Universidade de Brasília – UnB

Instituto de Ciências Biológicas – IB

Programa de Pós-Graduação em Nanociência e Nanobiotecnologia

Desenvolvimento de nanopartículas lipídicas sólidas para carreamento de curcumina e análise de sua atividade contra células de câncer colorretal murino CT26

Brasília

2020

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Tese apresentada ao Programa de Pós-Graduação em Nanociência e Nanobiotecnologia, do Instituto de Ciências Biológicas da Universidade de Brasília, como parte integrante dos requisitos para a obtenção de título de Doutor em Nanociência e Nanobiotecnologia.

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Dedico este trabalho a Deus, meu criador, Senhor, salvador e amigo

e ao meu pai, fonte de apoio, cuidado e amor.

Agradecimentos

A Deus, pelo amor incondicional, por me encorajar frente aos desafios, por ser meu refúgio e fortaleza.

Aos meus familiares, primos e tios (são tantos!), que sempre me apoiaram nesta jornada. Que sempre lembram de mim e se preocupam de como eu estou. Agradeço pelas orações, pela união e amor. É incrível sentir o amor e torcida de vocês pela minha vida. Em especial quero agradecer ao meu Pai Eduardo, por me amar, apoiar e cuidar de mim nos mínimos detalhes. Ao meu irmão Dudu, pelo companheirismo e por me fazer tão feliz. Sem dúvidas você é o melhor presente que Deus me deu. À minha preciosa Vovó Maria por todas as orações, cuidado e imenso amor. A saudade muitas vezes dói demais, mas o seu incentivo sempre me fez crer que toda luta vale à pena para o crescimento. Obrigada por ser o alicerce da minha vida. À minha madrasta Sandra por cuidar tão bem de nossa família. Por todo carinho e preocupação. Aos meus amigos da minha cidade, da graduação, da igreja e da vida, por sempre estarem na torcida por mim. Por desejarem o meu bem e por ficarem felizes com minhas vitórias. Obrigada por todo o encorajamento!

Acredito que somos um pouco de cada um que passou em nossas vidas. Sendo assim, não posso deixar de agradecer a todos que participaram na construção da pessoa e profissional que sou hoje. Em especial, gostaria de agradecer:

Ao meu orientador Luis Alexandre Muehlmann, por todos os ensinamentos, pela amizade e pela grande confiança depositada em mim. Por me dar apoio, orientação e os instrumentos para conclusão desta importante etapa da minha vida. Quero agradecer em especial pela paciência em me ensinar na teoria e na prática por estes 10 anos e por me estimular a agarrar as oportunidades que apareceram no meio do caminho.

Ao Professor Ricardo Bentes por acreditar em meu trabalho, por ter me dado a oportunidade de pesquisar em seu laboratório e me encorajar para os desafios da pesquisa. Agradeço também por todo o cuidado e carinho com a minha pessoa durante todos esses anos.

Ao Professor Sergio Moya por me receber calorosamente em seu laboratório. Agradeço a confiança e disponibilidade de equipamentos e reagentes. Essa oportunidade foi de imensa contribuição para a minha vida profissional.

À Professora Flávia Nader pela disponibilidade e paciência de me ensinar e auxiliar em experimentos de forma perfeita. Obrigada por tanto carinho e cuidado.

À Professora Patrícia Bento pelo exemplo de dedicação e amor à pesquisa, pelo constante incentivo e principalmente pelo companheirismo e amizade nesta jornada.

Agradeço a todos do grupo ImunoNano que participaram de forma direta ou indireta nesta etapa. Agradeço pela confiança no meu trabalho e pela oportunidade de compartilhar conhecimentos. Quero agradecer em especial aos membros Victor Mello e Athayde por me ajudarem neste trabalho. A participação de vocês foi essencial. Além da amizade que levarei para minha vida. Agradeço em especial também à minha ex-estagiária Giulia, agora mestranda, por tanta preocupação em me ajudar nos experimentos, por confiar nos meus ensinos e por ser meu braço direito neste trabalho.

Agradeço à minha amiga Amanda por estar sempre presente na hora de um conselho, de uma simples conversa, pela compreensão, pela ajuda e interesse em minha vida. Pela amizade. Ao Mosar pela companhia e parceria, pela confiança que sempre podemos fazer o melhor e por me fazer rir todos os dias. À Dani e Fernanda por toda ajuda, preocupação e carinho, tanto em relação ao profissional quanto pessoal. Ao Victor Hugo, pela amizade, palavras de ânimo, colaboração e ajuda neste trabalho. As meninas Raquel e Luiza por todo apoio e ajuda e por me proporcionarem momentos incríveis de muitas risadas no doutorado sanduíche, sem vocês eu não teria me divertido tanto. Agradeço à Jaque pelo acréscimo em conhecimentos científicos e por todos os conselhos na minha vida profissional. Agradeço à Karen pela paciência em resolver problemas e cuidar do laboratório. Agradeço a todos os meus companheiros e amigos do laboratório de Nanobiotecnologia por terem confiado no melhor que poderíamos fazer, pela cumplicidade e pelos tantos momentos de felicidades nos corredores do laboratório.

Também agradeço aos membros dos laboratórios de microscopia, nanociência e nanobiotecnologia (física) e genética toxicológica (Gtox) pelo auxílio e disponibilidade de equipamentos para realização dos meus experimentos.

Agradeço às instituições CAPES pela concessão da bolsa de mestrado e às instituições CNPq, FAP/DF e FINEP pelo fomento da minha pesquisa em nanobiotecnologia.

“Feliz aquele que transfere o que sabe e aprende o que ensina.”

(Cora Coralina)

RESUMO

A quimioterapia contra o câncer continua sendo um desafio devido aos mecanismos de resistência das células tumorais e à toxicidade dos fármacos anticâncer. Estudos clínicos sugerem que as respostas imunitárias específicas para células cancerosas podem ser responsáveis, pelo menos em parte, para o sucesso clínico dos esquemas terapêuticos que dependem de indutores de morte celular imunogênica. Neste contexto, a eficiência de agentes anticâncer que são indutores de morte celular imunogênica dependem da capacidade do sistema imunitário do hospedeiro em reconhecer os sinais desta morte e gerar uma robusta resposta imunitária contra as células cancerosas. Morte celular imunogênica é uma via proeminente para a ativação do sistema imunitário contra o câncer, podendo resultar no sucesso de terapias anticâncer em longo prazo, devido às respostas imunitárias específicas, aumentando assim a eficácia da terapia em comparação com os agentes anticâncer convencionais utilizados. O fitoquímico curcumina possui uma ampla gama de propriedades farmacológicas incluindo ações antioxidantes e anti-inflamatórias, antidiabéticas, antibacterianas, antiparasitária, cicatrização de feridas, antiateroscleróticas, hepatoprotetoras, e estudos indicam que é um potente inibidor da proliferação de células cancerosas. É conhecido que a curcumina inibe a bomba ATPase-Cálcio, aumentando a concentração de cálcio citosólico, causando assim, estresse no retículo endoplasmático e levando a célula a morte celular programada, apoptose. O estresse no retículo endoplasmático induz a exposição da calreticulina na membrana plasmática, num estágio pré- apoptótico, de ATP, durante a apoptose, e da morte celular associada a liberação da proteína HMGB1, sendo que estes mecanismos podem levar a morte celular imunogênica. Neste sentido, a curcumina apresenta potencial em promover MCI em células cancerosas.

Palavras-chaves: morte celular imunogênica, curcumina, DAMPs, sistema imune, estresse no retículo endoplasmático.

ABSTRACT

Cancer chemotherapy remains a challenge due to the resistance mechanisms of tumor cells and the toxicity of anticancer drugs. Clinical studies suggest that specific immune responses to cancer cells may be responsible, at least in part, for the clinical success of therapeutic regimens that depend on inducers of immunogenic cell death. In this context, the efficiency of anti-cancer agents that are immunogenic cell death inducers depends on the ability of the host's immune system to recognize the signs of this death and generate a robust immune response against cancer cells. Immunogenic cell death is a prominent pathway for the activation of the immune system against cancer and may result in the success of anti-cancer therapies in the long term due to specific immune responses, thus, increasing the effectiveness of therapy compared to conventional anti-cancer agents. The phytochemical curcumin has a wide range of pharmacological properties including antioxidant and anti-inflammatory, antidiabetic, antibacterial, antiparasitic, wound healing, anti-atherosclerotic, hepatoprotective, and studies indicate that it is a potent inhibitor of cancer cell proliferation. It is known that curcumin inhibits the calcium ATPase, increasing the concentration of cytosolic calcium, thus causing stress in the endoplasmic reticulum and leading the cell to programmed cell death, apoptosis. Stress in the endoplasmic reticulum induces exposure of calreticulin in the plasma membrane, at a pre-apoptotic stage, of ATP, during apoptosis, and of cell death associated with the release of the HMGB1 protein, and these mechanisms can lead to immunogenic cell death. In this context, curcumin has the potential to promote ICD in cancer cells.

Keywords: immunogenic cell death, curcumin, DAMPs, immune system, endoplasmic reticulum stress.

SUMÁRIO

INTRODUÇÃO

Diante dos crescentes novos casos de câncer é evidente a necessidade de se estudar novos esquemas terapêuticos na tentativa de encontrar soluções menos agressivas aos pacientes e com melhores resultados. Câncer é o nome dado ao conjunto de mais de 100 doenças que tem em comum o crescimento desordenado de células, que invadem tecidos e órgãos, formando metástase (INCA 2019). O câncer é caracterizado pela acumulação de um número variável de alterações genéticas e pela perda de processos regulatórios normais das células (Tian et al. 2011). O câncer começa a surgir quando as etapas de crescimento e morte das células estão fora de controle. Portanto, células cancerosas mantém o crescimento e geram novas células de forma desordenada (American Cancer Society 2016).

