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Low-Dose Chemotherapy Impact on Metastatic Breast Cancer: A Pre-clinical Study

Impacto da quimioterapia em baixas doses no câncer de mama metastático: um estudo pré-clínico

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BRASÍLIA, DF
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Doctoral Thesis presented to the
Program of Post-graduate in Animal Biology,
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the requirements to obtain the title of Doctor
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Supervisor : Prof. Dr. João Paulo Figueiró Longo

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I dedicate this work to my parents, brother, grandparents, and entire family for their unwavering love, support, and encouragement.

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List of publications and conference attended

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Dileep Kumar, Luana Cristina Camargo, Victor Carlos Mello da Silva, Luis Alexandre Muehlmann, Thyago Jose Arruda Pacheco, Joao Paulo Figueiro Longo. Low-Dose Chemotherapy Impact on Metastatic Breast Cancer: A Pre-clinical Study (in preparation).

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Dileep Kumar, Camila Cardador, João Paulo Figueiró Longo To target and decrease the myeloid-derived suppressor cells (MDSCs) population through the treatment of low dose chemotherapy with cyclophosphamide and 5-fluorouracil. II International Conference of Nanoscience and Nanobiotechnology, May, 26 - 28th, 2021 Brasília, Brazil, Virtual (Poster presentation).

Dileep Kumar, Luana Cristina Camargo, Victor Carlos Mello da Silva, Thyago Jose Arruda Pacheco, Luis Alexandre Muehlmann, João Paulo Figueiró Longo Effect of Low-dose Chemotherapy in a Metastatic Pre-clinical Breast Cancer Model. III International Conference of Nanoscience and Nanobiotechnology, August, 16 - 18th, 2023 Brasília, Brazil, (Poster presentation).

ABSTRACT

Metastatic breast cancer is a major cause of morbidity and mortality among women globally. While early-stage breast cancer is often diagnosed and treated effectively, advanced metastatic cases pose significant challenges. Treatment for metastatic breast cancer typically involves a combination of surgery, radiotherapy, chemotherapy, hormonal therapy, and immunotherapy. The primary goals are to control the disease, alleviate symptoms, prolong survival, and enhance the patient's quality of life. Treatment choice depends on cancer type, stage, hormone receptor status, HER2 presence, and individual patient factors, and it often evolves based on treatment response and disease progression. Despite available treatments, metastatic breast cancer remains difficult to manage and often requires personalized strategies. Researchers aim to understand the mechanisms of metastasis to develop more effective treatments. One key mechanism involves immunosuppression, where proliferating tumor cells evade immune detection. Myeloid Derived Suppressor Cells (MDSC) play a significant role in this process by suppressing the antitumor immune response, leading to treatment resistance.

This study investigates the effects of low-dose chemotherapy (LDC) using cyclophosphamide (CP) and 5-fluorouracil (5-FU) on the tumor microenvironment and immune response in a preclinical metastatic breast cancer model. The hypothesis is that LDC can reduce MDSC populations, enhancing the antitumor immune response. The main objective is to evaluate LDC's therapeutic effects on metastatic breast cancer in mice. Specific objectives include assessing overall body weight and survival, tumor progression, lung metastasis, spleen size, and MDSC population. The study used mice inoculated with 4T1-luc cells, derived from a murine mammary adenocarcinoma. Mice were divided into treatment and control groups, receiving different LDC

regimens with CP or 5-FU. Assessments included body weight, survival, primary tumor size, and bioluminescence imaging. Spleen, lung, and blood samples were analyzed for histopathology, immunophenotyping, and MDSC quantification. Results indicated that LDC was well-tolerated, with no significant impact on body weight or survival.

LDC effectively inhibited primary tumor growth and reduced lung metastasis progression, particularly with 5-FU. Histopathological analysis confirmed the absence of metastases in treated animals' lungs. LDC also reduced MDSC populations, suggesting an immunomodulatory effect. Hematological analysis showed changes in leukocyte parameters, indicating an active immune response. In summary, LDC demonstrates potential as an effective and well-tolerated treatment for metastatic breast cancer, with benefits in inhibiting tumor growth and modulating the immune response. These findings provide a foundation for further research to optimize LDC and explore its use in other cancers.

Keywords: Metastatic Breast Cancer, Immunosuppression, Myeloid Derived Suppressor Cells, Tumor Microenvironment, Low-Dose Chemotherapy, Cyclophosphamide, 5-Fluorouracil, Antitumor Immune Response.

RESUMO

O câncer de mama metastático é uma das principais causas de morbidade e mortalidade entre as mulheres em todo o mundo. Enquanto o câncer de mama em estágio inicial é frequentemente diagnosticado e tratado de forma eficaz, casos avançados e metastáticos apresentam desafios significativos. O tratamento para o câncer de mama metastático geralmente envolve uma combinação de cirurgia, radioterapia, quimioterapia, terapia hormonal e, mais recentemente, imunoterapia. Os objetivos principais são controlar a doença, aliviar os sintomas, prolongar a sobrevivência e melhorar a qualidade de vida do paciente. A escolha do tratamento depende de vários fatores, como o tipo de câncer de mama, o estágio da doença, a presença de receptores hormonais ou HER2, além das características individuais do paciente, e frequentemente evolui com base na resposta ao tratamento e na progressão da doença. Apesar dos tratamentos disponíveis, o câncer de mama metastático continua difícil de manejar e muitas vezes requer estratégias personalizadas. Pesquisadores buscam entender os mecanismos da metástase para desenvolver tratamentos mais eficazes. Um mecanismo chave envolve a imunossupressão, onde células tumorais proliferativas evitam a detecção imunológica. As células supressoras derivadas de mieloides (MDSC) desempenham um papel significativo nesse processo, suprimindo a resposta imune antitumoral e levando à resistência ao tratamento.

Este estudo investiga os efeitos da quimioterapia em baixa dose (LDC) usando ciclofosfamida (CP) e 5-fluorouracil (5-FU) no microambiente tumoral e na resposta imunológica em um modelo pré-clínico de câncer de mama metastático. A hipótese é que a LDC pode reduzir as populações de MDSC, aprimorando a resposta imune antitumoral. O objetivo principal é avaliar os efeitos terapêuticos da LDC no câncer de mama metastático em camundongos. Objetivos específicos incluem avaliar o peso corporal e a sobrevivência geral, a progressão do tumor, a metástase pulmonar, o tamanho do baço e a população de MDSC. O estudo utilizou camundongos inoculados com células 4T1-luc, derivadas de um adenocarcinoma mamário murino. Os camundongos foram divididos em grupos de tratamento e controle, recebendo diferentes regimes de LDC com CP ou 5-FU. As avaliações incluíram peso corporal, sobrevivência, tamanho do tumor primário e imagens de bioluminescência. Amostras de baço, pulmão e sangue foram analisadas para histopatologia, imunofenotipagem e quantificação de populações de MDSC. Os

resultados indicaram que a LDC foi bem tolerada, sem impacto significativo no peso corporal ou na sobrevivência.

A LDC inibiu efetivamente o crescimento do tumor primário e reduziu a progressão da metástase pulmonar, especialmente com 5-FU. A análise histopatológica confirmou a ausência de metástases nos pulmões dos animais tratados. A LDC também reduziu as populações de MDSC, sugerindo um efeito imunomodulador. A análise hematológica mostrou mudanças nos parâmetros de leucócitos, indicando uma resposta imunológica ativa. Em resumo, a LDC demonstra potencial como um tratamento eficaz e bem tolerado para o câncer de mama metastático, com benefícios na inibição do crescimento tumoral e na modulação da resposta imunológica. Estes achados fornecem uma base para futuras pesquisas visando otimizar a LDC e explorar seu uso em outros tipos de câncer.

Palavras-chave: Câncer de mama metastático, Imunossupressão, Células Supressoras Derivadas de Mieloides, Microambiente Tumoral, Quimioterapia de Baixa Dose, Ciclofosfamida, 5-Fluorouracil, Resposta Imunológica Antitumoral.

RESUMO ESTENDIDO

Introdução: O câncer de mama metastático é uma das principais causas de morbidade e mortalidade em mulheres em todo o mundo. Felizmente a maior parte dos diagnósticos do câncer de mama é realizado em estágios iniciais da doença, onde a característica metastática ainda não se desenvolveu. Porém, quando o diagnóstico é realizado em um processo mais avançados da doença, os métodos terapêuticos convencionais ainda não são totalmente efetivos, até o momento. Em termos de opções terapêuticas, o câncer de mama metastático geralmente envolve a combinação de diferentes terapias, incluindo a cirurgia, a radioterapia, a quimioterapia, a terapia hormonal e mais recentemente a imunoterapia. O objetivo do tratamento é controlar a doença, aliviar os sintomas, prolongar a sobrevida e melhorar a qualidade de vida da paciente. A escolha do tratamento depende de vários fatores, como o tipo de câncer de mama, o estágio da doença, a presença de receptores hormonais ou HER2, além das características individuais da paciente. A abordagem terapêutica pode ser modificada ao longo do tempo com base na resposta ao tratamento e na progressão da doença. Embora muitas opções terapêuticas estejam disponíveis, o câncer de mama metastático continua sendo uma condição desafiadora de tratar e muitas vezes requer uma abordagem personalizada e contínua para otimizar os resultados clínicos. Neste contexto, cientistas ao redor do mundo tentam compreender melhor os principais mecanismos desta doença com o objetivo de poder controlar de forma mais efetiva a progressão e evolução da mesma.

Dentre os mecanismos utilizados pelas células metastáticas de mama, a imunossupressão é uma bastante desafiadora do ponto de vista do manejo da paciente oncológica. Nestas situações, as células tumorais em proliferação criam um ambiente com baixa vigilância imunológica com o objetivo de proliferar sem as pressões das células de defesa. Entre os mecanismos celulares

envolvidos neste processo estão o desenvolvimento de algumas células mieloides da própria paciente, chamadas de células imunosupressoras imaturas, do inglês *Mieloid Derived Supressor Cells* (MDSC), que suprimem a resposta imune contra as defesas antitumorais. Estas células MDSC têm sido implicadas na promoção da progressão tumoral e na supressão da resposta imunológica antitumoral, contribuindo para a resistência ao tratamento. Diante disso, este projeto se propõe a investigar minuciosamente os efeitos terapêuticos de uma abordagem alternativa: a quimioterapia de baixa dose (QBD), especificamente utilizando ciclofosfamida (CP) e 5-fluorouracil (5-FU), na tentativa de modular o microambiente tumoral e potencializar a resposta imunológica contra o câncer de mama metastático. Esta hipótese é fundamentada em alguns estudos da literatura que indicam que a quimioterapia em baixa dose com estes quimioterápicos seria capaz de reduzir as populações de MDSC, permitindo assim uma recuperação da resposta imune antitumoral. **Objetivo Principal:** O objetivo principal desta pesquisa é avaliar, em profundidade, os efeitos terapêuticos da QBD no câncer de mama metastático em um modelo murino. Para alcançar esse objetivo, serão definidos os seguintes objetivos específicos: Em primeiro lugar, examinar os efeitos da quimioterapia em baixas doses no peso corporal geral dos ratos e na taxa de sobrevivência. Em segundo lugar, para avaliar a terapia com baixas doses na progressão do tumor. Em terceiro lugar, avaliar a terapia com baixas doses na progressão de metástases pulmonares à distância em camundongos portadores de tumor de câncer de mama. Além disso, analisar o peso do baço, o tamanho e as quantificações da população de MDSCs imunossupressoras. Finalmente, caracterizar a modificação da população de leucócitos sob o tratamento. **Metodologia**: Este estudo utilizou um modelo murino de câncer de mama metastático pré-clínico, no qual camundongos foram inoculados com células 4T1-luc (células transformadas com o gene da luciferase), derivadas de um adenocarcinoma mamário murino. Os animais foram

divididos em grupos de tratamento e controle, sendo submetidos a diferentes regimes de QBD com CP ou 5-FU. Foram realizadas avaliações periódicas do peso corporal, da taxa de sobrevivência e do tamanho do tumor primário, utilizando técnicas de imageamento in vivo, por meio do rastreio da bioluminência das células 4T1. Além disso, foram coletadas amostras de baço, pulmão e sangue periférico para análise histopatológica, imunofenotipagem e quantificação de populações de MDSC. A resposta imunológica foi avaliada por meio de ensaios de citometria de fluxo para fenotipagem das células MDSC. **Resultados e Discussão:** A evasão imune é uma característica típica do estágio metastático, durante a evolução do câncer. Durante este processo, as células tumorais desenvolvem vários mecanismos de supressão da resposta imune, que permitem que elas progridam, sem que haja uma repressão contra este progresso tumoral. Nesse sentido, abordagens terapêuticas, como a QBD são fundamentais para tentar frear esse processo, promovendo uma alternativa terapêutica às opções atualmente disponíveis.

