C) was higher in all animals inoculated with 4T1 cells compared to non-inoculated control animals ($P<0.05$). This parameter was also reduced in the BMDM+BCG and 5FU mice compared to the other inoculated groups ($P<0.05$).

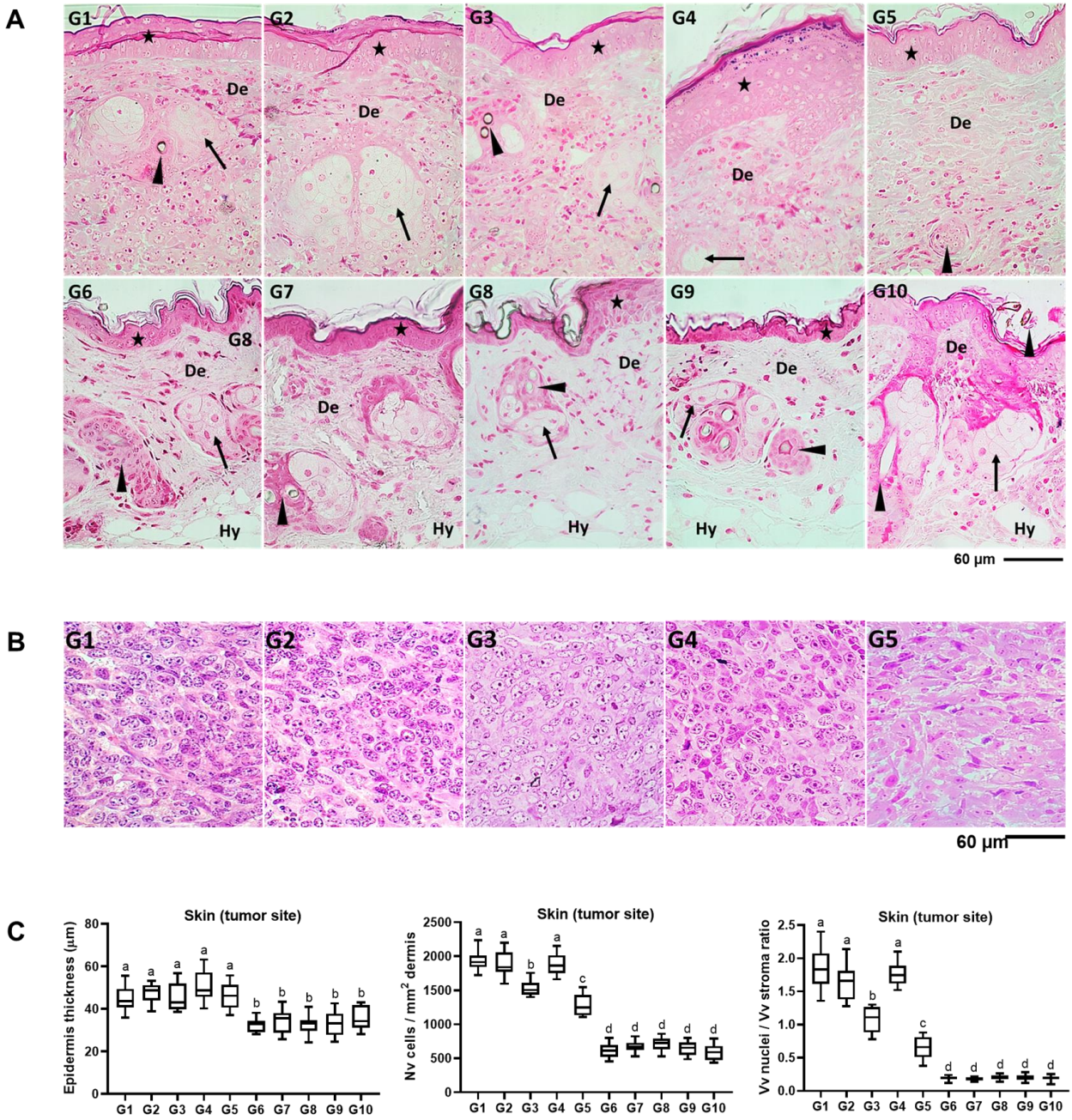


**Figure 8**. **Representative images of mouse breast skin subjected to treatments** (bright field microscopy, hematoxylin and eosin staining) (A). Groups= G1: Tumor + PBS, G2: Tumor + BMDM, G3: Tumor + BMDM+BCG, G4: Tumor + BCG, G5: Tumor + 5FU, G6: PBS, G7: BMDM, G8: BMDM+BCG, G9: BCG, G10: 5FU. Star: epidermis. De: dermis. Arrow: sebaceous gland. Arrowhead: hair follicle. Hy: hypodermis. Representative images of tumoral cells (B) and graphic of quantitative microstructural parameters (C). Data are expressed as median and interquartile interval. $^{a,b,c,d}$ Different letters indicate statistical differences among the groups ($P<0.05$), and groups with the same letter present similar statistical results ($P>0.05$).

**Discussion**

The effects of BCG in stimulating pro-inflammatory cells, which may aid in the treatment of bladder neoplasms, have been studied for over 30 years. The present study provides evidence that could expand its use for breast cancer control. Stimulation with BCG alone or combined with 4T1 proteins led to greater activation of macrophages resulting in lactate production, increased nitric oxide (NO), TNF-α, and delayed migration of 4T1 cells in vitro, unlike, stimulation with 4T1 cell proteins reduced the production of these components, increased IL-10 production and allow the migration of 4T1 cells. BCG stimulation promotes a metabolic state similar to M1 pro-inflammatory phenotype in immune cells, characterized by increased glucose uptake via glycolysis producing lactate. Recent studies have shown that BCG activates macrophages to an inflammatory state by binding to Toll-like receptors 2 and 4 (TLR2/4) through the MyD88 pathway, leading to increased NF-κB expression and secretion of TNF-α, IL-6, and NO (22). Corroborating these findings, recent studies observed increased production of TNF-α, IL-6, and IL-1β, as well as higher glucose consumption and lactate production when using BCG to stimulate macrophages (23-24).

Stimulation of BMDMs with 4T1 proteins, is associated with an M2 phenotype, favoring immune evasion (25). Similarly, studies have shown that co-culture of macrophages with 4T1 tumor cells induced a shift from M1 to M2 phenotype, with increased TGF-β and decreased TNF-α (26). In contrast, M1-profile BMDMs stimulated with endostatin (27) and BCG (28) exhibit high TNF-α expression and antitumor effects, such as inhibition of tumor cell migration and viability, reinforcing the role of M1 macrophages in the antitumor profile.