Ao longo da vida nos organismos surgem células alteradas que são identificadas e eliminadas por mecanismos de defesa (INCA 2019). O sistema imunológico é altamente específico na detecção e eliminação de agentes infecciosos (vírus, bactérias e fungos), e existem também, diversas evidências de que contribui significativamente para os mecanismos de defesa contra o câncer, apresentando uma grande complexidade de vias de ação. Porém, grande parte dos mecanismos de defesa do organismo contra células tumorais ainda não foram definidos. Neste contexto, a área da biologia do câncer é altamente promissora para a melhor compreensão dos eventos que geram as neoplasias e para o desenvolvimento de novas estratégicas terapêuticas contra o câncer (Weinberg 2014).

Quimioterápicos e radioterápicos podem oferecer vantagens diante de outros agentes anticâncer pelo fato de matar células cancerosas ativando uma resposta imunitária específica. Resultados pré-clínicos e clínicos sugerem que as respostas imunitárias específicas para células cancerosas podem ser responsáveis, pelo menos em parte, para o sucesso clínico dos esquemas terapêuticos que dependem de indutores de morte celular imunogênica (MCI) (Kepp et al. 2013, 2011). Isto implica que a eficiência dos agentes anticâncer que são indutores de MCI não se deve somente às suas propriedades farmacodinâmicas, mas também à capacidade das células cancerosas emitirem os sinais adequados para a morte celular ser interpretada como imunogênica, a capacidade do sistema imunitário do hospedeiro em reconhecer esses sinais e a capacidade de gerar uma robusta resposta imunitária contra as células cancerosas (Kepp et al. 2013). MCI ocorre de forma sequencial envolvendo mudanças na superfície celular e liberação de mediadores. É uma via proeminente para a ativação do

sistema imunitário contra o câncer, que pode resultar no sucesso de terapias anticâncer ao longo prazo, devido às respostas imunitárias específicas, aumentando assim a eficácia da terapia em comparação com os agentes anticâncer convencionais utilizados (Kroemer et al. 2013).

As células tumorais estudadas neste trabalho são da linhagem de carcinoma colorretal murino, pois são células modelo para se testar protocolos de imunoterapia (Tesniere et al. 2010; Humeau et al. 2019). O câncer colorretal é o terceiro mais comum em homens e o segundo mais comum em mulheres no mundo. As terapias existentes para este tipo de câncer são utilizadas para metástases locais, mas para metástases distantes apresentam baixa eficácia. Apesar de existirem diversos tratamentos para o câncer colorretal ainda há limitações incluindo resistência, eficácia limitada e efeitos colaterais severos, tornando necessário o desenvolvimento de novas terapias capazes de evitar metástases e aumentar a sobrevida dos pacientes (Gallagher and Kemeny 2010; Sanchez-Castañón et al. 2016). Neste contexto, compreender os mecanismos do sistema imunitário contra tumores é algo promissor para se desenvolver terapias mais eficazes.

Processos associados à MCI podem resultar na emissão de moléculas chamadas Padrões Moleculares Associados a Danos (do inglês, DAMPs), dentre as quais podem ser citadas aquelas que são expostas na membrana plasmática, como as proteínas de choque térmico (HSP70 e HSP90) e calreticulina (CRT), as que são secretadas para o ambiente extracelular, como proteínas HMGB1, ácido úrico e citocinas e aquelas que surgem como produtos de degradação, como ATP, DNA e RNA. Essas moléculas podem ativar células do sistema imunitário, tal como, as células apresentadoras de antígenos que apresentam antígenos para as células T (Garg et al. 2010). A CRT é a proteína mais abundante no lúmen do retículo endoplasmático (RE) e em resposta à ação de indutores de MCI esta transloca do lúmen do RE para a membrana da célula em estresse. Existe um consenso de que estresse no RE é necessário para que haja a exposição de CRT na membrana celular, mas o mecanismo exato ainda não está totalmente elucidado (Kroemer et al. 2013). Porém, sabe-se que o RE está envolvido na morte celular imunogênica.

Estímulos que interferem no funcionamento do RE induzem a formação de proteínas em resposta a esse estresse que promovem a sobrevivência e o crescimento das células cancerosas, ou seja, promovem a restauração do funcionamento normal do RE. Este mecanismo pode conferir resistência à ação de radiação e de quimioterápicos, no entanto, um efeito prolongado de estresse no RE pode superar estes mecanismos de proteção celular

levando a morte celular (Bakhshi et al. 2008). A curcumina, um fitoquímico isolado do rizoma da planta *Curcuma longa*, possui ação antioxidante e anti-inflamatória e estudos indicam que é um potente inibidor da proliferação de células cancerosas (Bakhshi et al. 2008; Ravindran, Prasad, and Aggarwal 2009; Pae et al. 2007). É conhecido que a curcumina desencadeia acúmulo de cálcio (Ca2+) no citosol aumentando o estresse no RE (Bakhshi et al. 2008). Estudam demostraram a capacidade da curcumina em causar apoptose devido ao estresse no RE (Pae et al. 2007). Além disso, existem estudos que demostram a ação sensibilizante da curcumina na terapia combinada com um agente anticâncer, provocando maiores efeitos em vias que levam à morte celular (Bava et al. 2005). Sendo assim, a curcumina apresenta potencial em promover MCI em células cancerosas.

Porém a eficácia terapêutica da curcumina é limitada devida sua baixa solubilidade em meio aquoso, rápida degradação e metabolização e baixa biodisponibilidade (Yallapu et al. 2014; Wang et al. 2015), por este motivo, faz-se necessário o desenvolvimento de um veículo de entrega adequado. Nanopartículas lipídicas são partículas coloidais compostas por diversos lipídeos que apresentam vantagens como um carreador de fármaco por possuir baixa toxicidade, capacidade de proteger fármacos de degradação química e por permitir uma liberação controlada destes fármacos (Wang et al. 2015). Neste contexto, a curcumina pode ter suas propriedades aprimoradas quando associada às nanopartículas lipídicas.

O objetivo deste trabalho foi investigar se a curcumina é capaz de gerar morte celular imunogênica e desenvolver uma nanopartícula biocompatível, sem o uso de solventes orgânicos, para aumentar sua biodisponibilidade por administração oral em testes futuros. O trabalho foi dividido em 3 capítulos, sendo cada capítulo um artigo científico. O primeiro capítulo é uma revisão da literatura sobre morte celular imunogênica e estresse no retículo endoplasmático. O segundo capítulo é sobre o desenvolvimento e caracterização de uma nanopartícula lipídica sólida contendo curcumina. O terceiro capítulo é sobre o estudo da morte celular imunogênica provocada por curcumina.

CHAPTER 1: IMMUNOGENIC CELL DEATH, DAMPs AND CANCER THERAPY

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Future publication in: Immunobiology

ABSTRACT

The interaction of tumors with the immune system has been proven crucial in cancer progression and treatment outcomes. Recently, the induction of immunogenic cell death (ICD) has been suggested as a candidate strategy for cancer immunotherapy. ICD is mainly characterized by the release and exposure of damage-associated molecular patterns, such as calreticulin and ATP, which help to elicit an immune response against the antigens of the affected cell. In this review, different aspects of ICD in cancer biology are discussed.

Keywords: immunogenic cell death, DAMPs, immune system, endoplasmic reticulum stress

1. Introduction

The immune system is highly specific in detecting and eliminating infectious agents, such as viruses, bacteria and fungi. Moreover, several studies show the immune system to be crucially involved in the defense against cancer. Although great advances in the field of tumor immunology have been achieved, mainly over the last three decades, it is quite probable that the majority of the immune mechanisms of defense against tumor cells, as well as the immune evasion strategies used by cancer, has not yet been uncovered (Weinberg 2014). In this context, the area of cancer immunotherapy is very promising for new therapeutic alternatives against cancer. The immune system is divided into two main branches: the adaptive immunity and the innate immunity. These two branches of immunity

usually work together, and depend upon each other, but can be didactically characterized by the way the pathogen is distinguished from the host (Weinberg 2014).

The main distinctive mechanism of the innate immunity is the capacity of recognizing infectious agents and abnormal cells due to their expression of molecular patterns, such as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). PAMPs are molecules shared by different pathogens, such as the lipopolysaccharide, certain glycolipids, and other molecules, while DAMPs are molecules of the host itself exposed to the immune system in a context of tissue or cell damage, such as extracellular ATP, calreticulin, and others. Rather than recognizing specific antigens, cells of the innate immune response recognize characteristic molecular patterns that are present on the surface of infectious agents, or tumor cells, that are not expressed in normal cells (Weinberg 2014). As bacterial and viral infections progress, they release increasing amounts of pathogenic material. The recognition of these PAMPs, such as bacterial RNA, by the innate Toll like receptors (TLRs) results in a robust amplification of $CD4^+$ and $CD8^+$ T cells. This stimulation of innate TLRs provides a non-specific warning to the immune system of the presence of invading pathogens and elicits appropriate non-specific innate immune response (Ludgate 2012). Death of cancer cells by medical therapy causes the release of endogenous danger signals known as DAMPS (Ma et al. 2011). These DAMPS augment the presentation of tumor antigens released from death tumor cells ultimately inducing the immune system to attack cancer and thereby mimicking an acute infection. DAMPs and PAMPs may share some commonality of expression and it may be possible to evoke an acute anticancer immune response using an 'endogenous' vaccine approach (Ludgate 2012).