A terapia QBD oferece vantagens significativas nesse contexto, pois não apenas reduz os efeitos adversos da quimioterapia convencional, mas também tem o objetivo de otimizar a resposta imunológica contra os tumores metastáticos. Para o presente estudo avaliamos a QBD contra modelos animais de câncer de mama metastático. Entre os resultados, observamos que o protocolo utilizado não impactou significativamente o peso corporal ou a sobrevivência dos animais tratados, demonstrando uma boa tolerabilidade dos regimes de tratamento selecionados. Além disso, observou-se uma inibição efetiva do crescimento do tumor primário e uma redução na progressão da metástase pulmonar nos grupos tratados com QBD em comparação com o grupo controle, que não recebeu tratamento. Este efeito terapêutico foi particularmente evidente com o uso de 5-FU, que apresentou uma atividade terapêutica com relação à presença de metástases pulmonares. Adicionalmente, a QBD com CP ou 5-FU levou a uma redução no peso tumoral e confirmou-se

essa redução através de imagens de bioluminescência. Os achados também mostraram que a QBD inibiu a progressão da metástase pulmonar, uma descoberta promissora dada a importância clínica de controlar a disseminação do câncer para outros órgãos.

Adicionalmente, a análise histopatológica corroborou essa observação, evidenciando a ausência de metástases nos pulmões dos animais tratados. Além disso, a QBD teve um impacto positivo na redução das populações de células supressoras do sistema imunológico, particularmente das células MDSC, que desempenham um papel crucial na promoção do crescimento tumoral. Esses resultados indicam um efeito imunomodulatório significativo da QBD, além de seus efeitos diretos sobre o crescimento tumoral. A análise hematológica revelou alterações nos parâmetros leucocitários, sugerindo uma modulação da resposta imune pela QBD. Em particular, observou-se uma redução nas populações totais de glóbulos brancos e na porcentagem de linfócitos nos grupos tratados, indicando uma resposta imunológica mais ativa. A QBD também afetou os parâmetros eritrocitários, sugerindo efeitos sobre a eritropoiese. Em resumo, os resultados deste estudo destacam o potencial da QBD como uma estratégia terapêutica eficaz e bem tolerada para o tratamento do câncer de mama metastático, com efeitos benéficos tanto na inibição do crescimento tumoral quanto na modulação da resposta imunológica do hospedeiro. Essas descobertas fornecem uma base sólida para pesquisas futuras visando otimizar ainda mais o uso da QBD e explorar seu potencial em outros tipos de câncer.

Palavras-chave: Câncer de mama metastático, Imunossupressão, Células Supressoras Derivadas de Mieloides, Microambiente Tumoral, Quimioterapia de Baixa Dose, Ciclofosfamida, 5-Fluorouracil, Resposta Imunológica Antitumoral.

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LIST OF ABBREVIATIONS AND ACRONYMS

BC	: Breast cancer
bFGF	: basic fibroblast growth factor
CP	: Cyclophosphamide
DMEM	: Dulbecco's modified Eagle's medium
EDTA	: Ethylene diamine tetra acetic acid
FITC	: Fluorescein isothiocyanate
5-FU	: 5-fluorouracil
GM-CSF	: Granulocyte-macrophage colony-stimulating factor
HCT	: Hematocrit
H&E	: Hematoxylin and Eosin
HGB	: Hemoglobin
HSCs	: Hematopoietic stem cells
IFN- γ	: Interferon-gamma
IL	: Interleukin
LDC	: Low-dose chemotherapy
LCR	: Large cell ratio
MCV	: Mean corpuscular volume
MCH	: Mean corpuscular hemoglobin
MCHC	: Mean corpuscular hemoglobin concentration
MDSC	: Myeloid-derived suppressor cells
MMPs	: Matrix metalloproteinases
NK	: Natural killer
QBD	: Quimioterapia de baixa dose

ROI	: Region of Interest
ROS	: Reactive oxygen species
RBC	: Red blood cells
RDW-CV	: RBC-distribution width variations co-efficient
RDW-SD	: RBC-distribution width standard deviation
SCR	: Small cell ratio
S.C	: Subcutaneous
TME	: Tumor microenvironment
4-T1 Luc	: 4-T1 Luciferase
TNF-α	: Tumor necrosis factor alpha
TGF-β	: Transforming growth factor-β
VEGF	: Vascular endothelial growth factor
W-MCR	: White Blood cell - Middle cell ratio

SUMMARY

BACKGROUND

1.1 Metastatic breast cancer

Cancer is a term used to describe a collection of more than 100 different malignant diseases. These diseases are characterized by the uncontrolled and disorderly growth of cells. The rapidly dividing cells can be highly aggressive and invasive, often spreading to nearby tissues or distant organs, leading to the development of tumors. Metastasis breast cancer is a process of cancer spreading, it happens when a breast cancer (BC) cell travel through the bloodstream or the lymphatic system to the other distant regions of the body. Among women globally, breast cancer metastasis stands as the prevailing concern. In the year 2020 alone, an estimated 2.3 million new cases of breast cancer were diagnosed worldwide (Łukasiewicz et al. 2021; Sung et al. 2021; Lei et al. 2021). Failure of treatment may result in the recurrence of the disease either locally or at distant sites and the proliferation of recurrent malignant cells, which evaded elimination during the initial therapeutic approach, can account for local recurrence (Mahvi et al. 2018).

Patients diagnosed with BC malignant tumors exhibiting metastases demonstrate advanced systemic illness, including splenomegaly, hepatic failure, alveolar hemorrhage, dyspnea, and hemoptysis (Kazawa et al. 2021; Gould Rothberg et al. 2022). Only a small percentage of patients survive beyond one year following a diagnosis of metastasis (Rothberg et al. 2022). Specifically, around 20% of individuals with solid tumors exhibit lung-limited metastases that are not detectable

in other organs (Kazawa et al. 2021). Managing the pulmonary dissemination of malignant cancers is therefore essential from a therapeutic perspective. One hallmark of tumor growth is immune evasion, which allows tumor cells to evade and suppress anti-tumor immune responses (Mortezaee 2020). As a result, the formation of primary tumors and subsequent metastases occurs. The selection of treatment is contingent upon several factors, including the subtype of breast cancer, the disease's stage, the presence of hormone receptors or HER2, as well as the individual characteristics of the patient (Almeida et al. 2024). One of the cellular mechanisms implicated in this process entails the generation of certain myeloid cells within the patient, known as immature immunosuppressive cells, specifically Myeloid Derived Suppressor Cells (MDSCs), which inhibit the immune response directed towards antitumor defenses.

1.2 MDSCs as immunosuppressive regulators in breast cancer progression

In the course of cancer development, diverse cellular productions are instigated within the tumor microenvironment (TME). Among these, during chronic inflammatory condition arises the generation of immature immunosuppressive myeloid cells by extreme reduction in peripheral myeloid cells, which perturb the equilibrium between the myeloid and lymphoid lineages. And, contributes to the production of immature myeloid cells with an immunosuppressive phenotype by coupling with raised cytokine levels and tumor-derived factors (Zhao, Du and Shen 2023; Calderon et al. 2023) and, known as MDSCs (Figure 1).

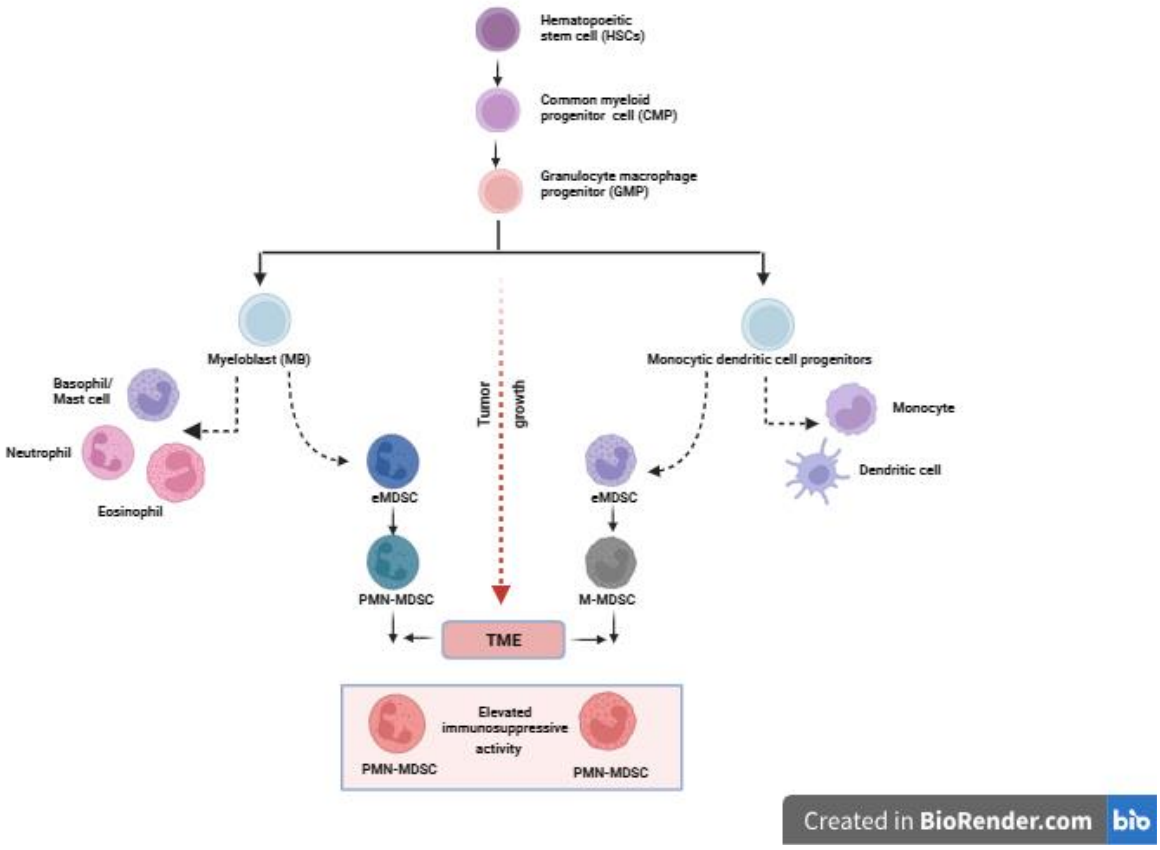


Figure 1. Schematic representation of the from the hematopoietic stem cells (HSCs) to the end point of altered myelopoiesis. The phenomenon of immature pathologically activated and differentiated immunosuppressive MDSCs in chronic inflammation condition in cancer. (Created with BioRender.com)

This deviation from normal myeloid cell development leads to the generation of MDSCs, which exhibit potent immunosuppressive capabilities within the TME (Wu et al. 2022). Its exerting immunosuppressive effects, constitute a pivotal element within the TME. Its role by contributory to tumor advancement via diverse non-immunological pathways secreting soluble factors MMPs, VEGF, TGF-β, and others, including the facilitation of neovascularization, proliferation, invasion,

creation of pre-metastatic niches and the metastasis (Li et al. 2021; Ya et al. 2022; Cheng et al. 2021). MDSCs release various factors, including VEGF, bFGF, TGF-β, MMPs, and S100A8/A9 a binding protein that modulate the inflammatory response and induce cytokine secretion. These factors promote cancer neovascularization, proliferation, invasion, and metastasis (Figure 2).