Since the mid-1980s, clinical trials have been conducted in cancer patients to study the adoptive transfer of macrophages generated from blood monocytes, demonstrating the safety and feasibility of macrophage reinfusion in patients (29). In the in vivo tumor model of the present study, treatment with BMDMs, whether unstimulated or stimulated with BCG, maintained 100% survival in the animals, suggesting safety of treatments. All tumor-bearing animals experienced weight loss and decreased food consumption, results associated with cancer-induced energy imbalance. This alteration, characterized by increased resting energy expenditure, is also linked to tumor competition for energy fuels and the tumor’s high intrinsic

metabolic rate (30). Animals treated with BCG showed higher cumulative food consumption but still lost weight, which can be explained by BCG-induced metabolic changes. BCG promotes a state of high cellular glucose utilization through anaerobic glycolysis, which demands more energy (31), potentially explaining weight loss despite higher caloric intake. Among treatments, the BMDM+BCG group showed the lowest mean weight loss and the second-highest total food intake, suggesting a lesser impact on energy metabolism, while the 5FU-treated group exhibited the highest weight loss, characteristic of cachexia observed during chemotherapy.

A previous study using a murine breast cancer model treated with tumor lysate combined with BCG observed a slight reduction in body weight (32). Similarly, studies reported that BCG treatment in bladder cancer did not result in significant body weight changes (33). The use of CAR-M macrophage immunotherapy in mice with breast cancer induced by 4T1 cells, also did not observe significant changes in body weight (34). Thus, the use of BCG may affect body weight differently depending on its combination with other therapies and the type of cancer studied.

The breast cancer can cause intestinal changes due to systemic inflammation (35). In this study, tumor-bearing animals showed greater fecal changes, especially those treated with 5FU. Were reported diarrhea and fecal bleeding in mice treated with 5FU, a chemotherapeutic agent associated with gastrointestinal toxicity, even in a single dose (36). Groups treated with BMDMs, with or without BCG stimulation, showed fewer changes, indicating a lesser impact on the gastrointestinal tract.