The adaptive immunity is based upon the generation of variable molecules, such as antibodies and T cell receptors (TCR), which can strongly interact with virtually all the possible pathogen molecules, named the antigens. Adaptive immunity can be divided into cell-dependent immunity and antibody-dependent immunity. The cell-dependent adaptive immunity is based on the interaction of T cells with the antigen presented on the surface of host cells. The antibody-dependent immunity is based on the interaction of antibodies, produced by B cells, with soluble or surface-associated antigens. As it specifically recognizes and eliminates infectious microorganisms, such as bacteria, fungi and viruses, through the activation of several distinct mechanisms, it is plausible to relate that the immune system has a mechanism that detects the presence of tumor cells. Once detected, the immune system

would be able to eliminate them (Weinberg 2014). Both the innate immune response and the adaptive immune response are necessary to prevent tumor development (Vesely et al. 2011).

The role of the immune system in in the elimination of cancer cells was suggested by Ehrlich in 1909, who speculated that it may suppress the growth of carcinomas. Fifty years later, Macfarlane Burnet and Lewis Thomas developed the concept of "immunovigilance", i.e., the ability of the immune system to promote an effective reaction to specific tumor cell antigens that eliminates the development of cancer before clinical expression, that is, is the ability of the immune system to promote an effective response against specific antigens in tumor cells that are not expressed in normal cells (Sanchez-castañón et al. 2016; Pernot et al. 2014; Dunn, Old, and Schreiber 2004).

Immunovigilance can function as a component of a process called cancer immuno-editing, which is described as a complex dynamics stablished between the immune system and the tumor (Pernot et al. 2014). The immuno-editing of cancer consists of three phases: elimination, equilibrium and escape. Elimination represents the classic concept of immunovigilance, because in this phase both the innate and the adaptive immunities can eradicate tumor cells and prevent carcinogenesis. If the elimination of all the potentially cancerous cells is not complete, then some altered cells may persist, in an equilibrium with the immune system, i.e., although these cells persist, they are not able to freely proliferate, and do not generate clinically apparent tumors. Some of the cells in equilibrium may develop immune evasion abilities, becoming then poorly or non-immunogenic. These cells eventually generate a tumor mass in the escape phase (Pernot et al. 2014; Dunn, Old, and Schreiber 2004; Sanchez-castañón et al. 2016).

Tumor cells may express neoantigens, which are different from any other normal protein in the host, or tumor-associated antigens, which are expressed in an unusual tissue/development stage, or even at higher than the normal amounts. These antigens can potentially be recognized by the immune system and trigger the activation of effector $CD8^+$ T cells that can eliminate the tumor cells. However, the immune evasion strategies used by cancer cells can avoid the elimination of the tumor. These strategies can be, for instance, a reduction on the expression of tumoral antigens, a lower expression of MHC, or the direct or indirect secretion of immune suppressors such as TGF-β or IL-10 (Escribese and Barber 2017).

2. Cancer immunotherapy: from William B. Coley to the modern immunotherapy

In the late 19th century it was observed that infection can have an effect on tumor regression (McCarthy 2006; Dobosz and Dzieciatkowski 2019). Case reports about the role of infections in stimulating the body to suppress tumors served as support for this hypothesis in 1891, when the American surgeon William B. Coley, treating patients with sarcoma, observed that some cancer patients with infectious diseases were getting greater success in cancer treatment (Oiseth and Aziz 2017; McCarthy 2006).

The seminal results from the investigation conducted by Coley are described in his work published in 1891 (Coley 1891). Coley reported patients who were diagnosed with sarcoma, contracted erysipelas after oncological surgery and, unexpectedly, had spontaneous remission of the tumor. This observed correlation brought to light the idea of injecting a mixture of bacteria (*Streptococcus pyogenes* and *Serratia marcescens*) inactivated by heat ("Coley toxin") to induce an antitumor immune response in patients with advanced and inoperable tumors(Dobosz and Dzieciatkowski 2019). This was one of the first anticancer immunotherapies described in the literature, consisting of a rational approach to eliminate cancer by targeting not the cancer cells, but rather the immune system.

The immunotherapy described by Coley was successful to some extent, with a number of patients supposedly cured from tumors. However, the standardization of the technique, based on the application of a mixture of bacteria, and patient safety concerns, made the idea not so popular by that time (Hobohm 2001; McCarthy 2006; Oiseth and Aziz 2017). The harsh criticism received by the work of Coley made this bacteria-based immunotherapy not popular in the clinical practice for some decades. However, in 1935, Codman analyzed the work of Coley and suggested that, in fact, there was a strong scientific basis in the published results (Codman et al. 1934; McCarthy 2006). This not only gave Coley the title of "Father of Immunotherapy", but also refreshed the interest of oncologists for immunotherapy (McCarthy 2006) . Later on, Macfarlane Burnet and Lewis Thomas developed the concept of "immunovigilance", which is the ability of the immune system to mount an effective reaction against tumor-specific or -associated antigens, eventually eliminating most of the potential cancer cells before their become a clinically apparent tumor (Coley 1891; Hobohm 2001; Codman et al. 1934).

Over subsequent decades, many advances in immunology contributed to the development of the modern immunotherapy (Dobosz and Dzieciatkowski 2019). Some of these advances are particularly noteworthy: (1) the identification and demonstration of the role of T lymphocytes in an animal models (Weiss 1967); (2) the demonstration of the

presence of dendritic cells in peripheral lymphoid organs (Steinman and Cohn 1973); and (3) the identification of natural killer cells (NK cells) (Kiessling, Klein, and Wigzell 1975; Kiessling et al. 1975). Many approaches to target the immune system as an anticancer strategy followed, such as the use of cytokines, vaccines, adoptive cell therapies and antibodies against immune checkpoints (Oiseth and Aziz 2017).

The more recent milestone in modern immunotherapy, the discovery of immune checkpoint inhibitors, led to the development and approval by the FDA of anti-PD-1/PD-L1 and anti-CTLA 4 antibodies, which were proved to be effective against melanoma and different other tumors (Yousefi et al. 2017). Finally, all the discoveries over time, coming from Coley's first observations to the approval of new drugs, contributed to the great visibility of modern immunotherapy and to the development of new research fields, representing a new alternative in cancer treatment, mainly for patients with metastasis and inoperable tumors.

3. Immunogenic cell death

The immune system is routinely exposed to dead cells during normal cell turnover. However, in certain cases, cells die because something is not right. Viruses, oncogenes, and unfavorable conditions, such as hyperthermia, can also induce cell death. Thus, normal and abnormal dead cells must be discriminated by the immune system. Understanding, and possibly manipulating, these processes can have important implications for the therapy of cancer (Green et al. 2009).

Depending on the initiating stimulus, cancer cell death can be immunogenic, nonimmunogenic or even tolerogenic (Kroemer et al. 2013a). Various factors work in concert to determine whether cell death is immunogenic or not. These parameters include the intrinsic antigenicity of the cells, the history of activation or stress before cell death, the nature of the cell death inducer, and the precise cell death pathway triggered (Kroemer et al. 2013a).

The innate immune system uses endogenous molecules released by cells under stress, injury or cell death known as DAMPs. DAMPs are responsible for activating immune and inflammatory mechanisms by binding to specific receptors (Showalter et al. 2017; Guo, Liu, and Bartlett 2014). In recent decades, some DAMPs have stood out and have been increasingly studied and characterized in different animal models, among them, the high mobility group box 1 (HMGB1), adenosine triphosphate (ATP), stress proteins in the

endoplasmic reticulum as calreticulin (CRT) and heat shock proteins (HSPs) (A D Garg et al. 2014; Kroemer et al. 2013b; Abhishek D Garg et al. 2012). Tests with these proteins have shown promising results in the induction of innate immune responses (Wang et al. 2018; Turubanova et al. 2019).

Naturally, DAMPs are considered intracellular molecules, however, under stressful conditions and pathological changes promote intra- or extracellular release of these proteins for defense and signaling to the surrounding cells to the system. Immediately after recognition of a molecule as DAMP the innate immune system, a signaling cascade is triggered which activates transcription factors, leading to the release or production of cytokines, chemokines which, cause structural and molecular changes in response to injury (Showalter et al. 2017).

Regarding tumor cells, DAMPs function as adjuvants enhancing the immunogenicity of the organism or activate antigen-presenting cells (APC) such as dendritic cells (DCs) facilitate the uptake of tumor antigens by DCs initiate the cross-presentation of antigens and, initiate an adaptive antitumor response that targets local and distant cancer cells (da Cunha, Antoniazi Michelin, and Cândido Murta 2016; W. Yang et al. 2019). In the absence of harmful conditions, cells are programmed to die without inflammation local or systemic. This process of homeostasis of cell death often occurs by tolerogenic or non-immunogenic apoptosis. However, in the case the cells are dying as a consequence of metabolic stress, for instance, immunogenic cell death (ICD) can be triggered (Kroemer et al. 2013a). ICD results in a complex cellular communication between dying and immune cells, eventually leading to the activation of a specific immune response against the antigens of the affected cell (Yatim, Cullen, and Albert 2017; Legrand et al. 2019). Legrand, A. J. et al, 2019 propose a different approach to the definition of ICD. They integrate it into the wider context of tissue repair, and not only as a cellular death event (Legrand et al. 2019).

In this context, ICD is said to be a cell death capable of assembling adaptive immune responses. Defense cells play a significant role in tumor protection and development of this type of immunity is the goal for the success of cancer treatment. ICD contributes efficiently to the positive result of chemotherapy treatment for cancer (Y. Yang, Hu, and Wang 2016).