MDSCs exert their influence by dampening the innate immune response and impeding the progression of the adaptive immune response. Additionally, they release cytokines, interact with various immune cells, and stimulate the proliferation of other immunosuppressive cell populations. Particularly, MDSCs hinder the anti-tumor activities of T and NK cells, thereby facilitating the immune evasion mechanisms employed by malignant tumors (Dong et al. 2022; Cheng et al. 2023).

The immunosuppressive mechanisms mediated by MDSCs are a major contributor to tumor progression and metastasis. Research indicate that growth factors produced by tumors are accountable for instigating the aforementioned conditions in individuals afflicted with cancer (Li et al. 2021).

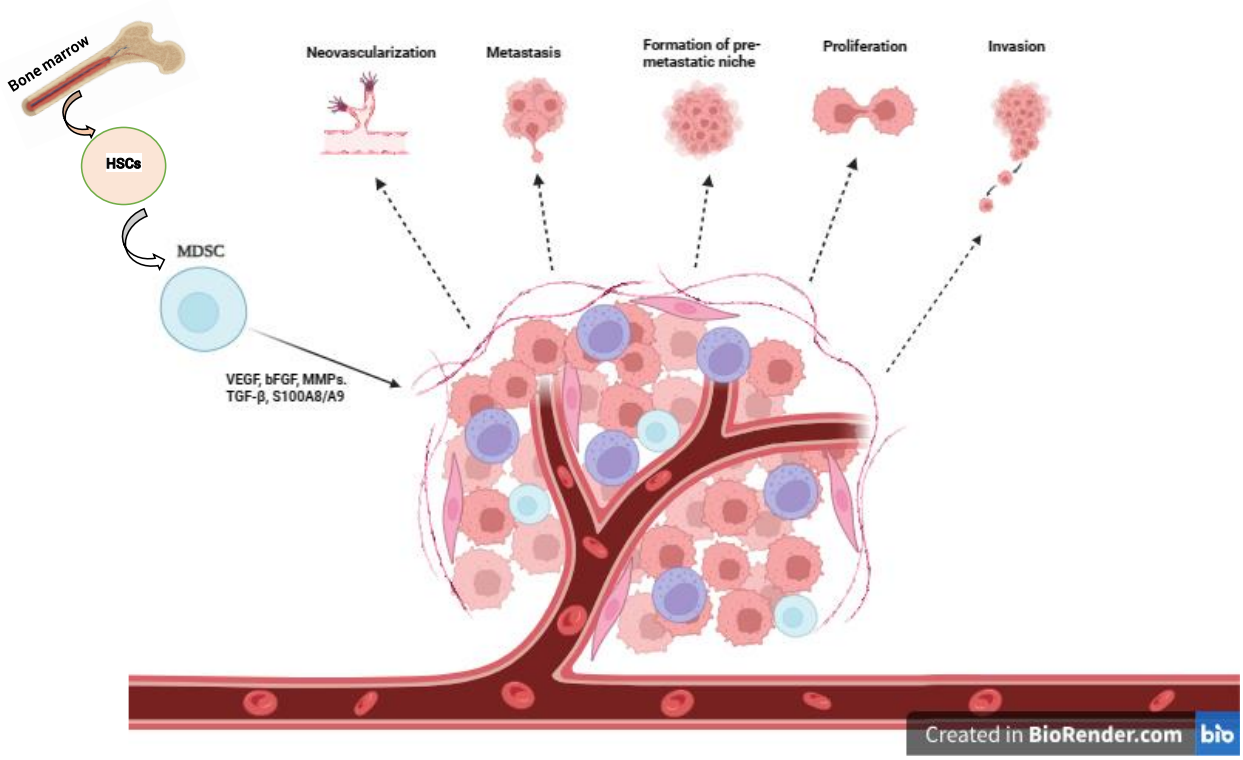


Figure 2. Development of tumor and metastasis reinforced by MDSCs. MDSCs releases factors Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), Transforming growth factor-β (TGF-β), Matrix metalloproteinases (MMPs), S100A8/A9 (binding proteins Ca^{2+} serves as modulating the inflammatory response and inducing cytokine secretion) which promotes cancer neovascularization, proliferation, invasion and metastasis. (Created with BioRender.com)

Phenomenon within the TME, increased production of inflammatory mediators, for example tumor necrosis factor alpha (TNF-α), colony-stimulating factor (GM-CSF), and IL1b, IL5, IL10, interferon-gamma (IFN- γ) due to the rise the number of suppressive MDSCs ($Gr1^+CD11b^+$) (Krajnak et al. 2020) Markers CD33 and CD11b show a positive expression from MDSCs in humans, whereas representative reduced HLA-DR expression levels, in humans (Zhao, Du and Shen 2023). CD11b and $Gr1^+$ display a positive expression from MDSCs, where the

Ly6C/Ly6G epitopes of the Gr1 antigen distinguish between monocytic (mMDSCs) ($Ly6G^{-}$ $Ly6C^{hi}$) and polymorphonuclear MDSCs (PMN-MDSCs) or granulocytic myeloid-derived suppressor cells (gMDSCs) ($Ly6G^{+}Ly6C^{low}$) subpopulations, respectively, in mice. Generating of cytokines, arginase and reactive oxygen species (ROS) by these cells, they employ suppressive activity to the immune system (Li et al. 2021). The quantity of MDSCs tends to escalate with the burden of cancer, while the suppression of MDSCs leads to enhanced disease outcomes in murine models (Cicco, Ercolano and Ianaro 2020). Addressing these immunosuppressive MDSCs, which contribute to tumor progression and metastasis, necessitates the development of targeted therapeutic strategies.

1.3 Low-dose chemotherapy

Certain therapeutic approaches aim not only to eliminate tumor cells but also, to target abnormal immune cell populations, thereby impairing the systemic immunosuppression facilitated by these myeloid cells. For instance, chemotherapy kills rapidly growing cells such as cancer cells. Despite substantial advancement and the developing use of immunotherapies, chemotherapy continues to be a widely chosen therapeutic approach for treating BC (Anand et al. 2023).

Low-dose chemotherapy (LDC) is a targeted approach that helps to control cancer by administering low doses of chemotherapy drugs that causes less side effects, much safer than standard conventional chemotherapy (Xie et al. 2017; Krajnak et al. 2020). LDC has drawn

attention in part as a result of mounting evidence that certain chemotherapy drugs can specifically inhibit the suppressive arm of the immune response (Yuan et al. 2021). Furthermore, by modulating the tumor microenvironment, inhibiting angiogenesis, and stimulating immune responses, LDC produces indirect effects on tumor cells (Krajnak et al. 2020)

1.4 Cyclophosphamide

Cyclophosphamide (CP) ($C_7H_{15}Cl_2N_2O_2P$, Figure 3) is a member of the oxazaphosphorine family of mustard-alkylating agents, this particular chemotherapy drug is widely regarded as one of the most effective and successful medications in the field. Moreover, it is included in the World Health Organization's essential medicines list. Nevertheless, cyclophosphamide is still employed, either as a standalone treatment or as adjuvant therapy, for conditions such as lymphomas, breast cancer, and ovarian cancer at reduced doses (Madondo, Quinn and Plebanski 2016; Ali et al. 2024). The effectiveness of low-dose cyclophosphamide mainly stems from its capacity to enhance anti-tumor immune response through the targeted reduction of regulatory T cells and the augmentation of effector T cell activity (Mortezaee 2024; Madondo, Quinn and Plebanski 2016). Transitioning from immunosuppressive to immunostimulatory effects by LDC also alters the tumor microenvironment (Kikuchi et al. 2024).

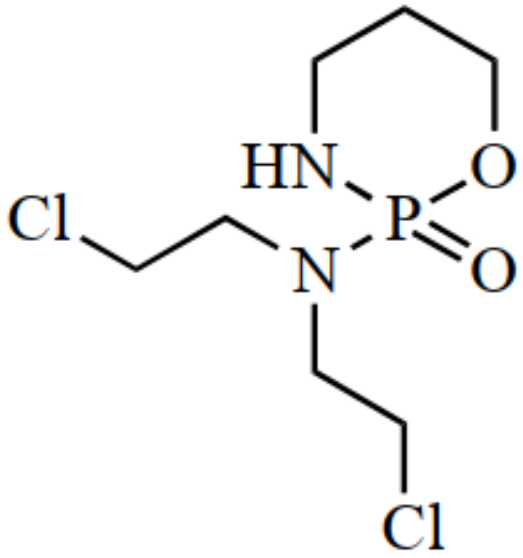


Figure 3. Chemical structure of cyclophosphamide ($C_7H_{15}Cl_2N_2O_2P$) representation. Adapted from (Nascimento, Aquino and Silva-Júnior 2021)

1.5 5- Fluorouracil

5- Fluorouracil (5-FU) ($C_4H_3FN_2O_2$, Figure 4) is a cytotoxic chemotherapy drug and approved by FDA, which comprises as a pyrimidine derivative. It is utilized in the chemotherapy treatment of carcinomas for example breast cancer, stomach cancer, skin cancer and head and neck cancer (Mafi et al. 2023). 5-FU, has been presented to preferentially induce apoptosis in MDSC when administered to mice with established subcutaneous (s.c.) tumors at a size of around 100 mm^2. Considering that 5-FU showed its destructive consequences for growth of cancer cells, and a selective ability to deplete MDSC and to increase antitumor immunity in tumor-bearing mice *in vivo* (Yang et al. 2023).

When low-dose 5-FU treatment was administered to primary tumors, the number of lung-accumulating MDSCs and lung metastases decreased along with the improvement in survival (Mathew et al. 2023). Based on this background, this study aimed to evaluate the presence of these MDSC cells after the treatment of 4T1-tumor-bearing mice with LDC of CP and 5-FU, administrated independently.

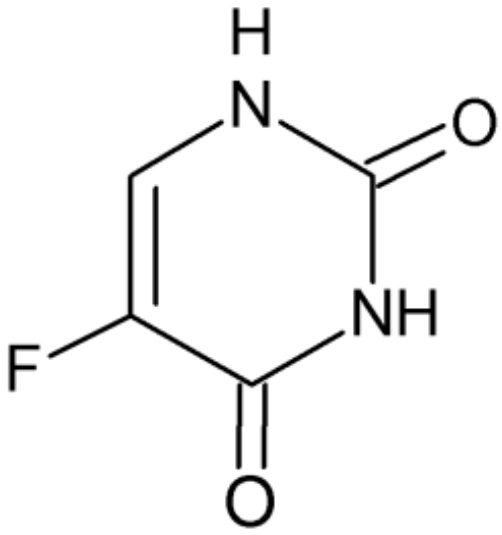


Figure 4. Chemical structure of 5-fluorouracil ($C_4H_3FN_2O_2$) representation. Adapted from (Šuleková et al. 2019)

1. Objective

The aim of this study is to employ low-dose chemotherapy utilizing two independent drugs, cyclophosphamide, and 5-fluorouracil, to regulate the immune response against breast cancer tumors in balb/c mice**.**

2.1 Specific objective

1. To examine the effects of low-dose chemotherapy on overall mice body weight and survival rate.
2. To evaluate the low-dose therapy on tumor progression.
3. To evaluate the low-dose therapy on distant lung metastasis progression in a breast cancer tumor-bearing balb/c mice.
4. To analyze spleen weight, size, and immunosuppressant MDSCs population quantifications.
5. To characterize the leucocytes population modification under the treatment.