Recent studies have demonstrated the promising role of macrophage-based immunotherapies in combating various types of cancer. Macrophages modified to express IFN-α (37) macrophages modified by chimeric antigen receptor (CAR-M) (38) and CAR-M targeting CCR7-positive (39) demonstrated reduce tumor progression and inhibited metastasis in a murine breast cancer model with 4T1 cells. In the present study, treatment with BMDMs stimulated with BCG showed the best results in reducing tumor volume and weight, followed by treatment with unstimulated BMDMs.

The antitumor mechanisms of macrophages, including BMDMs and CAR-M, are widely discussed (40). Key mechanisms include direct cytotoxicity, NO release, and production of pro-inflammatory cytokines such as TNF-α. NO release can inhibit metabolic activities such as mitochondrial respiration and DNA replication, and TNF-α

production can activate pathways that induce apoptosis and increase ROS release, damaging DNA and leading to tumor apoptosis (41).

This study the treatment with BCG showed greater tumor volume compared to others treatments. Studies demonstrated the immunostimulatory effect of BCG as a treatment, recruiting macrophages and lymphocytes to the TME, mainly after its success in bladder cancer. Contrary to our findings, a recent study observed tumor reduction when combining radiotherapy and BCG in a murine tumor model induced by 4T1 cells (42). The use combined treatment of BCG with autologous tumor cells (ConvitVax) in the same tumor model and reported reduced tumor growth and greater immune cell infiltration into the TME (32). Thus, using BCG in breast cancer induced in animal models with 4T1 cells may be more effective when combined with other therapies.

Studies on BCG in different types of cancer seem controversial. One study reported greater tumor volume in mice with lung adenocarcinoma xenografts treated with BCG, (43) while other, observed tumor reduction in murine melanoma (44). Despite its consolidated use in bladder cancer, its mechanism of action is still not well understood. Adverse effects such as cystitis, hepatitis, and increased PD-L1 expression in tumor cells may limit its efficacy. Additionally, the strain used may influence therapeutic results, as according to some in vitro studies (45).

The results of this study demonstrate the importance of primary immune organs, such as the spleen, in antitumor immunity control. Increased spleen size in tumor-bearing animals, especially in treated groups, may indicate high local immune activity involving antigen presentation, recruitment, proliferation, and activation of lymphocytes, as also found in previous studies (46). Furthermore, spleen cells restimulated with BCG and 4T1 tumor proteins demonstrated different immune responses among treatments. The group treated BMDM and BMDM+BCG induced a pro-inflammatory response, with higher IFN-γ production, which is known to induce T cell activation signals and inhibit tumor suppressor cells in the tumor microenvironment, contributing to reduced tumor growth (47). In contrast, treatments with BCG and 5FU showed higher IL-10 production, characterizing an anti-inflammatory response. This immunosuppressive profile, with predominance of IL-10, may explain the lower efficacy of BCG treatment in reducing tumor volume and weight, as observed in this study. In contrast to our findings, previous studies

observed increased IFN-γ production after restimulation with BCG and tumor antigens in spleen cells from animals previously treated with BCG in melanoma (44) and bladder cancer models (48).

The analysis of blood smears reinforces our findings. Treatments with BMDM and BMDM+BCG showed increased peripheral lymphocytes and monocytes, which may result from the high immune cell load inoculated, indicating greater recruitment of immune cells beneficial in tumor combat. However, the increase in monocytes should be interpreted with caution, as it may be associated with worse prognosis in some contexts (49). The 5FU-treated group demonstrated hematotoxic effects, with reduced neutrophils and increased basophils, consistent with common side effects of chemotherapy treatments (50).