ICD is a prominent pathway for the activation of the immune system against cancer, which may result in the success of anti-cancer therapies in the long term, due to the specific immune responses that increase therapy effectiveness compared to commonly used

anticancer agents (Krysko et al. 2012; Green et al. 2009). In the body, cell death is a normal process of tissues, most cells are programmed to die without inflammation local or systemic. This process of homeostasis of cell death often occurs through apoptosis, being a tolerogenic (tolerates itself) or null (does not impact the immunity system). While necrosis were associated to immunogenic cell death, which is a modality of cell death that stimulates an immune response against antigens of the dead cell, particularly when derive from cancer cells (Green et al. 2009).

Many studies using natural and synthetic compounds of pharmacological interest have been described as triggering mechanisms of cell death leading to the emission of danger signs. This immune modulation mechanism, mainly by dying cancer cells after cytotoxic stress, can be considered as an essential component of immune-mediated anti-cancer approaches (Park et al. 2019; Alessandra da Cunha; Marcia Antoniazi Michelin; Eddie Fernando Cândido Murta 2016; Guo, Liu, and Bartlett 2014).

Contrary to what was thought, current studies show that some types of apoptosis can also initiate immunogenic cell death in tumors, depending on the composition of the cell surface and the products secreted by the dying cells (Pitt, Kroemer, and Zitvogel 2017; Radogna, Dicato, and Diederich 2019). Therefore, cancer cell death can be immunogenic or nonimmunogenic, depending on the initiating stimulus (Kroemer et al. 2013b). Various factors work in concert to determine whether cell death is immunogenic or not. These parameters include the intrinsic antigenicity of the cells, the history of activation or stress before cell death, the nature of the cell death inducer, the precise cell death pathway that is engaged and the availability of immune cells that can respond (Green et al. 2009).

So far, several anti-cancer agents are capable of triggering or not the ICD. Drugs such as mitoxantrone, oxaliplatin, and cyclophosphamide appear as ICD inducers, whereas cisplatin, etoposide and mitomycin C do not. Interestingly some of these drugs are well characterized and are used as ICD control groups in several studies. Additionally, the ICD can act in synergy with immunostimulatory treatment including checkpoint inhibitors, T-cell recruitment and inhibition of MDSCs (myeloid-derived suppressor cells) and T reg (regulatory T lymphocytes) (Sukkurwala et al. 2014; Diederich 2019; Park et al. 2019).

The composition of immune cells in a tumor changes with the use of chemotherapeutic drugs, this may be essential to obtain therapy results. In patients with colorectal cancer and human breast, treated with oxaliplatin and anthracyclines, respectively,

showed an increase in the number of T lymphocytes, and thus an increase in the proportion of lymphocytes T $CD8^+$ on FOXP3+ regulatory T lymphocytes in the tumor after chemotherapy, predicting favorable therapeutic responses (Kroemer et al. 2013a). Human tumor cells killed by anthracyclines are also apparently immunogenic and have been used successfully for therapeutic vaccination of cancer patients (Zappasodi et al. 2010). The immune response stimulated by chemotherapy involves the presence of dendritic cells, which phagocytize, process and present antigens from dead tumor cells (Kroemer et al. 2013a).

In this context, the researchers suggest some chemotherapeutic drugs (those that considerable success rates) can induce stress and immunogenic death in cancerous cells. (Kroemer et al. 2013a). Thus, cancer cells from patients works as vaccines that stimulate a specific immune response to tumor that can control or eradicate residual tumor cells (Zitvogel, Kepp, and Kroemer 2011). Researchers considering ICD can follow two factors: tumor cells that affect ICD *in vitro* and are administered in the absence of any adjuvant can cause a response immune system that protects mice from a subsequent graft of tumor cells viable from the same lineage (Green et al. 2009); and a second serious event in ICD that occurs *in vivo* must cause an immune response with the recruitment of effector immune cells at the tumor site, and thus result in the inhibition of tumor growth via mechanisms that depend (at least in part) on the immune system (Michaud et al. 2011). Pre-clinical studies have shown that changes in various mechanisms of the immune system compromise one or both phenomena (Kroemer et al. 2013a).

Not all chemotherapeutic drugs have the ability to induce ICD (Kroemer et al. 2013b). In addition to that biochemical analyzes show different mechanisms of ICD: the pre-apoptotic exposure of calreticulin (CRT) and other proteins from the endoplasmic reticulum, ATP secretion, and cell death associated with HMGB1 release (high-mobility group box 1 protein). The identification of cell death mechanisms immunogenic drugs are important to identify the ability of drugs to induce this type of death (Zappasodi et al. 2010; Abhishek D. Garg et al. 2010). However, in most cases, all these hallmarks are not detected at once, only a few are found together. That is why a predicted ICD score for compounds present in the FDA-approved drug library was calculated based on the correlation between biological and physicochemical characteristics using five biological signs of ICD: phosphorylation of eukaryotic initiation factor (eIF)2α, CRT exposure, HMGB1 release, formation of RasGAP SH3 domain binding protein (G3BP) stress granules, and microtubule-associated proteins

1A/1B light chain 3A (LC3) puncta (Tesniere et al. 2010; Radogna, Dicato, and Diederich 2019; Bezu et al. 2018).

However, summarizing the main characteristics of an immune response to cell death are determined by the precise molecular signaling between dying cells and local immune cells (Pitt, Kroemer, and Zitvogel 2017). The main mechanisms to the activation of the immune system are: calreticulin (CRT) exposure; the release of HMGB1; the autophagy-dependent release of ATP; viral mimicry that induces IFN type I signaling and stress in the endoplasmic reticulum (ER), which can be caused by its oxidative stress, leading to unregulated Ca^{2+} release, changes in the accumulation of lipids or carbohydrates that will result in protein malformation (Abhishek D. Garg et al. 2010; Pitt, Kroemer, and Zitvogel 2017). Even so, ICD inducers are primarily classified by their ability to selectively trigger ER stress that drives cancer cell death by apoptotic (Rufo, Garg, and Agostinis 2017; Radogna, Dicato, and Diederich 2019).

When proteins are malformed during stress in the ER, occur a response known as UPR (Unfolded Protein Response) due to a signaling cascade that is activated by three transmembrane proteins: ATF6 (activating transcription factor 6), PERK (protein kinase RNA-like ER kinase) and IRE1 (inositol-requiring enzyme 1) (Bezu et al. 2018). The UPR response is nothing more than the ER trying to restore homeostasis in the organelle, this restoration will depend on the amount of unfolded proteins found in the endoplasmic reticulum. Another pathway to be taken is that of cell death by apoptosis, if there is no repair in the organelle (Calvet et al. 2014; Abhishek D. Garg et al. 2010; Rufo, Garg, and Agostinis 2017).

4. Damage-associated molecular patterns (DAMPs)

Processes associated with ICD can result in the emission of molecules called DAMPs, among which can be mentioned those that are exposed on the plasma membrane, such as heat shock proteins (HSP70 and HSP90) and calreticulin (CRT), those that are secreted to the extracellular environment, such as HMGB1 proteins, uric acid and cytokines, and those that appear as degradation products of unfolded proteins, such as ATP, DNA and RNA (Abhishek D. Garg et al. 2010, 2012).

Normally these DAMPs perform specific functions while remaining hijacked by the cells. But, outside the cells they can activate various immune cell receptors, which include

families of standard recognition receptors. They are also capable of potentiating pro-inflammatory effects such as the maturation and activation of antigen-presenting cells, such as dendritic cells and macrophages, which ultimately activate T cells ($CD8^+$), creating an anti-tumor immunity (Zindel and Kubes 2020; Pitt, Kroemer, and Zitvogel 2017; Abhishek D. Garg et al. 2010).

Calreticulin is the most abundant chaperone protein in the lumen of the ER and your exposure is an early event required for immune stimulation following ICD on dying cells (Panaretakis et al. 2009; Pitt, Kroemer, and Zitvogel 2017). Under normal conditions, this protein is related to the function of modulating the calcium homeostasis process and to vital and specific functions within the cell, such as: regulation of most proteins; functioning as hormone receptors; performance in signaling pathways and consequently also in response to cell stress (Krysko et al. 2012; Abhishek D. Garg et al. 2010).

Garg at al, 2010 was the first to divide the DAMPs into the three major categories mentioned above : (1) DAMPs exposed on plasma membrane (e.g. calreticulin, HSP70 and HSP90), (2) DAMPs produced as end-stage degradation products (e.g. ATP, DNA and RNA) and (3) DAMPs secreted extracellularly (e.g. HMGB1, uric acid, IL-1α and other pro-inflammatory cytokines) (Abhishek D. Garg et al. 2010). Backing to exposed on plasma membrane the HSPs are a family are chaperone proteins like calreticulin which are important to refolding of proteins affected due to various stress conditions and also like CRT during stress are translocate to the plasma membrane (Rufo, Garg, and Agostinis 2017; Panaretakis et al. 2009; Calvet et al. 2014; Abhishek D. Garg et al. 2010)**.**

In response to ICD inducers, CRT translates from the lumen of the ER to the cell membrane under stress, being a pre-apoptotic response that acts as an “eat me” signal for phagocytes and functions as an engulfment signal for dendritic cells (Calvet et al. 2014; Pitt, Kroemer, and Zitvogel 2017). There is a consensus that ER stress is necessary for CRT exposure in the cell membrane, but the exact mechanism is not yet fully understood (Kroemer et al. 2013a; Panaretakis et al. 2009).

Exposed in membrane HSP70 and HSP90 act as DAMPs and determine the immunogenicity of stressed/dying cells. This is due to their ability to interact with a number of APC surface receptors like CD91, LOX1 and CD40 and to facilitate cross presentation of antigens derived from tumor cells (i.e. tumor associated antigens or TAAs), on MHC class I molecule, capable of evoking ($CD8^+$) T-cell response (Abhishek D. Garg et al. 2010).