3. Materials and methods

3.1 Animals and Experimental Design

Female BALB/c mice (20 ± 1 g, body weight) were obtained from the Animal Resources Centre *Universidade Federal de Goiás, UFG* (Goiania, Basil) and housed under specific pathogen-free (SPF) conditions (F-block Animal Facility House, Department of Genetics and Morphology, University of Brasilia). Mice were between 8 and 10 weeks of age for these studies. All animal handling and surgical procedures were conducted in strict accordance with the Ethics Committee for the Use of Animals (CEUA) Guidelines for the Care and Use of Laboratory Animals. All experiments were conducted according to the University of Brasilia Animal Ethics Committee CEUA approvals (Protocols, 026/2020). Mice were fed standard chow cubes and housed on regional wood granulate with extremely dust-free bedding. The animal facility temperature was kept between 21 ℃ and 22 ℃. After one week of housing (quarantined), the animals were divided into four experimental groups.

The experiment was conducted with 20 mice divided into the following experimental groups:

(1) Healthy mice (n=5)

(2) Tumor-bearing mice treated with cyclophosphamide alone (n=5)

(3) Tumor-bearing mice treated with 5-fluorouracil alone (n=5)

(4) Positive control – Tumor-bearing mice, without treatment (n=5)

The experimental design was planned by tumor inoculation day 0, tumor growth and metastasis development day 1 to 7. Body weight and tumor growth (by caliper measurement) was taken day 0 to day 42 at three days of intervals period. Drug administration was performed for 4 days continuously day 8 -11. Bioluminescence imaging of tumor and metastatic region conducted on day 7 and 41. Follow-up and survival rate were conducted day 12 to 42. Mice were euthanized on day 42 and collected biological organ samples followed by taking hematological analysis and splenic cell extractions for MDSCs quantification. Afterwards, collected organ samples lung, liver was analyzed for histopathological evaluations.

3.2 Cell culture Conditions

The murine breast cancer cell line 4T1 modified to express firefly luciferase (4T1-Luc) using cell culture medium from Luc-expressing 4T1 cells cultivated in a 96-well cell culture plate to enable cell tracking and quantification *in vivo* (Tao et al. 2008) was maintained in Dulbecco's modified Eagle's medium (DMEM), which is enhanced with 10% (v:v) fetal bovine serum and 1% (v:v) antibiotic solution (100 IU/mL penicillin and 100 mg/mL streptomycin). The cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2. Adherent cells were incubated with 0.25% trypsin-EDTA solution (trypsin-ethylenediaminetetraacetic acid, Sigma-Aldrich),

centrifuged, and then rinsed with sterile phosphate buffer before cell resuspension in sterile media. Cell suspensions were counted at a final concentration of 4 x 10^4 cells/ml.

3.3 Chemotherapy dosing

The following chemotherapeutics were used in these studies: CP and 5-FU were purchased from Merck Sigma-Aldrich (São Paulo, Brazil). Using a twenty-nine-gram insulin syringe was used to administer doses intraperitoneally (I.P) to each mouse (Figure 5). According to the manufacturer's recommendations, we performed CP and 5-FU chemotherapy and diluted in sterile conditions using either phosphate-buffered saline (PBS). Chemotherapy concentration of 80 mg/kg of both drug CP and 5-FU separately were diluted for 20-gram mice to receive a 100 µl intraperitoneal infusion (Aston et al. 2017). All other materials were of pharmaceutical or analytical grade.

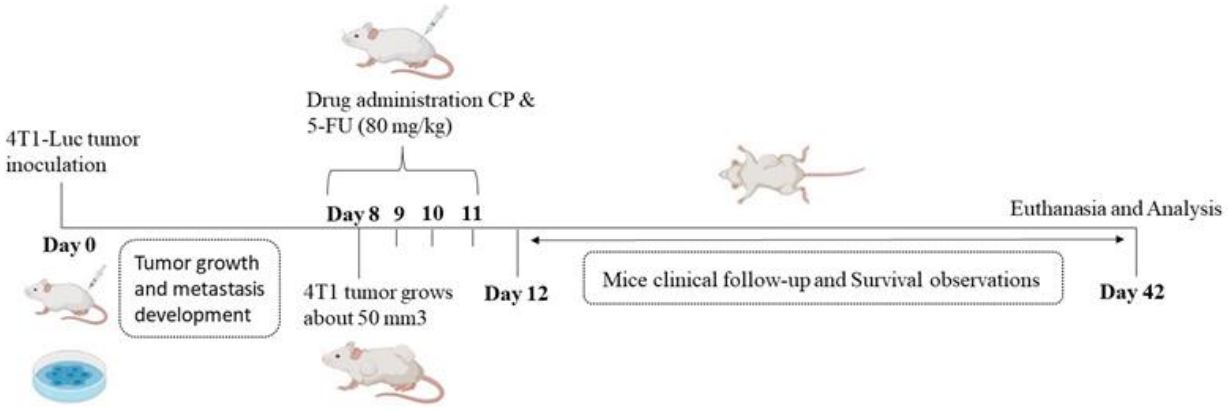


Figure 5. *In-vivo* 4T1-Luc breast cell lines induction. Schematic representation of treatment and analysis of tumor-bearing mice and experimental data endpoints.

3.4 Establishment of subcutaneous ectopic tumor model and pharmacological study

The 8-10 weeks old female balb/c mice were subcutaneously injected with 4×10^5 cells/ml 4T1-Luc cells into the mice's left dorsal hip to establish the tumor model (Fig.1). The xenograft/tumor volume (V) is measured with a caliper and calculated as length × breadth $^2 \times 1/2$. where L is the largest superficial diameter and B is the smallest superficial diameter of the xenograft/tumor. The tumor sizes were measured and recorded every three days of intervals from eighth day. The drugs were initially applied for continuous for 4 days to the all treatment groups of mice at the tumor sites intraperitoneally once daily after the tumor size reached about 50 mm^3 (Xiong et al. 2021). The tumor-bearing mice were divided into three groups of five mice in each group. Additionally, one group (n=5) was composed by healthy animals. (Group I) was composed of negative control as a healthy with saline only. The other groups included the tumor-bearing mice that were treated with cyclophosphamide [80 mg/kg] (Group II), or 5-fluorouracil [80 mg/kg] (Group III), and tumor-bearing mice without treatment and served as the positive control (Group IV). The mice were euthanized on 42 days. The tumors were dissected and weighed to calculate the tumor inhibition rates. An *in vivo* experimental model illustrating low-dose chemotherapy in a pre-clinical metastatic breast cancer setting is made. (Figure. 6)

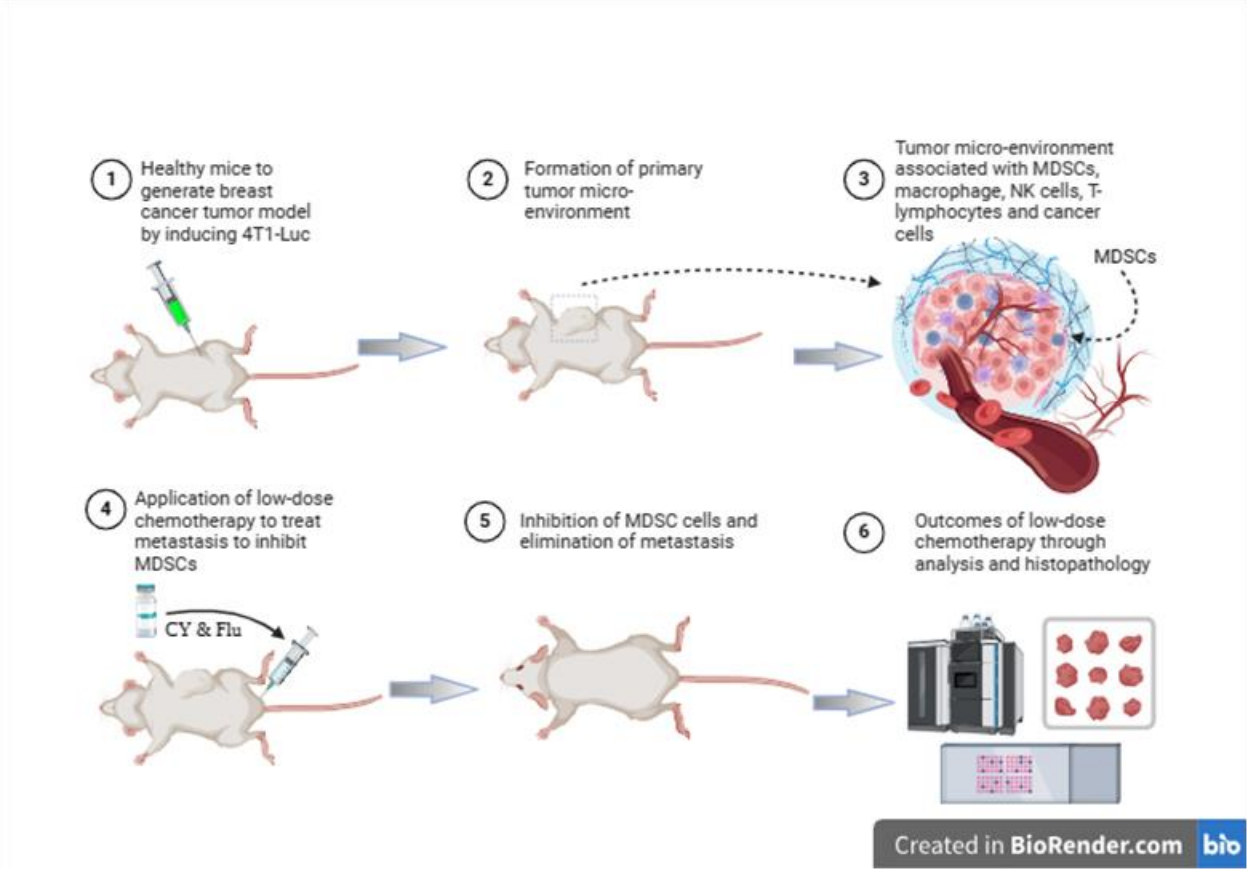


Figure 6. Illustration depicting *in vivo* low-dose chemotherapy in a pre-clinical metastatic breast cancer model. (created in biorender.com)

3.5 Bioluminescence-based evaluations of 4T1 primary tumor and lung metastasis

To assess the development of lung tumors and metastases, we employed *in vivo* bioluminescence imaging. For the *in vivo* quantifications, 100 µl of 15 mg/ml concentration, of D-luciferin (Sigma) was administered intraperitoneally per animal, and images were taken using the IVIS Lumina® XR Series III (Caliper Life Sciences). Animals were kept under isoflurane anesthesia after luciferin administration, and 20 bioluminescent photos were taken every 20 minutes one photo per minute. The open emission filter took pictures for 60 seconds for each image capture. The bioluminescent

data for each area of interest was calculated using the highest bioluminescence measurement (tumor or thorax region) two times firstly day 7 and lastly day 41 during whole experiment (Fig. 3 c, d and 4 b). Living Image 3.0 software was used to process each picture (Caliper Life Sciences, CA, USA).

3.6 Collection and analysis of biological samples

Mice were euthanized with an excess of anesthetic (xylazine -10 mg/kg and ketamine - 90 mg/kg). Blood samples (1 mL) were collected by using insulin syringe 1 mL (BD Ultra-Fine II) through cardiac puncture and then transferred to Vacutte® microtubes containing Ethylene Diamine Tetra acetic Acid (EDTA) an anticoagulant. Blood (leukogram, platelet, and erythrogram counts) was analyzed for cell count and cell type in a multiple automated hematology analyzer for veterinary use, the Sysmex pocH-100iV Diff (Curitiba/Paraná, Brazil). After blood collection, the spleen, lung, and tumor were dissected, and weighed.