The histological analyses confirm the inflammatory response profile previously observed among the treatments. The group treated with BMDM+BCG exhibited a higher cellular infiltrate in the liver, demonstrating a more robust inflammatory response, whereas the group treated with BCG alone showed cellularity similar to the control groups, indicating a more controlled immune response. Regarding pulmonary tissue damage, both treatments displayed a significant degree of cellular infiltrate, with alveolar septal narrowing and potential tissue damage. In contrast, the groups treated with unstimulated BMDM, and the untreated group showed the most severe alterations and the greatest tissue damage compared to the other treatments. The 5FU treatment revealed greater hepatocyte vacuolization, indicating direct toxicity, a common systemic adverse effect of chemotherapeutic agents (50). In the lung, 5FU also demonstrated a significant reduction in inflammation and greater preservation of alveolar tissue, likely due to its direct cytotoxic effect on cancer cells. However, this treatment exhibited an immunosuppressive effect, consistent with previous findings of increased IL-10 production, an immunosuppressive cytokine, which limits the ability to generate a robust local immune response. Previous studies using the same in vivo tumor model with RAW264.7 macrophage inoculation demonstrates of metastasis in the lung and not in the liver (26).

In the histopathological analyses of mammary skin, a significant reduction in the accumulation of tumor cells was observed in the groups treated with BMDM+BCG and 5FU, with an even more evident decrease in the numerical density of these cells in these groups. The ratio between the proportion of the cellular

nucleus and the tissue stroma was also reduced in these groups, demonstrating effectiveness in eliminating cancer cells.

Thus, the robust inflammatory response observed with the BMDM+BCG treatment proved effective in locally combating cancer cells but may result in tissue damage that compromises the architecture and normal function of the liver and lungs. On the other hand, treatment with BCG partially preserved the tissues, with less apparent inflammatory infiltration, suggesting a more controlled inflammatory response that protects tissue integrity. However, it was insufficient to prevent local tumor growth.

Therefore, we can conclude that treatment with BMDMs stimulated with BCG shows promise in reducing tumor growth and improving clinical parameters, such as body weight and food intake. However, strategies must be combined to modulate the exacerbated inflammatory response, reversing possible tissue damage that may impair the integrity and function of organs such as the liver and lungs. This study contributes to a better understanding of the immunomodulatory role of BCG, confirms the antitumor efficacy of macrophages activated to a pro-inflammatory profile, and paves the way for exploring safe and effective therapeutic strategies using these approaches against triple-negative breast cancer.

## Potential Conflicts of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Authors' Contribuitions

**Joyce Alves dos Santos:** Conceptualization; investigation; development methodology; validation; analysis and interpretation of data, writing - original draft; review of the manuscript. **Evandro Neves da Silva:** Methodology. **Thiago Caetano Andrade Belo:** Methodology. **Ana Clara Moreira:** Methodology. **Yasmin Vieira Braida:** Methodology. **Leonardo Augusto de Almeida:** Conceptualization; Formal analysis; Funding acquisition; Project administration; Resources; Review. **Patrícia Paiva Corsetti:** Project administration; Resources; Supervision; Conceptualization; Data curation; Acquisition of animals; Formal analysis; Funding acquisition; Investigation; Supervision; Validation; revision of the manuscript.