Concerning DAMPs produced as end-stage degradation products ATP is one of the most important. The release of ATP associated with ICD occurs through a complex mechanism. Extracellular ATP operates as a strong chemoattractant and promotes not only the recruitment of immune cells to the ICD sites, but also their differentiation (Abhishek D. Garg et al. 2010; Kepp et al. 2013).

In addition to serving as one of the main sources of intracellular energy, ATP also acts in the extracellular signaling mechanisms. ATP is secreted in response to the cytotoxic and cytostatic effects of some aggressive agents such as chemotherapy drugs (Martins et al. 2014; Michaud et al. 2011). ATP release occurs during the process of apoptosis and the formation of the apoptotic bodies. In apoptosis, different mechanisms can lead to the release of ATP from the intracellular to the extracellular enviroment: the caspase-dependent activation of Rho-associated, coiled-coil containing protein kinase 1 (ROCK1)-mediated, myosin II-dependent cellular blebbing, as well as the opening of pannexin 1 (PANX1) channels, which is also triggered by caspases e autophagy (Martins et al. 2014). Of these three, opening pannexin 1 channels and autophagy appear to occur in immunogenic cell death. During the formation of apoptotic bodies, caspases 3 and/or 7 act in the cleavage in the C-terminal portion leading to the opening and activation of the PANX 1, then releasing the ATP into the extracellular region. Apoptotic activation of caspases is also necessary for the recruitment of macrophages to the tumor microenvironment (Michaud et al. 2011; Martins et al. 2014). In autophagy the cytoplasmic constituents are degraded in double membrane organelles, the autophagosomes and subsequently fused with lysosomes, resulting in the degradation of the autophagocytic content by acid hydrolases and recycling towards energy metabolism or anabolic reactions, releasing the ATP for the extracellular portion (Michaud et al. 2011; Martins et al. 2014; Calvet et al. 2014).

Michaud et al 2011., demonstrated the relationship between autophagy and ATP release, when autophagy is inhibited by the pharmacological route, extracellular ATP levels are reduced, canceling the immunogenic process. ATP in the tumor microenvironment starts to emit “find-me” signals to attract DC to the tumor and phagocyting the apoptotic bodies generated (Michaud et al. 2011). Once released into the extracellular medium, the ATP binds to the purinergic dendritic cells receptors P2RY2 and P2RX7, resulting in the influx of K^+ and Ca^{2+} ions, activating the inflammasome NLRP3, followed by activation of caspase-1, consequently stimulating proteolytic maturation and secretion of interleukin 1 (IL-1) and interleukin 18 (IL-18), contributing to the immunogenic response (Martins et al. 2014).

And regarding DAMPs secreted extracellularly the HMGB1 protein is one of the main DAMPs related to ICD, however, its release and action on the activation of cells in the immune system will occur long after apoptosis is triggered by stress, because it requires the permeabilization of nuclear and plasma membranes, constituting then an event post mortem (Kang et al. 2013). HMGB1 is in the nucleus linked to chromatin, having intra and extracellular functions. Before being released by some process that triggers ICD, the HMGB1 protein performs specific functions within the nucleus of eukaryotic cells, which are replication, repair, recombination, DNA transcription and genomic stability. HMGB1 works as a chaperone, helping to shape the structure and dynamics of nucleosomes, to increase the affinity of gene transcription (eg: p53, p73, NF-kB and estrogen receptors), and the protein also helps in telomerase homeostasis (Kang et al. 2013).

However, HMGB1 that trigger ICD is extremely selective in activating the immune response. This protein can be released by cells of the innate immune system without the need for cell death in response to pathogens or secreted by cells in response to some type of damage in the late phase of apoptosis (necrosis) (Abhishek D. Garg et al. 2010).

When secreted into the extracellular environment, HMGB1 stimulates the production of pro-inflammatory factors by binding with toll-like receptors TLR4 e TLR2 present in the dendritic cells. Among the agents that cause damage are some antineoplastic agents with anthracyclines, oxaliplatin and some photosensitizers (Kang et al. 2013). The release of HMGB1 is associated with integrity loss of the plasma membrane and with translocation of the cytoplasm nucleus into the extracellular environment. The HMGB1 once secreted in the extracellular environment emits "danger signals" stimulating the immune response and leading to the activation of cytokines by monocytes and macrophages, contributing to the death is in fact immunogenic (Kepp et al. 2014).

Both the activation of pre-apoptotic translocation of CRT, as well as the release of ATP and HMGB, results in the attraction of dendritic cells (DCs) to the tumor microenvironment. Once there, the DCs phagocytes the tumor cells and starts to release pro-inflammatory substances in the tumor (for example, interleukin IL-1β, IL-18, interferon-gamma (IFN-γ), among others). In addition, DCs also can present the tumor antigens for the antigen presenting cells, culminating mainly in the activation of $CD8^+$ cytotoxic T lymphocytes, which are fundamental in the activation of the tumor retraction immune antitumor response (Krysko et al. 2012).

In addition to these DAMPs, there are many others that contribute to the immunogenicity of cell death: immunostimulating cytokines such as interferon α (IFN-α), sphingomyelin metabolites, for example, ceramide and sphingosine-1-phosphate, a series of mitochondrial products such as mitochondrial DNA, N-formylated peptides and cardiolipin, cytosolic components such as urate, as well as products from the extracellular matrix breakdown, such as hyaluronan fragments (Abhishek D. Garg et al. 2010; Krysko et al. 2012). But we can still classify that the three main DAMPs that need to be present for the immune system to be actually recruited after cell death are calreticulin, HMGB1 e ATP (Krysko et al. 2012).

5. ER stress and immunogenic cell death

The endoplasmic reticulum (ER) is a complex intracellular organelle, that performs an important role in eukaryotic cells, such as: protein folding and translation, storage of Ca^{2+} and metabolism of lipids and carbohydrates. This organelle has been gaining evidence when it comes to cell death. In normal cells, oxidative stress promotes the accumulation of malformed proteins in the lumen of the ER and therefore it initiates the activation of the Unfolded Protein Response (UPR), this response is intended to maintain the homeostasis of the ER. However, when the stress stimulus persists for a long time and is not resolved, UPR can activate genes that lead to cell death (Abhishek D Garg and Agostinis 2014a; Kepp et al. 2013).

The stressors of a cell can be defined as any agents or events capable of disturbing the folding of proteins in the ER, many cellular alterations included hypoxia, viral infections, toxic insults, such as some substances used in the treatment of cancer (mitoxantrone, doxorubicin and TFD) or even air pollution, chronic alcohol consumption and smoking habit, can cause protein folding disorder, generating protein accumulation and in turn inducing ER stress and subsequent unfolded protein response (UPR) (Abhishek D Garg and Agostinis 2014b; Bravo et al. 2013).

The improperly folded and accumulated proteins in the ER induce a response in the reticulum, this response has practically two purposes: (1) Try to restore homeostasis in the organelle through the UPR response, where this restoration will depend on the amount of malformed proteins that are found in the ER; (2) It can lead to cell death by apoptosis, if there

is no repair in the organelle, thus inducing an immunogenic response (Abhishek D Garg et al. 2010).

One way to reverse the stress condition in the ER is by cleaving the transmembrane protein ATF6, this cleavage causes the ATF6 protein to release itself from the ER. Therefore, it travels to the Golgi complex and is fragmented into ATF6N and retained in that organelle. After the cleavage of two proteases, S1P and S2P (sphingonise-1 or 2 phosphatase), these fragments are released. These proteases cleave in the transmembrane cytosolic region (Golgi complex) releasing fragments of ATF6N which, in turn, are translocated to the nucleus. At the nucleus, ATF6N will drive a gene transcription to form more chaperones (eg: calreticulin, GRP78/BiP and GRP94) in the ER, in order to optimize the folding of proteins in the ER and also generate more associated protein breakdown components to RE (ERAD), to try to stabilize the homeostasis in the organelle (Abhishek D Garg et al. 2010).

Another form used by UPR's is the transmembrane protein IRE1α. During ER stress, this protein dimerizes and autophosphorylates, favoring the conformational change that will activate endoribonuclease domains. Therefore, the activation of IRE1α catalyzes the unconventional processing of the mRNA encoding XBP-1 in XBO-1S (splicing). This coding activates lipid synthesis through transcription factors in the nucleus. Therefore, the regulatory system that control gene expression is related to the impulse of improperly folded proteins, it also facilitates the entry of proteins into the ER, such as ERAD, which degrade the proteins resulting from stress (Miyagi, Kato, and Lin 2020).

The PERK protein also participates in the attempt to regulate homeostasis. This protein also dimerizes and autophosphorylates when stress occurs in the endoplasmic reticulum, its autophosphorylation phosphorylate another protein called elF2α (eukaryotic initiation factor 2α - elF2αs). Thus, elF2αs triggers the selective translation of the transcription factor known as AFT4. ATF4 will then regulate genes involved in protein folding, amino acid metabolism and regulation of oxidative stress (Miyagi, Kato, and Lin 2020).

However, even with all these repair mechanisms, the stress in the ER can be so intense that instead of repairing the cell, another UPR pathway is activated: induction of cell death by apoptosis. At that moment, the cell decides that repairing is no longer the viable solution, so via PERK signaling through the UPR's eIF2α-ATF4 produces the pro-apoptotic transcription factors called CHOP. Consequently, CHOP will mediate apoptosis by activating

the overexpression of the BIM/BH-3 protein, which activates the BAX and BAK proteins. These two activated proteins bind to the mitochondria membrane and released the cytochrome C complex in the cell's cytosol. The cytochrome C complex activates apoptosomes, which then activate caspases (eg, caspases 3), thus initiating cell apoptosis. CHOP also acts on the Bcl-2 protein (autophagy activation factor), inhibiting its action (A D Garg et al. 2013; Quentin et al. 2012).