3.7 Splenic cell extractions for MDSCs quantification

MSDCs populations were evaluated in spleen samples. Female balb/c mice spleens were harvested under sterile conditions. The spleen was placed in a sterile culture dish and 2 mL PBS was added, then the spleen was quickly cut into small pieces of about 1 mm^3 and crushed gently, the process was repeated for each treatment group of mice. Using a serological pipette (10 mL) applied the

cell suspension using a 70 μm cell strainer (BD Falcon) and allowed it to filter. Prepared and dissociated single-cell suspension was incubated with ACK (Ammonium Chloride Potassium) lysis buffer (10 mL, Thermo Fisher Scientific) at RT for 7 minutes to remove red blood cells. The lysis buffer was removed, and the reaction was stopped with the addition of 10 mL ice-cold PBS. Counting of cells was performed using trypan blue and a hemocytometer Neubauer chamber. Next, a cell suspension containing 1×10^6 splenocytes was prepared in 100 µl for phenotype characterization. Splenocytes cells suspension was labeled with a 20 µl aliquot of fluorescently FITC (Fluorescein isothiocyanate) conjugated anti-bodies anti-CD11b (rat monoclonal anti-mouse), and PE (Phycoerythrin) conjugated anti-Ly6C, anti-Ly6G (rat monoclonal anti-mouse) 1:100 dilution fixed and incubated under dark condition for 20 minutes at 4 °C. Cells were washed twice with PBS buffer and analyzed in a FACSCalibur™ flow cytometer (BD Biosciences). A total of 10,000 events were counted per sample, and data were analyzed using the FlowJo® software.

3.8 Histopathological evaluation

The whole organs (lung and liver) were thoroughly rinsed in phosphate-buffered saline solution to effectively eliminate any excess blood and then subjected to fixation in 10% buffered formalin (Sigma-Aldrich located in Sao Paulo, Brazil) for a total of 24 hours at room temperature. Following this, the organs were treated with alcohol solutions (100%, 100%, 70%) to achieve dehydration,

then diaphanized in xylol, and ultimately included and embedded in paraffin through the utilization of a tissue processor. Manual microtome (Leica Microsystems, Nussloch, Germany) Leica RM2235, was employed to obtain 5 μm thick sections, which were subsequently stained with H&E, and prepared for light microscope analysis.

3.9 Statistical analysis

Data are reported as the mean ± SD. The Statistical significance evaluation of data was performed using a t-test, and one way ANOVA analyses of variance, followed by XY non-linear regression using the GraphPad Prism Prism® 8.0 software. Survival curve analysis was performed using log-rank (Mental-Cox) test. The significance level (α) adopted in statistical comparisons was 0.05.

4. Results

4.1 Effects of low-dose chemotherapy on overall mice body weight and on survival rate

An initial set of experiments was conducted to assess 4T1 tumor growth after LDC CP-80 mg/kg and 5-FU-80 mg/kg alone treatment. As observed in Fig. 1(a), from day 8^{th} to 11^{th}, continuous low-dose treatments of CP and 5-FU intraperitoneally (i.p) were given. We used only PBS as a healthy negative control and with only tumor without treatment as a positive control. During the first 24-hour period of observation and end of the treatment, it was noted that the animals displayed neither signs of skin ulceration nor any behavioral changes. Following treatment, body weights were maintained in all groups.

The body weight and survival rate of the 5-FU mice showed that little influence on the body weight due to the death of one animal caused by compromised health condition while effectively improving the survival time of other groups which include healthy, CP and tumor bearing mice. As shown in Fig. 7(a), there was no significant difference in body weight during the experiment time between treated and untreated mice as well as in healthy group. The overall mice survival (Fig. 7b) shows one animal death with an 80 % of mice survival rate in the group treated with 5-FU with 20 % loss of survival, however, this data is not statistically different according to the Mantel-cox test.

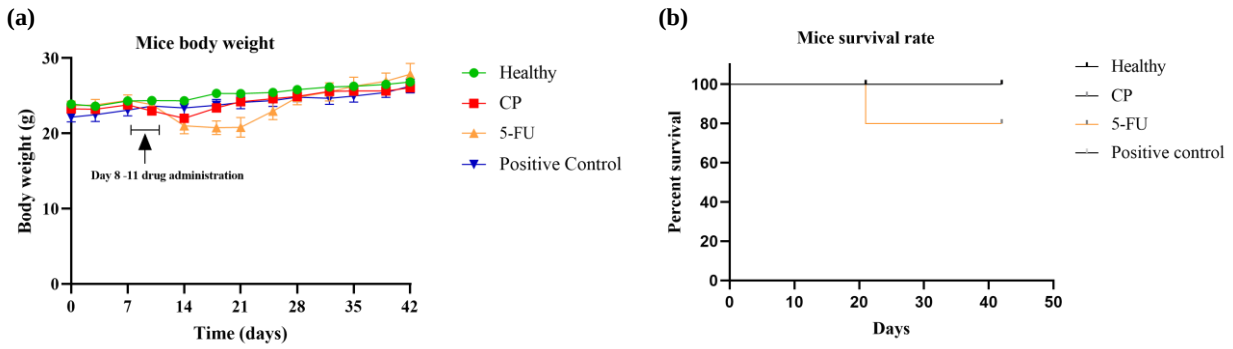


Figure 7. Treatment effect on body weight and percent animal survival. (a) Mice body weight from the day 0 to 42 long days and days 8-11th low-dose drug administration is indicated. Overall mice body weight variation during the experimental period had no observation of significant difference (b) Mice percentage survival curve with one animal death of the 5-FU group has an 80 % survival rate among all. However, this survival rate has no statistical significance difference according to the Mantel-cox test P value – 0.3916 (n=5).

4.2 Evaluation of low dose therapy on tumor progression

The experimental results of tumor volume expressed in Fig. 8(a) indicate that the treatment of two independently CP or 5-FU had primary tumor inhibition capability, compared with non-treated positive control group, following initially delayed effects probably due to the complexity and heterogeneity of 4T1 tumor induction. Moreover, 5-FU exhibited better therapeutic activity in the last 3 days (38th to 41) in comparison to CP. Fig. 8 (b) showed that the tumor weight in treated group 5-FU had the lower mass then CP group of animals comparing with the non-treated positive control group.

The subsequent comparison of quantified primary tumor growth by using bioluminescence imaging measurement (Fig. 8c) showed and detects the reduction of tumor growth in treated mice

with 5-FU relative to CP group of animals along with untreated positive control. Fig. 2(d) showing the bioluminescence *in-vivo* imaging of mice of day 7 and day 41 time period in all groups, where in day 41 5-FU group showing a gap due to death of one animal. However, all treated groups do not have any significant differences.

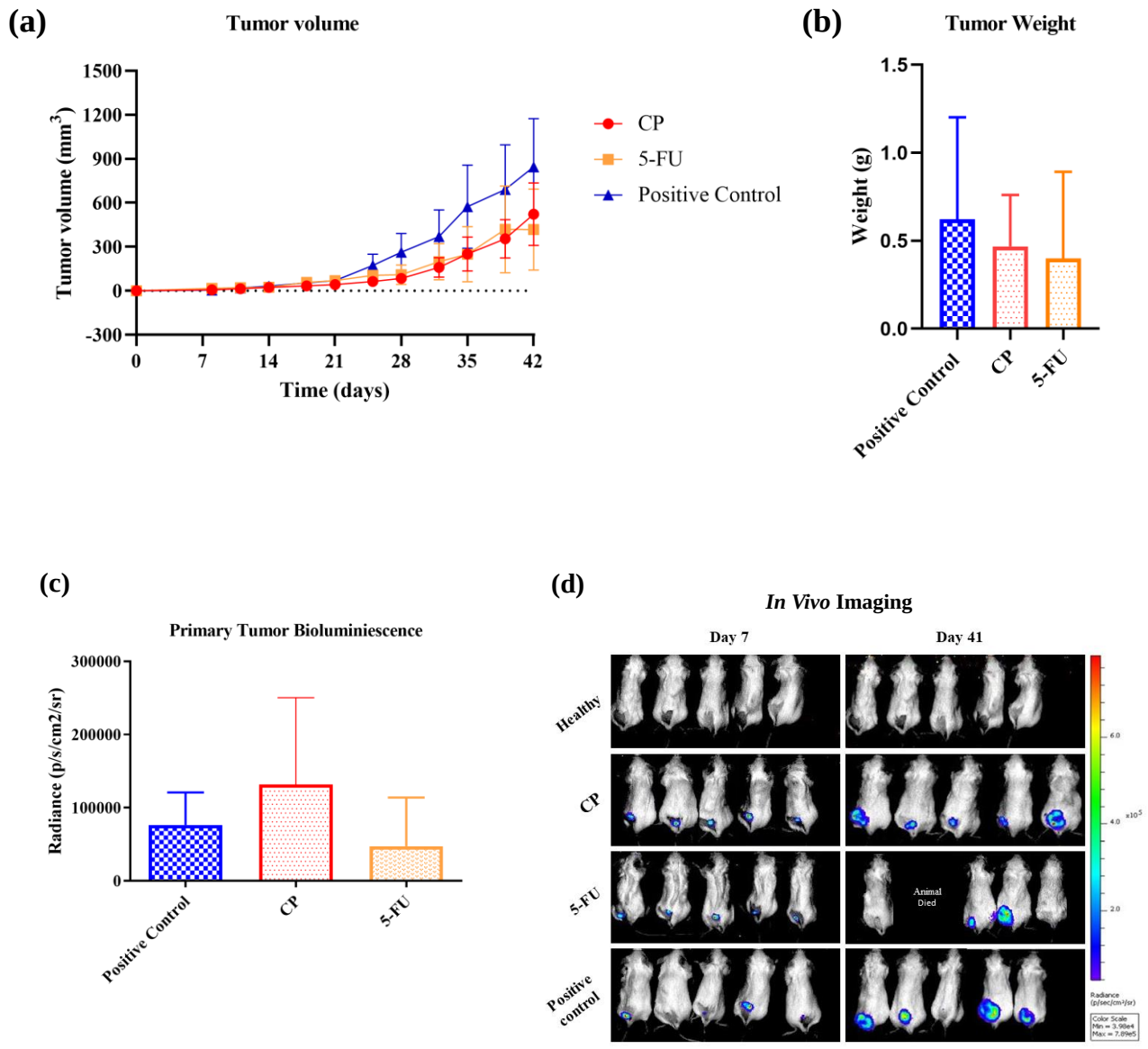


Figure 8. Evaluation of *in vivo* tumor progression and therapy effects (a) The volume of tumors in each administration group was measured at 3-day intervals with a caliper. (b) The quantification tumor weight of the excised tumor of all treated groups after 42 days of tumor induction. (c) Region of Interest (ROI) analysis to conduct a thorough quantification of tumor load among treatment groups in comparison to untreated controls after 42 days of tumor induction. (d) Bioluminescence *in-vivo* imaging of mice of day 7 and day 41 time points. The mean bioluminescence radiance +/−SD generated as photons/sec/cm^2/sr is presented in a bar graph for display. All data are presented as mean ± SD. No significant difference has been found in tumor volume, tumor weight and primary tumor bioluminescence.

4.3 Evaluation of low dose therapy on distant lung metastasis progression in a breast cancer tumor bearing mice

After mice had been euthanized on 42 days, the lungs were weighed to complement the *in-vivo* analysis. As presented in Fig. 9 (a), all treated groups had similar lungs weight. A significant difference between the treated and positive control groups was not observed. The lungs of CP or 5-FU-treated mice presented a similar morphology to that observed in healthy mice. Bioluminescence imaging of thorax region (Fig. 9b) demonstrates a trend of reduction in metastasis among CP or 5-FU groups compared to the positive control, the observation is the treatment low-dose therapeutics impair the secondary metastasis in the thorax breast region. However, these data were not statistically significant. The metastasis was further confirmed by histopathological H&E staining analysis. As shown in Fig. 9 (c), many tumor metastasis loci (black square presentation) were observed in lungs of positive control group, while there was no metastasis in the lungs of CP or 5-FU treated group. These results suggest that CP as well as 5-FU can inhibit distant tumor metastasis to lungs.