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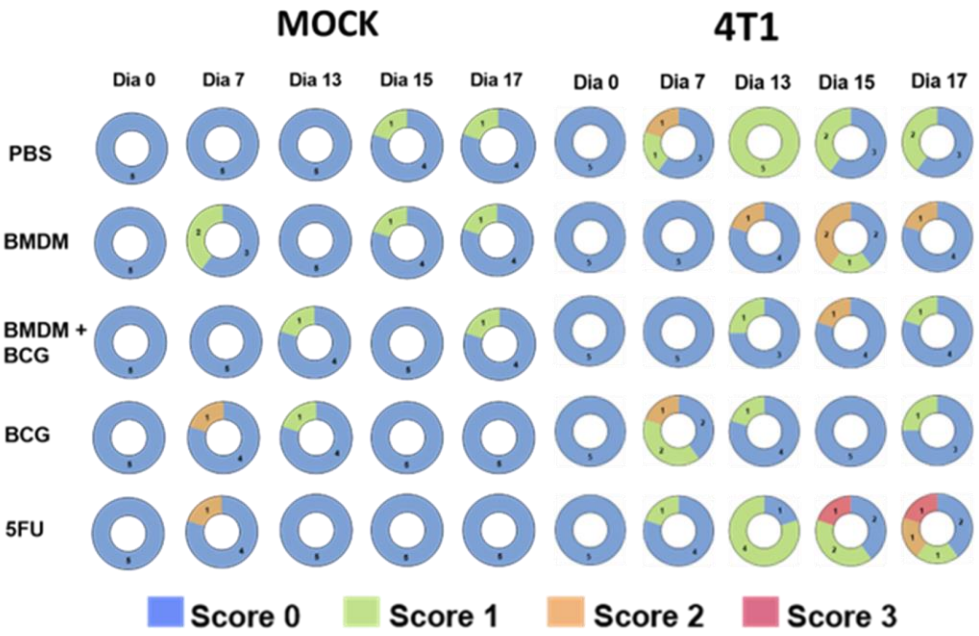
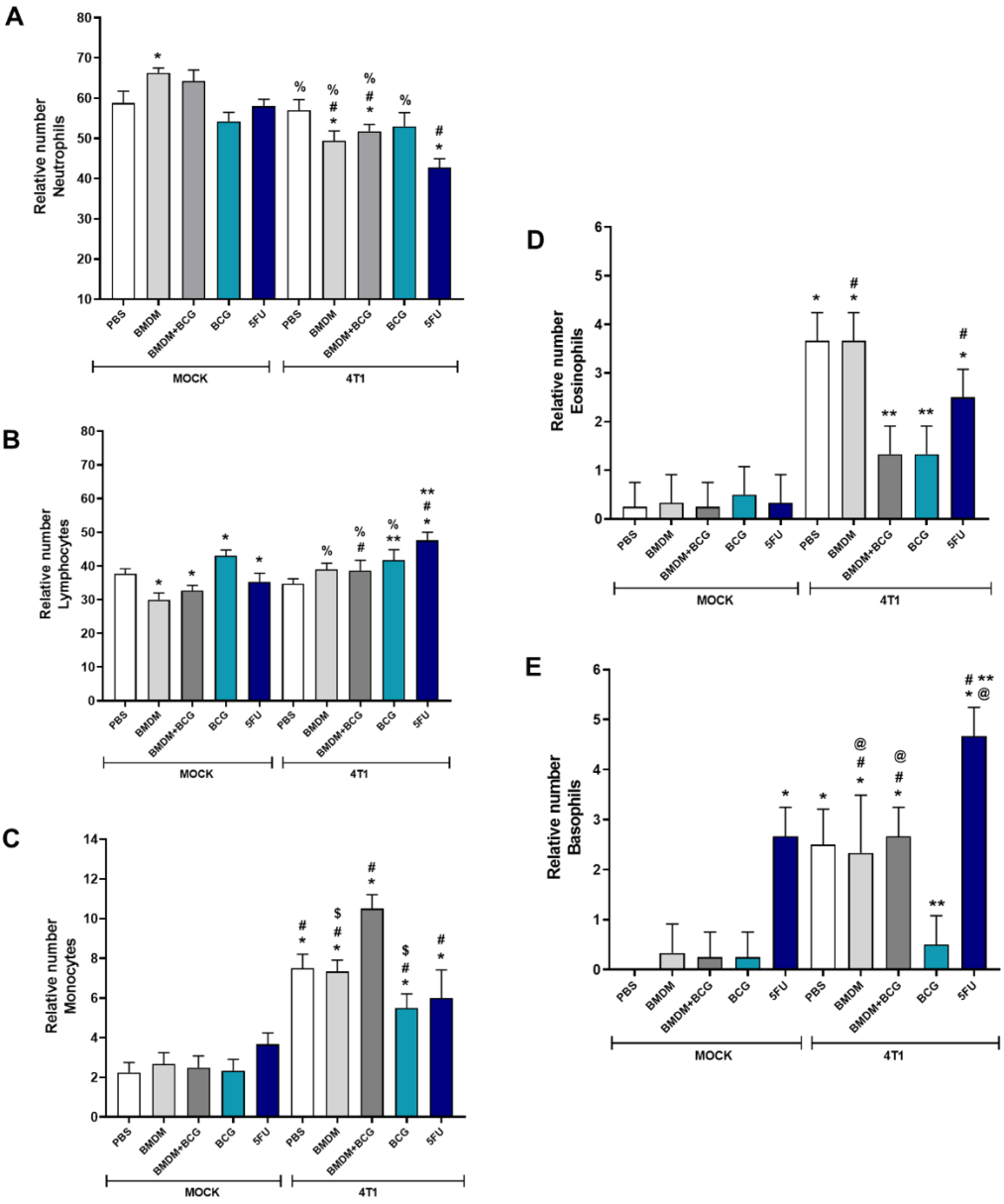
## Supplementary figures




**Supplementary figure S1. Qualitative fecal score evaluation on days 0, 7, 13, 15, and 17 in control and 4T1 groups.** Score 0: normal (firm stools); 1: soft consistency (soft and formed stools); 2: mild diarrhea (fluid stools); and 3: severe diarrhea (watery and profuse stools). All values are means ± SD (n=5).