The normal functioning of the ER is essential to promote normal cellular activities and survival. Any condition that interferes with the normal functions of the ER triggers the accumulation and aggregation of folded or unfolded proteins and the induction of a response to restore normal function. Microenvironment of cancer cells includes resistance to stress in the ER so that the tumor can continue to develop and grow. Stress in the ER can lead to the induction of proteins that promote the blocking of stress signals, maintain its integrity and function, ensuring the correct folding of proteins and protecting the cell from the toxicity of poorly folded proteins, maintaining survival and/or growth of cancer cells. This resistance mechanism allows tumor progression, metastasis and resistance to radiotherapy and chemotherapy. It is reported that prolonged stress in the ER impairs these protection mechanisms, overcoming them in cancer cells, leading to organelle dysfunction and programmed cell death (Bakhshi et al. 2008).

6. Other forms of cell death that can activate the immune system

Immunotherapy is a therapeutic approach that aims to activate the immune system for response against tumor-associated antigens (TAAs) and finally destroy these tumor cells (Sanchez-castañón et al. 2016). Specific anticancer agents can cause immune control or tumor cell death and as residual cells, but if the agent is unable to stimulate this type of immune response, such as tumor cells as they die no immune signals emitted, the immune system is not activated, as effector cells are immunosuppressed and finally a therapeutic failure occurs. More recent studies use the blocking of checkpoints via CTLA-4 and PD-1 proteins, respectively, expressed by lymphocytes, so that T cells can circumvent the escape of cancer cells and thus immune system to recognize and combat them (Goto et al. 2019; Wei, Duffy, and Allison 2018).

Another method of immunotherapy in cancer, not so recent but very effective are the use of vaccines aims to recruit the immune response in addition to eliminating the tumor

generating protection against a possible recurrence. In vaccines, peptides derived from antigens are used associated with tumors, entire tumor cells, dendritic cells generated *in vitro* or based on in viral vectors. Immunotherapy can also be used to prevent suppression of the response immune response to tumor cells using checkpoint inhibitors with specific antibodies (examples: anti-CTLA, anti-PD-1, anti PD-L1). These specific antibodies are intended to restore the immune system against cells tumoral. Considering all the strategies used in oncology, the sipuleucel-T (Provenge®) vaccine for prostate cancer has been approved by the FDA (Sanchez-castañón et al. 2016).

Some studies used to say that chemotherapy that induces cell death immune system, for example, studies indicate that oxaliplatin reduces cell activity dendritic and anti-VEGF agents inhibit the expansion of regulatory T cells (Sanchez-castañón et al. 2016). Contrary to what was said in previous studies, in murine models and tissues oxaliplatin-based chemotherapy can trigger pre-apoptotic exposure of calreticulin and post-apoptotic release of the high mobility group protein box 1 (HMGB1), two signals that are required for immunogenic cell death. Dendritic cells have several receptors for HMGB1, including the toll-like receptor 4 (TLR4). In a murine model using dead CT26 cells oxaliplatin it was not possible to elicit an anti-tumor immune response in TLR4 deficient mice, whereas mice without this depletion were protected against a new graft with the same tumor lineage (Pernot et al. 2014). Other dendritic cell receptor related to immunogenic cell death is P2X7 which has a high affinity for ATP released by dead tumor cells, being able to modulate the susceptibility of tumor cells to anticancer treatments (Zitvogel, Kepp, and Kroemer 2011).

It has been much questioned about what type of cell death influences the activity of the immune system, that is, which cell death is immunogenic or not.(Wu and Waxman 2019; Fan et al. 2018) It was thought that apoptosis was directly responsible for ICD, however, it is known that other forms of cell death are also responsible. It is still unclear, but the potential of the anticancer immune response is not directly correlated with the type of cell death, but rather due to the biochemical and molecular changes that occur in cells (Galluzzi et al. 2016; Fan et al. 2018).

Necrosis induces activation of the immune system more frequently than apoptosis, due to the sudden release of pro-inflammatory mediators. Studies also show that necrosis induces the extracellular release of some DAMPs as in the work by Saito et al., 2005 (Saito, Dai, and Ohtsuka 2005), using HeLa, which detected HSPs outside cells after treatment with acrylamide indicating that these HSPs are released from cells killed by necrosis. Scaffidi et

al, 2002,(Scaffidi, Misteli, and Bianchi 2002) demonstrated that HMGB1 is released by HeLa cells after necrosis induction, besides showing that HMGB1 (- / -) necrotic cells have a reduced capacity to promote inflammation. Iyer et al., 2009, (Iyer et al. 2009) showed that necrotic cells produced by pressure disruption, hypoxic injury, or complement-mediated damage were able to increase the release of ATP produced by the mitochondria of damaged cells.

Not all cell death inducers cause the exposure of immunogenic factors on the cell surface or the release of immunogenic signals in the extracellular space. Additionally, the anticancer agent can even cause the release of immunogenic signs of a tumor, but not to others because release requires the intervention of specific signal transduction pathways. Cell death of tumor cells is a complex, multi-factor process and can induce immune responses, which can range from immunosuppression to induction of tumor-specific immunity. Studies also show that the location of tumor cell death and the local cellular environment of dying tumor cells can affect antitumor immunity (Wang et al. 2018).

7. Conclusion

ICD is of great importance in cancer immunology, as it can activate the immune system against tumor antigens. Although the induction of ICD has not been rationally used as a clinical treatment modality, it a great potential as an immunotherapy tool. Although recent advances in the immunotherapy of cancer resulted in good clinical outcomes against unresectable melanoma, for instance, the efficacy of this approach is still low, suggesting that the interaction of tumors with the immune system is far from being clearly understood. Studies on the immune evasion strategies deployed by tumors, as well as on the mechanisms underlying the activation of immune cells against abnormal host cells, can give over the next decades new weapons against cancer. The current knowledge allows to suggest that the induction of ICD combined with other immune-activating strategies, such as immune checkpoint blockade, is a viable immunotherapy approach to treat cancer.

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CHAPTER 2: DEVELOPMENT, CHARACTERIZATION AND *IN VITRO* CYTOTOXITY OF CUBOID SOLID LIPID NANOPARTICLES LOADED WITH CURCUMIN

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Future publication in: Chemical Materials Engineering C

ABSTRACT

Curcumin has shown inhibitory and cytotoxic activity against different cancer cells. Curcumin has a wide range of pharmacological activities including antioxidant, antiinflammatory, antidiabetic, antibacterial, antiatherosclerotic, hepatoprotective and anticarcinogenic. However, its clinical applications are limited owing to its poor aqueous solubility and rapid clearance. Thus, the purpose of this work was to develop a new solid lipid nanoparticle formulation to be used as a vehicle for curcumin administration by oral or parenteral route. A simple, low-energy, scalable method was used, which yielded solid lipid nanoparticles stable in size for at least 300 days at room temperature and at 4 ºC. Their lipid core consisted of a mixture of murumuru butter and Compritol® 888 ATO, which enabled to load a high concentration of curcumin. Interestingly, these nanoparticles shown symmetrical cube-like morphology, as evidenced by transmission electron microscopy. The blank nanoparticle, with no curcumin, did not show any evident *in vitro* cytotoxicity. However, the curcumin-loaded nanoparticles are more toxic than free curcumin to murine colon tumor cells (CT26). The curcumin encapsulation efficiency was 100%, as expected, since the hydrophobicity of curcumin facilitates its loading into the lipid matrix of the nanoparticle. It was further observed by XPS analysis that the presence of curcumin in nanoparticles did not change the surface composition when compared with nanoparticles without curcumin,

reinforcing the homogeneous matting of curcumin and matrix lipids into the core of nanoparticles. The fluorescence microscopy images evidenced that curcumin-loaded nanoparticles are taken up by CT26 cells *in vitro*, both in monolayer and spheroids cultures. Thus, the present work propose a novel formulation of solid lipid nanoparticles which is promising carrier system for hydrophobic molecules such as curcumin.

Keywords: murumuru butter, Compritol® 888 ATO, drug delivery, encapsulation, spheroids

1. Introduction

Curcumin, a highly hydrophobic phenolic compound found in the rhizome of turmeric (*Curcuma longa*), has a wide variety of pharmacological effects, such as anti-inflammatory, antiprotozoan, antiviral, hypoglycemiant, antioxidant, wound-healing, and also anticancer activities [1–4]. Particularly, recent studies show curcumin to inhibit cell growth, to affect the cell-cycle and to induce apoptosis in various cancer cell lines [5–7]. However, the therapeutic use of curcumin is limited mainly because of its low solubility in aqueous media, rapid metabolization and low bioavailability by the oral route [8, 9]. Several colloidal drug carrier systems, mostly based on polymeric and lipid materials, have been proposed to disperse and to improve the bioavailability of curcumin [7, 10]. In that regard, solid lipid nanoparticles (SLN) can be used as colloidal curcumin carriers.

SLN were invented about 25 years ago and, since then, used as drug carriers in pharmaceutical technology [11]. Better stability and ability to modify the drug release may be cited as advantages of SLN over other systems, such as nanoemulsions and liposomes; moreover, as the usual SLN formulations are composed of natural lipids and other biodegradable materials, SLN are more biocompatible than a number of polymeric nanoparticles [12]. Usually, SLN show diameters below 1 μm, and present a core of lipids that are solid at room temperature, stabilized by nonionic surfactants. Hydrophobic drugs may be associated to the solid lipid matrix and to the surfactant layer of SLN sand thus they can load [7, 13]. These colloidal systems may be used not only to disperse hydrophobic drugs in aqueous media, but also to alter the drug release profile and to protect the drug from degradation [14].