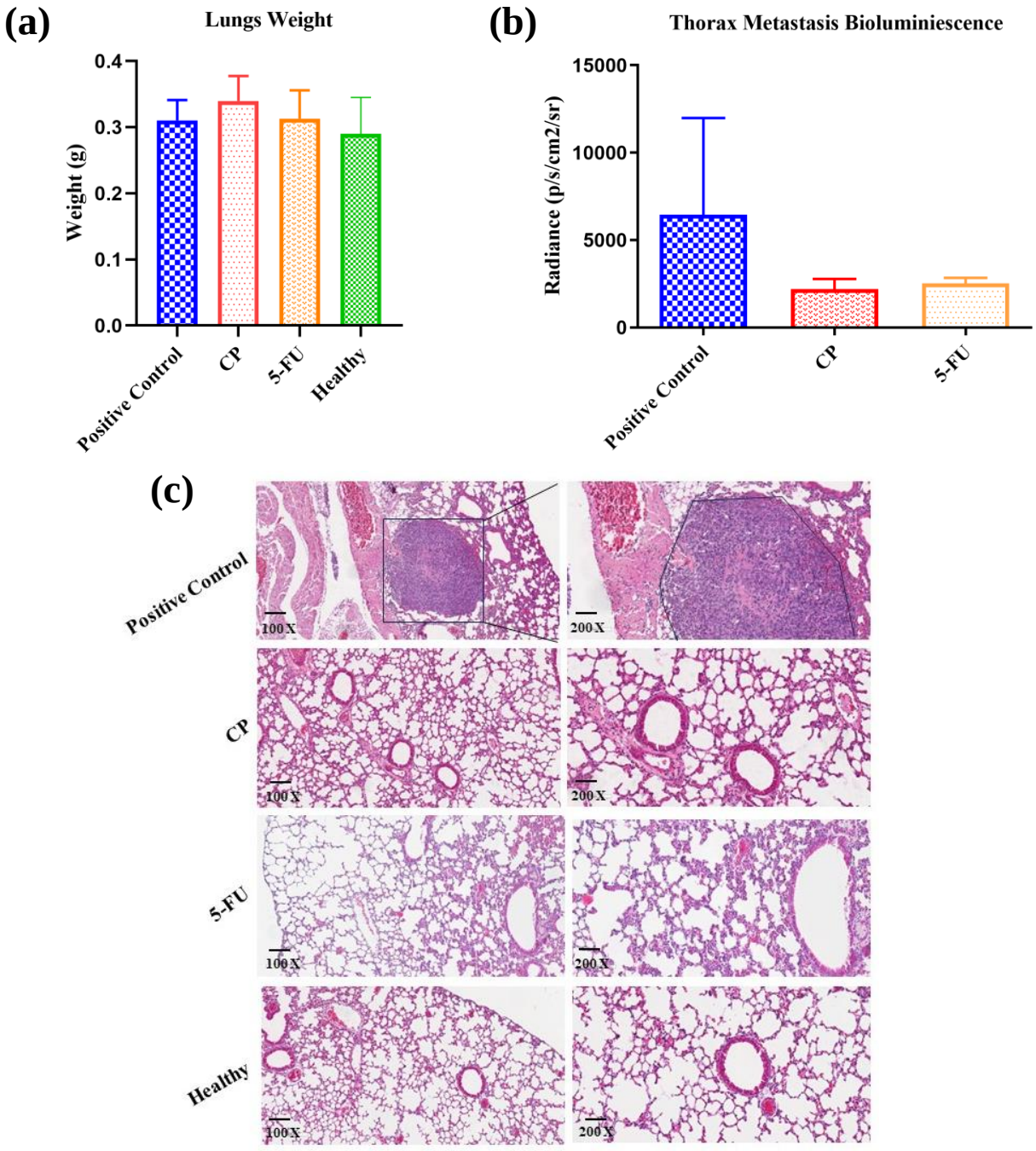


Figure 9. Effective lung metastasis prevention (a) Average lung weight after animal euthanasia on day 42 after tumor induction of all treated groups (n = 5). (b) Quantification of bioluminescence with the mean fluorescent intensity of the thorax regions, observed inhibition of metastasis in CP and 5-FU groups. (c) Histopathological examination of the lungs: representative images of H&E staining (100x and 200x) of

isolated lung of different administration groups to evaluate the tissue histological conditions. No significant difference has been found in lung weight and in thorax metastasis bioluminescence.

4.4 Treatment effects on spleen weights and immunosuppressant MDSCs population quantifications.

Spleens were collected, photographed and weighted. It's been observed a variation between the spleen weight (Fig. 10a) in all treated groups compared to the healthy. The spleens of untreated tumor-bearing mice positive control presented a 3 spleen from 5, enlarged in size (Fig.10b) having weight increase compared with the spleens of mice healthy without tumors. Likewise, difference was observed between animal healthy with CP or 5-FU treated mice. As reported, the enlarged spleen is an indicative of complications and inflammation which caused by suppression of the immune cells (Wu, Hua and Zheng 2020). And, for 4T1 tumor, splenomegaly is also related to the hyperproduction of myeloid-derived suppressor cells.

Following that, the splenocyte suspensions were directly double stained with Ly6C/Ly6G and $CD11b^+$ to detect the MSDCs, a subpopulation of immature myeloid cells that are present in tumor-bearing mice. According to the findings shown in Fig. 10 (c), the tumor-bearing mice without treatment had a higher number of these cells compared with healthy and CP/5-FU treated mice, having no significant difference. Although no significant difference was found, this finding suggests that the low-dose treatment with CP or 5-FU reduces the Ly6C/Ly6G and $CD11b^+$ populations.

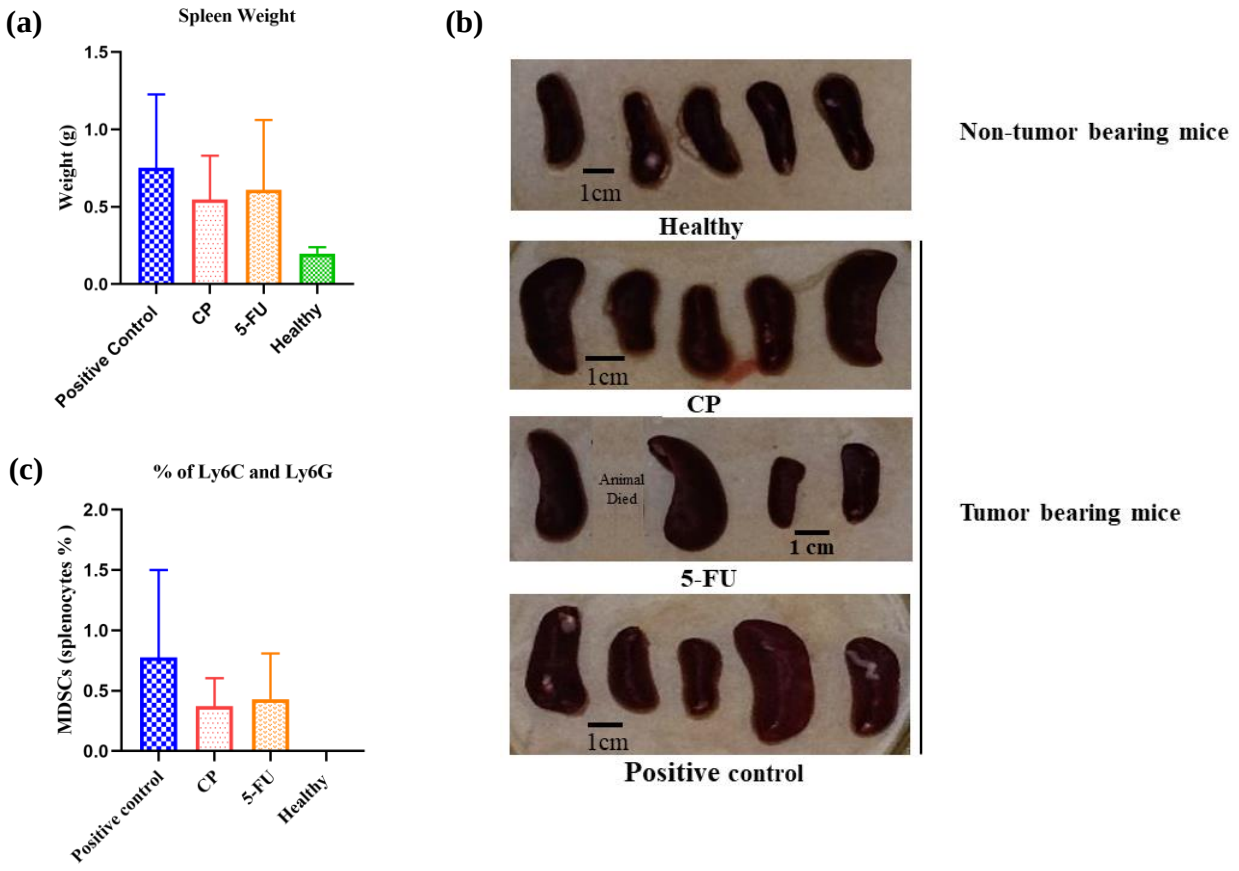


Figure 10. Overall treatment effects on spleen weights, morphology and splenocytes population quantifications using flow-cytometry. (a) Average spleen weights variation of experimental mice. (b) Macroscopic aspects of spleen excised from non-tumor bearing mice and tumor bearing group of animals. (c) MDSCs splenocytes populations percentage among all animal group. Each group included n = 5 mice, expect the 5-FU group n=4 mice. Mean and SEM is shown. Spleen weight and MDSCs % population which comparatively having no statistically significant difference with positive control.

4.5 Characterization of overall blood cell population modification under the treatment

The blood cell count was also evaluated at the end of the experiment. Hematological analysis showed significant alterations in leukogram of the treated animals compared with untreated positive control group. Total white blood cell populations (Fig. 11a) are higher in untreated positive control group due to inflammation, while the value of CP or 5-FU have reduction in the cell count comparing to positive control. Production of lymphocytes (SCR-small cell ratio) percentage (Fig. 11b) were significantly lower between healthy and in treated group along with untreated, indicate that occurrence of chronic inflammation due to tumor, during the treatment period. White Blood cell - Middle cell ratio (W-MCR - basophils, eosinophils and neutrophil) were significantly higher (Fig. 11c) in CP treated group with healthy, likewise 5-FU with healthy along with untreated group, indicating the activation of immune cells to fight with immunosuppressant cells. LCR (Large cell ratio - Monocyte) population (Fig. 11d) showed comparatively equal in treated groups and higher in untreated group, findings suggest that the treatment might be affecting the immune system, potentially by reducing inflammation or suppressing overall activity, however this parameter do not have significance difference. Platelets count (Fig. 11e) 5-FU group were significantly higher count among group CP along with healthy and untreated positive control, it's possible due to the stimulation in bone marrow response which produces early megakaryocyte

precursors or changes in the body inflammatory response after 5-FU chemotherapy and turned to the elevated production of platelets (Li and Slayton 2013) or platelets rebound in that mice group.