**Supplementary Figure 2. Leukocyte alterations related to the presence of breast cancer in a murine model.** From the analysis of blood smears, the relative counts of neutrophils (A), lymphocytes (B), monocytes (C), eosinophils (D), and basophils (E) were performed. * p<0.05 compared to PBS/control; # p<0.05 compared to the corresponding control; ** p<0.05 compared to PBS/4T1; @ p<0.05 compared to BCG/4T1; % p<0.05 compared to 5FU/4T1; $ p<0.05 compared to BMDM+BCG/4T1. All values are expressed as mean ± SD (n=5).

## 5 CONSIDERAÇÕES FINAIS

O estímulo com BCG em BMDMs levou os macrófagos a um perfil pró-inflamatório M1, impedindo parcialmente a migração de células 4T1 in vitro, enquanto o estímulo com PTN4T1 levou a um perfil anti-inflamatório M2 que favoreceu a migração celular. In vivo, o tratamento com BMDMs estimulados com BCG evitou maior perda de peso, melhorou consumo alimentar dos animais e foi eficaz na diminuição do crescimento tumoral local, caracterizado por um perfil de resposta imune mais pró-inflamatório, eficaz para eliminação das células cancerosas. A histopatologia local do tecido mamário, confirmou sua eficácia, demonstrando diminuição da presença de células 4T1 no tecido mamário após tratamento com BMDM+BCG, semelhante ao observado no grupo tratado com 5FU. Por outro lado, o tratamento com BCG isolado não foi eficaz na redução do volume tumoral, caracterizado por um perfil de resposta anti-inflamatório que preservou integridade tecidual dos tecidos hepático e pulmonar. A resposta gerada com o tratamento com BMDM+BCG, apesar de eficaz na redução do volume tumoral, levou a um maior dano tecidual no fígado e pulmão, colocando em risco a função dos órgãos e saúde geral. Dessa forma, pode-se concluir que o tratamento com BMDMs estimulados com BCG possui eficácia no controle do crescimento do câncer de mama triplo negativo induzido em modelo murino, no entanto estudos adicionais são necessários para avaliar os efeitos a longo prazo desse tratamento bem como explorar abordagens para controlar a inflamação de forma a minimizar danos nos órgãos. Este estudo abre caminhos futuros para o desenvolvimento de terapias a partir de células imunes eficazes contra o câncer de mama triplo negativo.

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# ANEXOS

## ANEXO A – CEUA UNIFAL

**MINISTÉRIO DA EDUCAÇÃO**
**Universidade Federal de Alfenas - UNIFAL-MG**
Lei nº 11.154, de 29 de julho de 2005

**Comissão de Ética no Uso de Animais - CEUA/UNIFAL-MG**

# Certificado

Certificamos que a proposta intitulada **Avaliação da Imunoterapia celular adotiva induzida pelo Mycobacterium bovis (bacilo Calmette-Guérin)-BCG contra o tumor de mama triplo negativo em modelo murino**, registrada com o nº **0039/2022**, sob a responsabilidade de **Patricia Paiva Corsetti de Almeida**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de **pesquisa científica**, com vigência de **01/03/2023 a 28/02/2027**, 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 de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA-UNIFAL) DA UNIVERSIDADE FEDERAL DE ALFENAS.

| Espécie/linhagem/raça | Total de animais | Total de machos | Total de fêmeas | Origem |
|---|---|---|---|---|
| Roedor / Balb/c | 109 | 0 | 109 | Biotério central da ufmg |

Alfenas, 17 de Fevereiro de 2023

***Prof(a). Dr(a). Pollyanna Francielli de Oliveira***
Coordenador(a) do CEUA/UNIFAL - MG

Para verificar autenticidade acesse: http://sistemas.unifal-mg.edu.br/app/ceua/autenticidadecertificado/ e digite a chave: d9d270ecd334e36f536502d017e4f91e

**ANEXO B – Normas da Revista *Cancer Research***

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