The goal of this work was to develop a curcumin-loaded SLN by a simple, organic solvent-free method, using biocompatible lipids. The experiments described in the present work show the matrix of the developed SLN to be non-cytotoxic *in vitro*. Moreover, this

nanoparticle presented a 100% curcumin encapsulation efficiency, with long-term stability, and increased the toxicity of curcumin *in vitro* against murine colorectal CT26 cancer cells.

2. Methods

2.1 Materials

The murine colon carcinoma cell line (CT26) were purchased from American Type Culture Collection (ATCC), Manassas, VA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), curcumin (*Curcuma longa*, >65%), Compritol® ATO 888, Span® 20, Tween® 80 and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich, USA. Roswell Park Memorial Institute medium (RPMI), fetal bovine serum (FBS), penicillin, streptomycin and trypsin were all purchased from Gibco, Carlsbad, CA. Phosphate buffered saline (PBS) was purchased from Laborclin, Pinhais, Parana, Brazil. The murumuru butter was purchased from Amazon Oil (Brazil).

2.2 Development of solid lipid nanoparticles of curcumin (SLN-C)

The development of the solid lipid nanoparticles of murumuru and curcumin (SLN-C) was based on a phase-inversion temperature method. A solid matrix composed by murumuru butter and Compritol® ATO 888 (7.5:2.5 w/w) was placed with a mixture of surfactants Span® 20 and Tween® 80 (2:8 w/w), at a proportion of lipid mixture and surfactant mixture 1:1 w/w. After melting the organic phase at 75 °C, the curcumin was added, forming a clear, yellowish solution. Then, 40mL of water at 65 °C was added to the organic phase, and the temperature was raised above the cloud point, to 91 °C, and remained in magnetic stirring for 30 minutes. Next, the formed emulsion was left to cool down to the room temperature, forming the SLN-C., The final formulation had 500 µg curcumin/mL. The blank SLN was prepared by the same method, but without the curcumin.

2.3 Nanoparticle size and morphology

The solid lipid nanoparticles were analyzed in light scattering apparatus (ZetaSizer, Malvern) for the determination of the hydrodynamic diameter (z-average) and polydispersity index (PDI). In addition, the nanoparticles were analyzed by transmission electron

microscopy (JEOL, JEM-2100F UHR, 80kV - 200kV, USA) for their diameter and morphology. Negative contrasting was performed with ammonium molybdate.

2.4 Encapsulation efficiency

The encapsulation efficiency was assessed after extraction of the curcumin loaded in SLN-C. Briefly, SLN-C and SLN were centrifuged at 15000 G for 30 min, supernatant was collected and then diluted 1:10 (v/v) in DMSO and submitted to ultrasonic bath for 3 minutes at room temperature. The result suspension was diluted (1:100 v/v) in mobile phase (aqueous 1% citric acid pH 3, and acetonitrile, 55:45 v/v), and submitted to a slight centrifugation at 500 G for 3 min, than the supernatant was collected to perform the analysis by high performance liquid chromatography method (HPLC) (Shimadzu, Tokyo, Japan) with detection by fluorimetry. The encapsulation efficiency was calculated with the equation 1, below.

Equation 1

$$\frac{\text{Obtained concentration}}{\text{Theoretical concentration}} \cdot 100$$

2.5 Curcumin release kinetics SLN-C

The release of curcumin from SLN-C was evaluated by a dialysis method previously described by Behbahani et al. [15], with adaptations. The dialysis was performed with Spectra/Pro® dialysis membranes with 14 kDa pores containing 1 mL of formulation, and immersed in the dialysis medium (water:ethanol, 50%, v:v). For every predetermined time point, a sample of the dialysate was collected and replaced with the same volume of the dialysis medium. The concentration of curcumin was measured by HPLC as previously mentioned. Different kinetic fitting models were used to examine the curcumin release results in order to establish the *in vitro* profile kinetic (Zero-order Model, First-order Model, Hixson-Crowell and Higuchi models). Both the R^2, to indicate the strength of correlation, and the Akaike information criterion (AIC), to indicate the loss of information by the model, were used as criteria of choice of the best fitting model.

2.6 XPS Analysis

XPS experiments were performed with a SPECS Sage HR 100 spectrometer with a non-monochromatic X-ray source (Magnesium K_α line of 1253.6 eV energy and 250 W) and calibrated using the $3d_{5/2}$ line of Ag with a full width at half maximum (FWHM) of 1.1 eV. The selected resolution for the spectra was 15 eV of Pass Energy and 0.15 eV/step. All Measurements were made in an ultra-high vacuum (UHV) chamber at a pressure around $8 \cdot 10^{-8}$ mbar. An electron flood gun was used to compensate for charging during XPS data acquisition. In the fittings Gaussian-Lorentzian functions were used (after a Shirley background correction) where the FWHM of the peaks were constrained while the peak positions and areas were set free. The peak position of each band was compared with the values available in the literature.

2.7 Fourier-transformed infrared spectroscopy

Fourier-transform infrared spectroscopy (FTIR-ATR) was used to assess the interactions between functional groups level of the curcumin and the carrier system components. The technique was performed with a Bruker brand Vertex 70 equipment with attenuated total reflectance (ATR), with a spectral resolution of 4 cm^{-1}, and 48 scans in the range of 375 to 4500 cm^{-1}. The isolated components, the physical mixture (PM), the SLN and the SLN-C were evaluated.

2.8 Raman spectroscopy

Complementary to the FTIR, Raman spectroscopy was performed in order to evaluate the possible interactions between nanoparticle components. The analysis was performed with a resolution of 0.7 cm^{-1} in the range of 150 to 3200 cm^{-1} by the LabRam HR Evolution equipment of the Horiba brand with the samples listed in the previous item. The measure was performed at different wavelengths for different samples: for SLN the analysis was analyzed at the green line, 532 nm; for the curcumin and SLN-C, the samples were analyzed in the red line, 633 nm.

2.9 Cell culture

CT26 cells were cultured in RPMI-1640 medium (Roswell Park Memorial Institute of Medicine) supplemented with 10% (v:v) fetal bovine serum (FBS) and 1% (v:v) antibiotic solution (100 units penicillin/mL and 100 mg streptomycin/mL). Cells were maintained in an incubator under humidified atmosphere with 5% CO_2 at 37 ºC. The spheroids were made using MicroTissues® 3D Petri Dish® micro-mold spheroids (Sigma Aldrich, EUA) according to the manufacturer protocol.

2.10 *In vitro* analysis of cytotoxicity

The MTT assay was used to verify cell viability [16]. The test was performed on cells (1×10^4 cells /well, in a 96-well plate) treated for 4 or 24 h with: 1) free curcumin; 2) SLN-C; 3) SLN and 4) no treatment (control). Each treatment was performed in triplicate of six different concentrations of curcumin (10.0, 5.0, 2.5, 1.2, 0.6, 0.3 μg/mL). After 4 or 24 hours the treatments containing were removed and the cells were washed with PBS and performed the MTT assay protocol. The cells were incubated with 0.5 mg/mL of MTT in culture medium for 2.5 hours at 37 °C in humid atmosphere and 5% CO_2. The MTT solution was discarded and the formazan was extracted from the cells with 200 μL of DMSO. The plates were analyzed by spectrophotometer (Spectramax M2, Molecular Devices, USA) and the absorbance was measured at the wavelength of 595 nm.

2.11 Analysis of cell morphology by flow cytometry

CT26 cells were seeded at a concentration of 6×10^4 cells/well in 24-well plates. After 24 hours the following treatments were performed: 1) curcumin; 2) H_2O_2 (positive control); 3) SLN-C; 4) SLN; 5) no treatment (control). The curcumin concentration was 10.0 μg/mL. After 24 hours of treatment, the cells were washed with PBS and treated with received trypsin. The cells were centrifugated at 500 G for 5 minutes. At each sample, 10,000 events were analyzed with a cytometer (FACS Canto II, BD, USA) to verify changes in cell size and granularity.

2.12 Analysis of internalization

CT26 were seeded at a concentration of 6×10^4 cells/well in 8-well plates of specific for the confocal microscope. The cells were treated for 4 or 24 h with: 1) 10 μg/mL curcumin, 2) SLN-C (equivalent to 10 μg curcumin/mL) and 3) no treatment (control). Next, the cells were washed twice with RPMI culture medium and analyzed by confocal laser scanning microscopy (LSM 880, Zeiss, Germany) for verification of the curcumin fluorescence using the 405 nm laser for excitation and 520 nm to emission.

2.13 Statistical analyzes

Data were analyzed with GraphPad Prism® version 6.0 software (GraphPad Software, La Jolla, CA). Significant differences between data sets were assessed by one- or two-way ANOVA, with post-tests of Tukey or Sidak ($\alpha = 0.05$). Quantitative results were expressed as mean ± standard error of the mean or mean ± standard deviation.