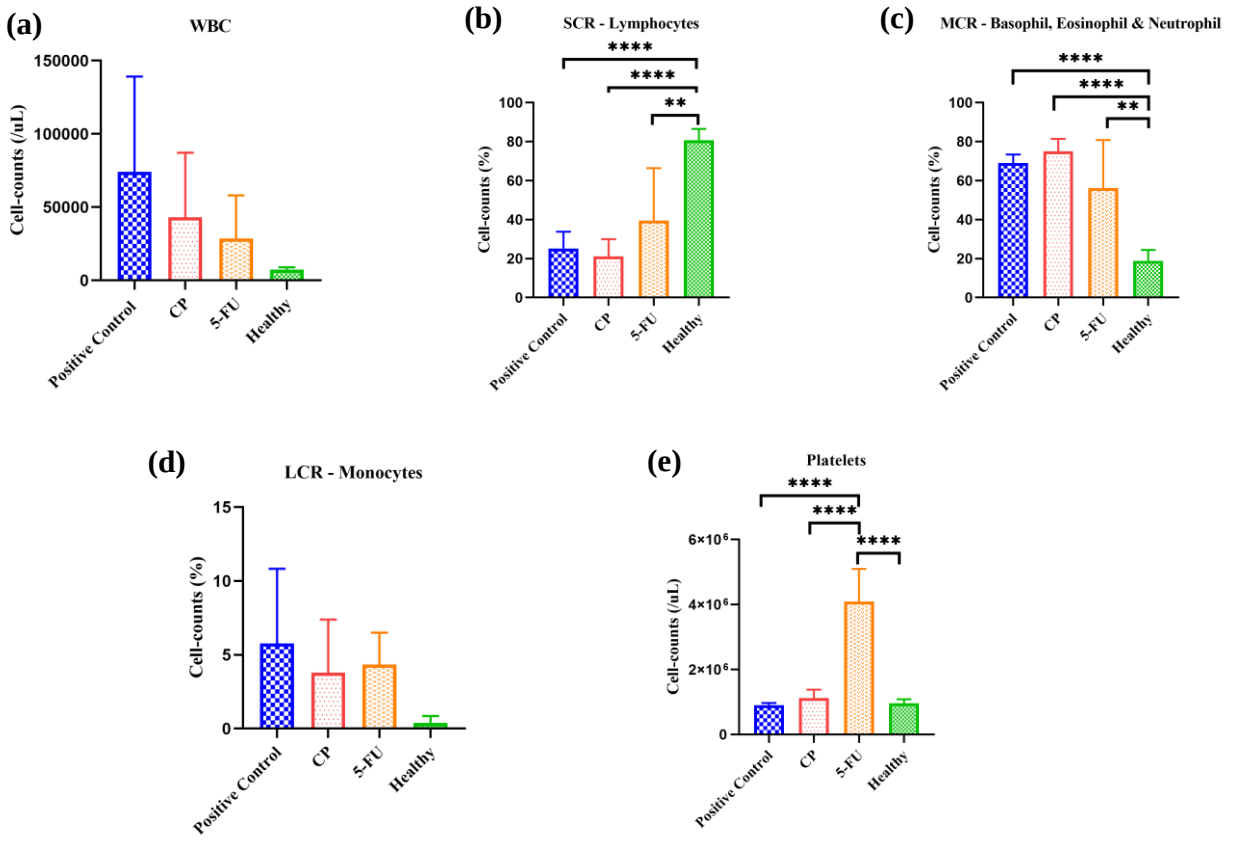
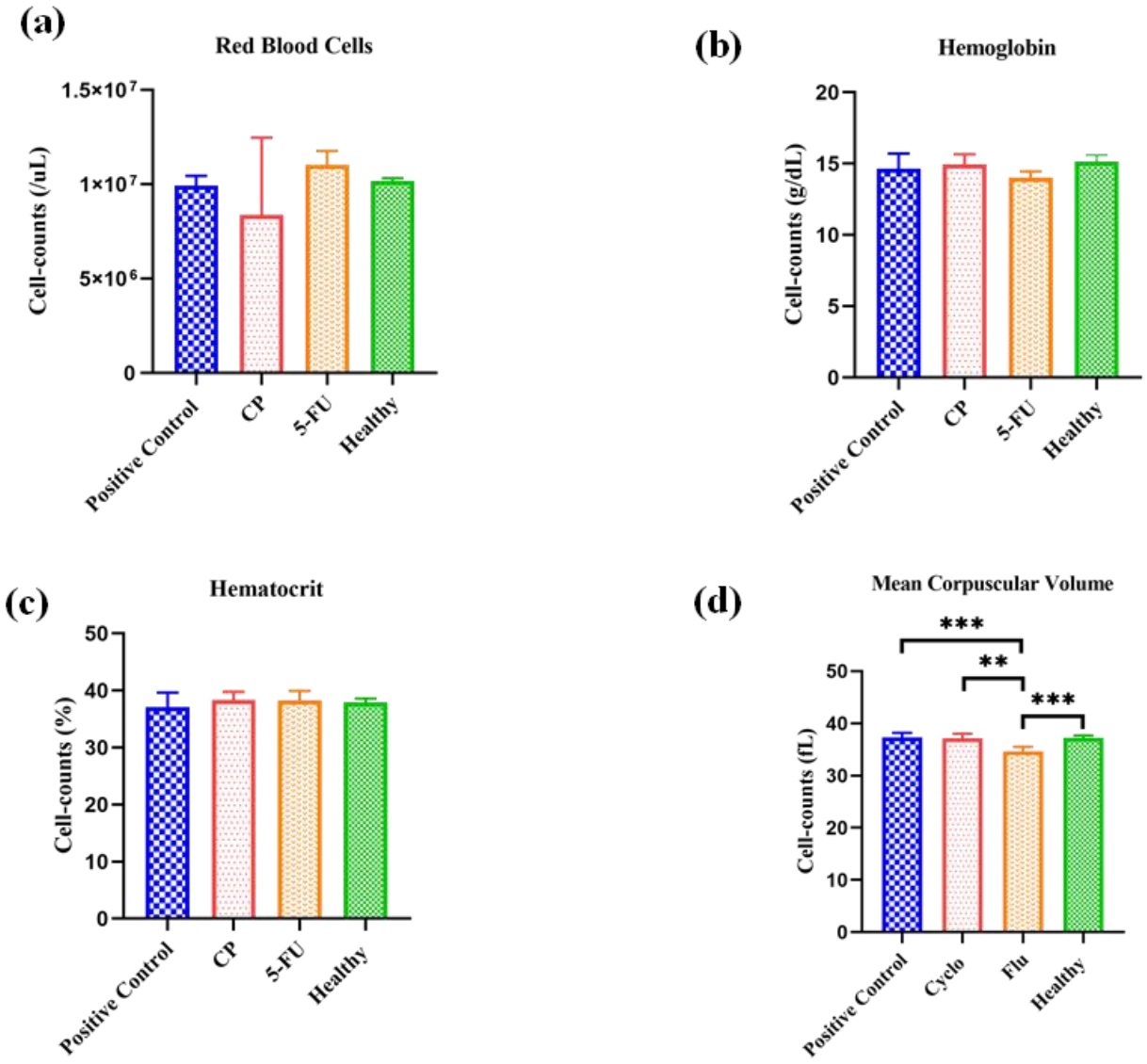


Figure 11. Leucocytes and platelets cell counts (cells/mL). (a) White blood cells - parameter has enhanced production of white blood cell count compared to the healthy group because of inflammation. (b) SCR-lymphocyte production in treated groups CP, 5-FU, and positive control have lower counts compared to the healthy group, ($^{****}p < 0.0001$, $^{**}p < 0.0025$, $^{****}p < 0.0001$) (n=5, 5-FU: n=4). (c) MCR -basophil, eosinophil, neutrophil production in treated groups CP, 5-FU, and positive control has higher counts compared to the healthy group, ($^{****}p < 0.0001$, $^{**}p < 0.0018$, $^{****}p < 0.0001$) (n=5, 5-FU: n=4) (d) LCR-monocyte production in treated groups CP, 5-FU, and positive control has higher counts compared to the healthy group having no significance difference. (e) Platelets production in treated groups CP have a little variation comparatively to healthy and positive control but group 5-FU has enhanced higher counts compared to the healthy group, ($^{****}p < 0.0001$, $^{****}p < 0.0001$, $^{****}p < 0.0001$) (n=5, 5-FU: n=4).

Addtionally, the hematological evaluation (Figure 12) revealed notable alterations in the erythrogram among all treated groups in comparison to the positive control group of animals. The red blood cell (RBC) count, hemoglobin (HGB) level, and hematocrit (HCT) values exhibited similar variations among the treated and untreated groups, indicating no significant treatment-related differences in total cell numbers. However, the mean corpuscular volume (MCV) values significantly have the consistent population across all groups. Additionally, both the mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) demonstrated consistent population counts, with significant differences observed among all treated groups compared to both the healthy and untreated groups. Notably, the RBC-distribution width variations coefficient (RDW-CV) and RBC-distribution width standard deviation (RDW-SD) count values were statistically lower across all groups when compared to the 5-Flu treated group.


(a)
Red Blood Cells
Cell-counts (/uL)
1.5×10^7
1×10^7
5×10^6
0
Positive Control
CP
5-FU
Healthy
(b)
Hemoglobin
Cell-counts (g/dL)
20
15
10
5
0
Positive Control
CP
5-FU
Healthy
(c)
Hematocrit
Cell-counts (%)
50
40
30
20
10
0
Positive Control
CP
5-FU
Healthy
(d)
Mean Corpuscular Volume
***
**
***
Cell-counts (fL)
50
40
30
20
10
0
Positive Control
Cyclo
Flu
Healthy

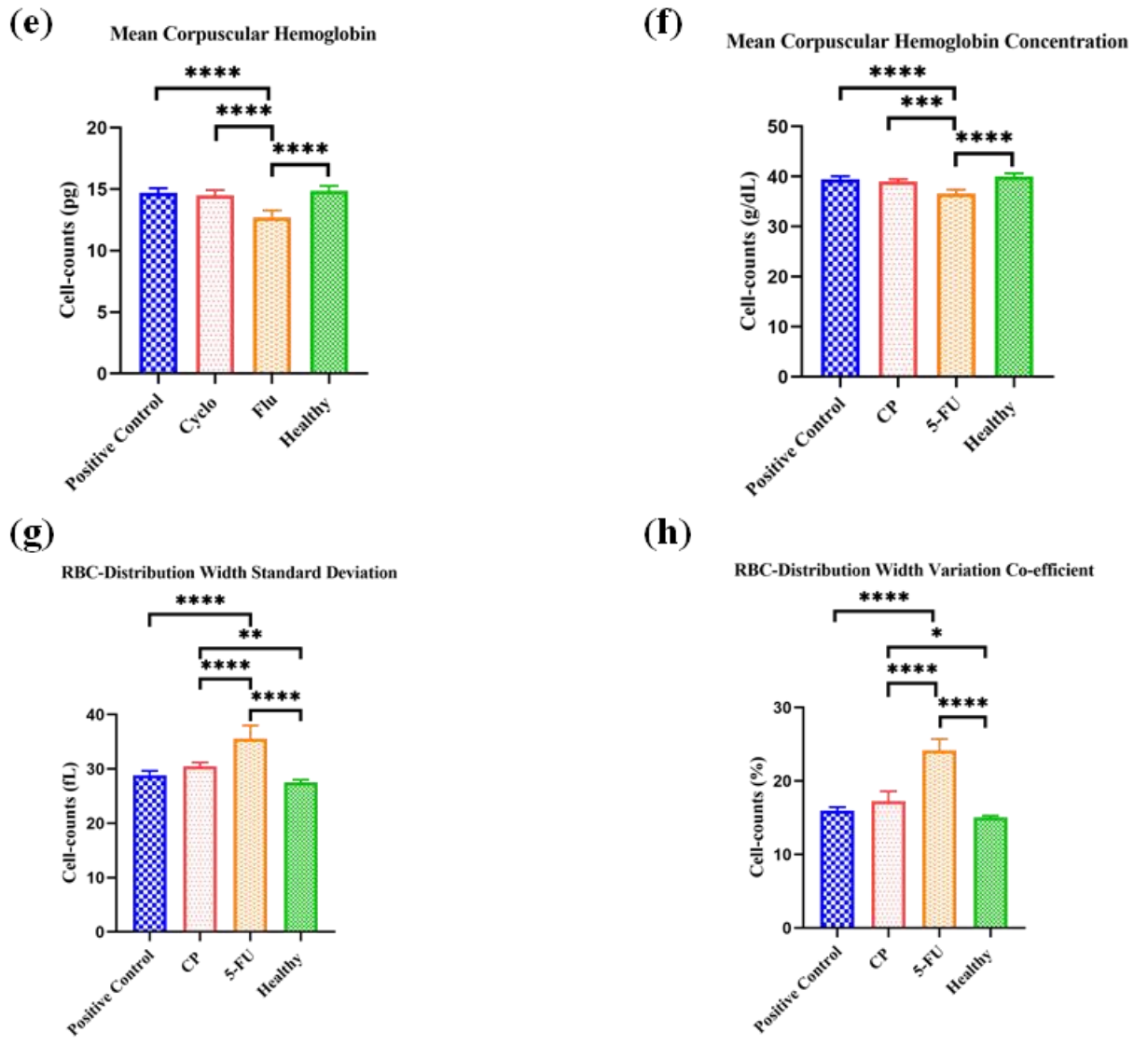


Figure 12. Absolute erythrocytes count (cells/mL). (a) Red blood cell - counts have almost similar high ratios in all groups except CP, respectively. (b) Haemoglobin counts have a similar cell counts ratio in all treated groups (c) Haematocrit counts have a similar number of cell productions in all treated groups (Fig. a-c) and found no significant difference (d) Mean corpuscular volume in group Flu has a lesser count having a significant difference in other groups, (***p < 0.0010, **p < 0.0013, ***p < 0.0008) (n=5), respectively. (e) Mean corpuscular haemoglobin in treated group Flu has a low number of counts comparatively to other groups, (****p < 0.0001, ****p < 0.0001, ****p < 0.0001) (n=5). (f) Mean corpuscular haemoglobin concentration in treated group Flu has also a low number of counts comparatively to other groups, (****p < 0.0001, ***p < 0.0003, ****p < 0.0001) (n=5) (g) RBC-distribution width variation co-efficient number is higher in the group Flu comparing to other treated groups, (*p < 0.0172, ****p < 0.0001, ****p < 0.0001,

****p < 0.0001) (n=5) (h) RBC-distribution width standard deviation number is higher also in the group Flu comparing to other treated groups, (**p < 0.0100, ****p < 0.0001, ****p < 0.0001, ****p < 0.0001) (n=5).

Additionally, we also observed a positive correlation (Figure 13) between % MDSC and the lung metastasis (p=0.0001), spleen weight (p=0.0005), and white blood cells quantification (p=0.0009). However, this positive correlation was not observed when comparing the % MDSC and primary tumor quantification (p=0.12), evidencing a systemic immune effect of the treatment.

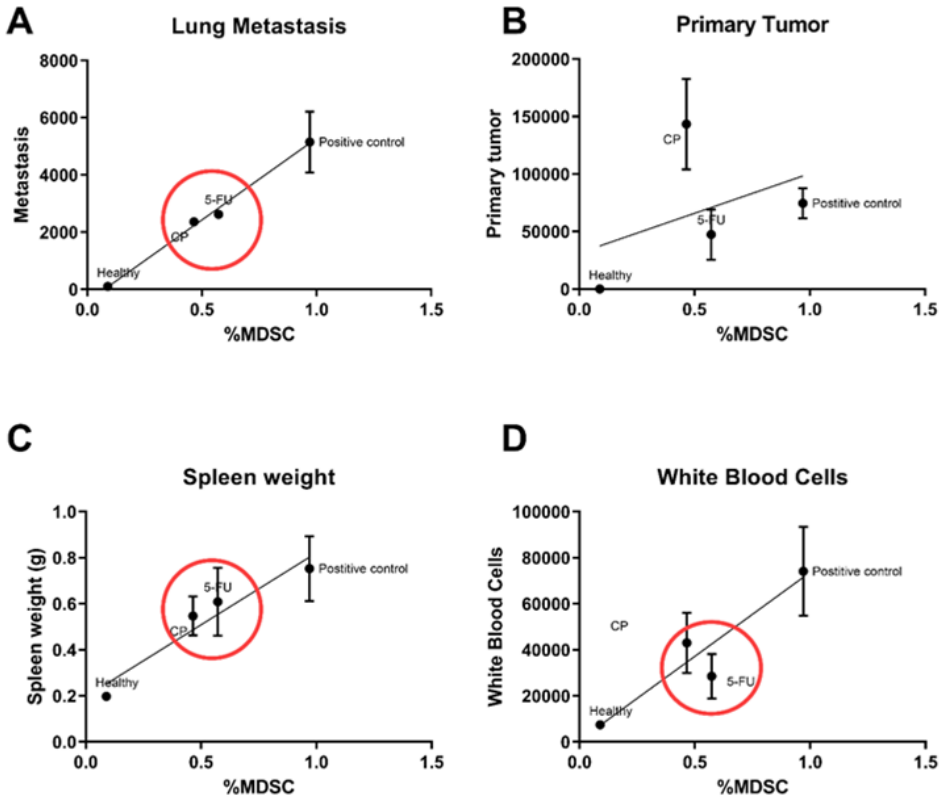


Figure 13. Linear correlation between (a) metastasis and % MDSC (p=0.0001). (b) Primary tumor and % MDSC (p=0.12) (c) Spleen weight and % MDSC (p=0.0005) (d) White Blood Cell and % MDSC (p=0.0009). Values are mean ± SEM (n=5 animals per group and n=4 animal for 5-FU group). P-values (p>0.05) were generated by Linear regression.

5. Discussion

One hallmark of tumor growth is immune evasion, which allows tumor cells to evade and suppress anti-tumor immune responses (Mortezaee 2020). LDC provides a twofold advantage by diminishing adverse effects while also offering benefits such as decreased tumor blood vessel growth, lower development of resistance to treatment, and importantly, enhancement of the body's immune responses against tumors (Lin et al. 2022; Krajnak et al. 2020), as well to boosts vascular regulation (Kikuchi et al. 2024). To explore our research, a tumor model of lung metastasis 4T1-based using in an animal was established, followed by an assessment of the therapeutic effects of administering low doses of CP or 5-FU.

The results highlight major findings after LDC application against experimental BC tumor model (4T1 Luc): We first observed that LDC with CP-80 mg/kg or 5-FU-80 mg/kg did not significantly impact the body weight of the mice over the course of the experiment, except a transitory reduction in treated group with the 5-FU or in CP group reduction occurred for one day and subsequently returned to baseline level. Furthermore, the survival rates of the treated groups were comparable to the control groups, indicating that LDC did not adversely affect survival outcomes. Despite one death in the 5-FU group, the survival rates were not statistically different. These findings suggest that the selected LDC regimens were well-tolerated and did not induce acute toxicity or significant changes in body weight or survival rate.

The results demonstrated that both CP or 5-FU treatment inhibited and reduction in primary tumor growth compared to the untreated control group. Notably, 5-FU exhibited better therapeutic activity in the later stages of the thorax bioluminescence experiment, which means the presence of bioluminescent tumor cells. Additionally, both CP or 5-FU treatment led to a reduction in tumor weight compared to the control group. Bioluminescence imaging further confirmed the reduction in tumor growth in the treated groups relative to the control group. These findings highlight the efficacy of LDC in inhibiting tumor progression.

Our findings showed that both CP or 5-FU treatment impaired distant lung metastasis progression compared to the untreated control group, as evidenced by reduced metastatic lesions in the lungs. Histopathological analysis also confirmed the absence of metastasis in the lungs of treated mice, further supporting the anti-metastatic effects of LDC. Low-dose protocols, have a better ability to impair distant metastasis to the lungs by developing systemic immunological protection. This result is quite important since one of the goals of contemporary treatments is to reduce or impair the distant lung metastasis, giving better health conditions to patients diagnosed with advanced disease. Although statistical significance was not achieved, the trend towards reduced metastasis in the treated groups is promising.

Research has provided clarity on the pathological functions of MDSCs in the promotion of cancerous tumors (Ren et al. 2023). MDSCs have been shown to play a crucial role in suppressing

the anti-tumor immune response and promoting tumor progression (Li et al. 2021). Low-dose 5-FU or CP have demonstrated the ability to impair the generation and accumulation of MDSCs in hosts with tumor bearing, leading to tumor growth inhibition and enhancing antitumor immunity by reducing lung metastasis formation (Yang et al. 2023; Liao et al. 2023). Studies showed that low-dose CP have the ability to enhance anti-tumor immune response through targeted depletion of regulatory T cells and augmentation of effector T cell activity and that is likely to hinder the best possible immune response is the MDSC induction (Leong et al. 2019; Madondo, Quinn and Plebanski 2016).

5-FU, when administered alone, swiftly reduces the number of circulating MDSCs, contributing to their depletion within the systemic circulation (Tang et al. 2021). Also, the administration of 5-FU resulted in notable reductions in both M-MDSCs and PMN-MDSCs within the spleen, this observation highlights the efficacy of 5-FU in diminishing the presence of these MDSC subsets in this immune organ and CP has slight effect on the growth of MDSCs in tumor locations or the spleens of mice with tumors, along with at a dosage of 50 mg/kg body weight, CP exhibited minimal impact on the accumulation of MDSCs at tumor sites, as demonstrated by the study. (Wang, Till and Gao 2017).

The results indicated variations in spleen weights among the treated groups compared to healthy controls, with splenomegaly observed in the untreated tumor-bearing mice due to the neoplastic

cells to infiltrate the spleen (Wu, Hua and Zheng 2020). Furthermore, LDC with CP or 5-FU reduced the population of MDSCs in the spleen of the tumor-bearing mice associated with metastasis, suggesting a potential immunomodulatory effect of the treatment, there is no detection of MDSCs in healthy animals. In addition to our finding MDSCs are associated with lung metastasis formation, their pivotal in facilitating the development of lung metastasis through the establishment of an immunosuppressive microenvironment (Cui et al. 2023; Zhang et al. 2022). Additionally, correlation is calculated between MDSCs and various parameters such as lung metastasis and spleen weight highlight the systemic immune effects of LDC, which that MDSCs is enough to promote important systemic effects between these cells and the metastatic progression.

Hematological analysis revealed significant alterations in leukogram parameters among the treated groups compared to the untreated control group. Notably, LDC with CP or 5-FU led to reductions in total white blood cell populations and lymphocyte percentages, indicating modulation of the immune response. Additionally, platelet counts were significantly higher in the 5-FU group, potentially due to from either the stimulation of bone marrow response, leading to the production of early megakaryocyte precursors, or alterations in the body's inflammatory response following 5-FU chemotherapy (Wu, Hua and Zheng 2020; Li and Slayton 2013).

Further analysis of the erythrogram revealed notable alterations in red blood cell parameters among the treated groups compared to the control group. While no significant differences were observed in total cell numbers, variations in mean corpuscular volume and hemoglobin concentration suggest potential treatment-related effects on erythropoiesis. These findings contribute to our understanding of the hematological effects of LDC in cancer therapy.

These findings underscore the complex interplay between LDC and the immune system in the context of cancer treatment. Together, our findings suggest LDC used in this study has the potential to attenuate distant lung metastasis as well as to reduce the immunosuppressive MDSC populations.

6. Conclusion

The findings of this study provide valuable insights into the multifaceted effects of LDC on tumor progression, distant lung metastasis, immune modulation, and hematological parameters in a murine model of breast cancer. These results support the potential utility of LDC as a therapeutic strategy with reduced systemic toxicity and highlight avenues for further research to optimize its efficacy and safety profile. These data could potentially provide valuable insights for the formulation of a novel approach aimed at regulating the immune responses associated with malignant tumors.

7. References

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