3. Results and Discussion

3.1 Colloidal characteristics of nanoparticles

The nanoparticles are cubic and monodisperse, as evidenced in figure 1, and stable at least for 300 days at 4 or 25 ºC (room temperature), as only little variations in hydrodynamic diameter (50 to 60 nm) and polydispersity index (0.11 to 0.22) were observed under these conditions (Figure 2). By analyzing the images, the average nanoparticles size distribution was 130,413 ± 17,450 nm (Figure 1). The nanoparticles stored at 37 ºC had significant changes in colloidal characteristics from the 15th day. The SLN were prepared using a phase-inversion temperature, consisting of dispersing the melted oil matrix in water then raising the temperature of the mixture to 91ºC, above its cloud point, and next cooling it down to room temperature. As the formulation cools down, causing the formation of solid nanoparticles, it becomes translucent. Particles that are translucent are smaller than opaque particles [10]. The decrease in temperature induces lipid crystallization and prevented the agglomeration and aggregation of nanoparticles [17]. The lipid particles are adequately dispersed in the aqueous solution due to the use of a stabilizer (surfactant) offering excellent physical stability, protection of environment sensitive labile drugs, and targeted drug delivery [18]. In this work it was possible to observe that the formulation stored at 4ºC and room temperature proved to

be stable in relation to size and PDI for at least 300 days. Lingling et al. 2016 developed Asiatic acid loaded SLNs (AASLN) such system with excellent stability at 4°C for 1 month [19]. Due to low risk/benefit ratio, several therapeutic patents are emerging out due to versatility of SLNs in various areas of research and development how improved therapeutics or cosmetic applications or for nutraceutical applications [18].

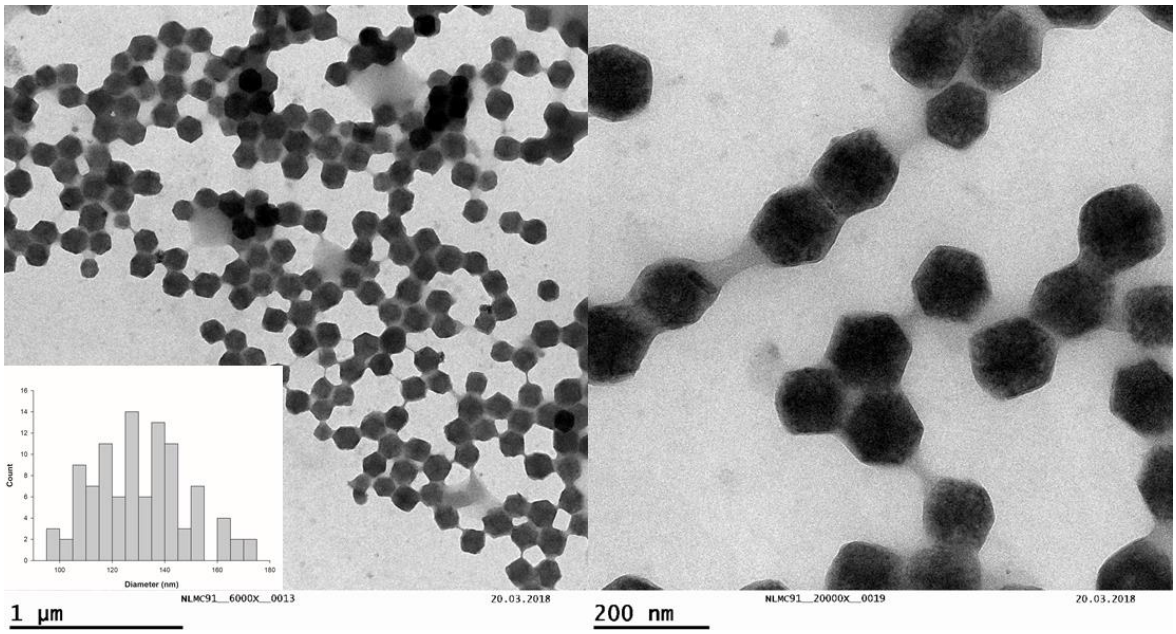

Figure 1: Transmission electron microscopy of nanoparticles with curcumin (SLN-C). Magnification of 6000 x on the left and 20000 x on the right. The box on the left shows size distribution of the nanoparticles.

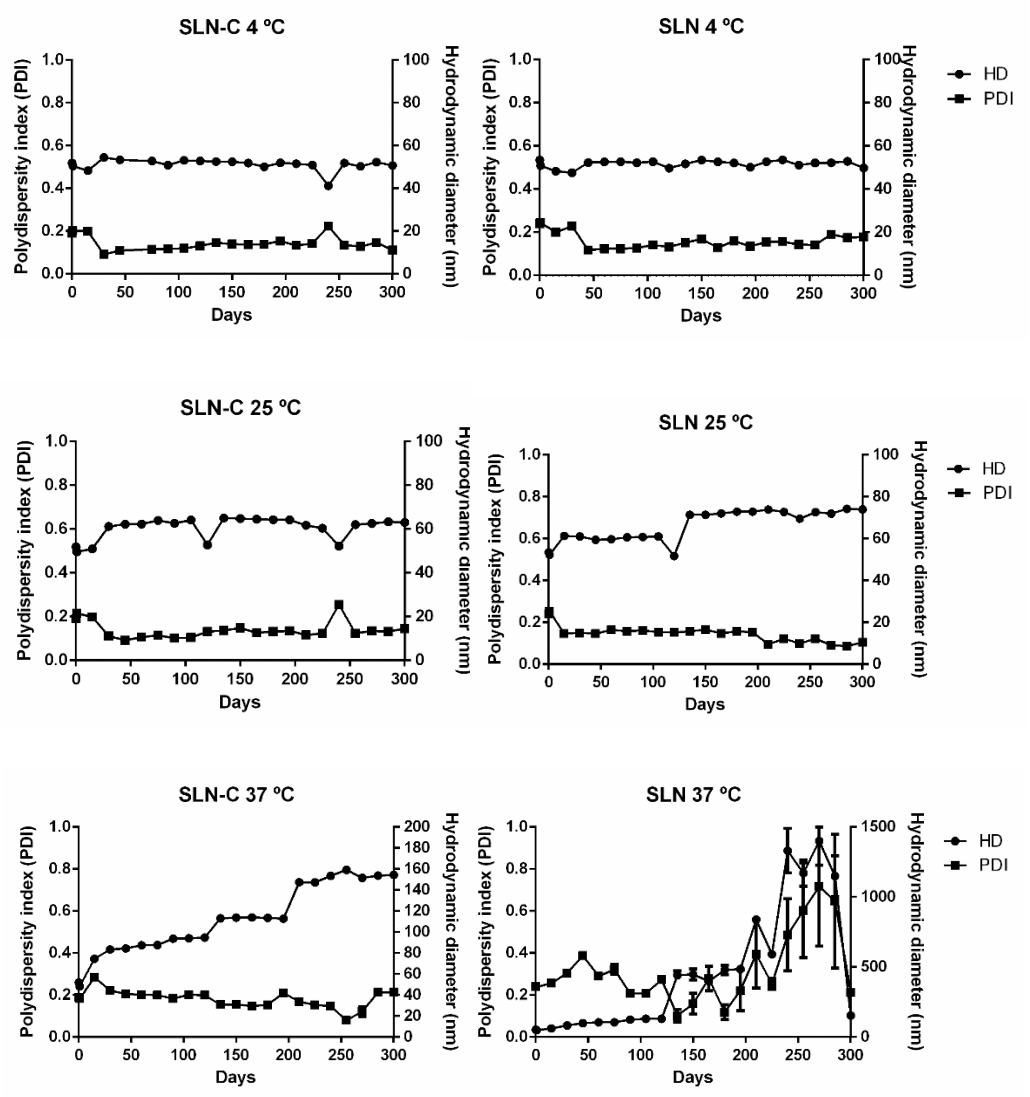

Figure 2: Polydispersity index (y-axis on the left) and hydrodynamic diameter (y-axis on the right) of nanoparticles with curcumin and blank nanoparticles (SLN-C and SLNM, respectively) for 300 days.

3.2 Encapsulation efficiency and kinetics of curcumin release from SLN-C

The curcumin was efficiently loaded into SLN-C, with a 100% encapsulation efficiency. This result can be attributed to the mixture between Compritol® ATO 888 and murumuru butter, as well as the complexity of components present in the second cited component. It is suggested that this mixture favored the formation of a system with more amorphous characteristics with greater ability to incorporate curcumin, favoring better results even found with nanostructured lipid carrier (NLC) containing curcumin [20–22].

The release profile of curcumin from SLN-C, obtained by dialysis method, can be observed in Figure 3.

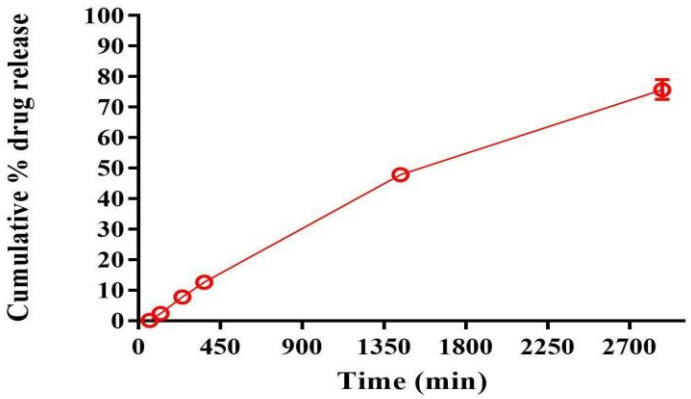

Figure 3. *In vitro* release profile of curcumin from solid lipid nanoparticles of curcumin (SLN-C).

Table 1 shows the tested mathematical models to *in vitro* release profile of curcumin.

Table. 1: Tested mathematical models, R^2, AIC and k values of release rate constant.

Model	R^2 value	AIC
Zero-order	0.9833	49.8230
First-order	0.9923	40.2570
Higuchi	0.8911	66.7162
Korsmeyer-Peppas	0.0748	91.8974
Hixson-Crowell	0.9972	30.4705

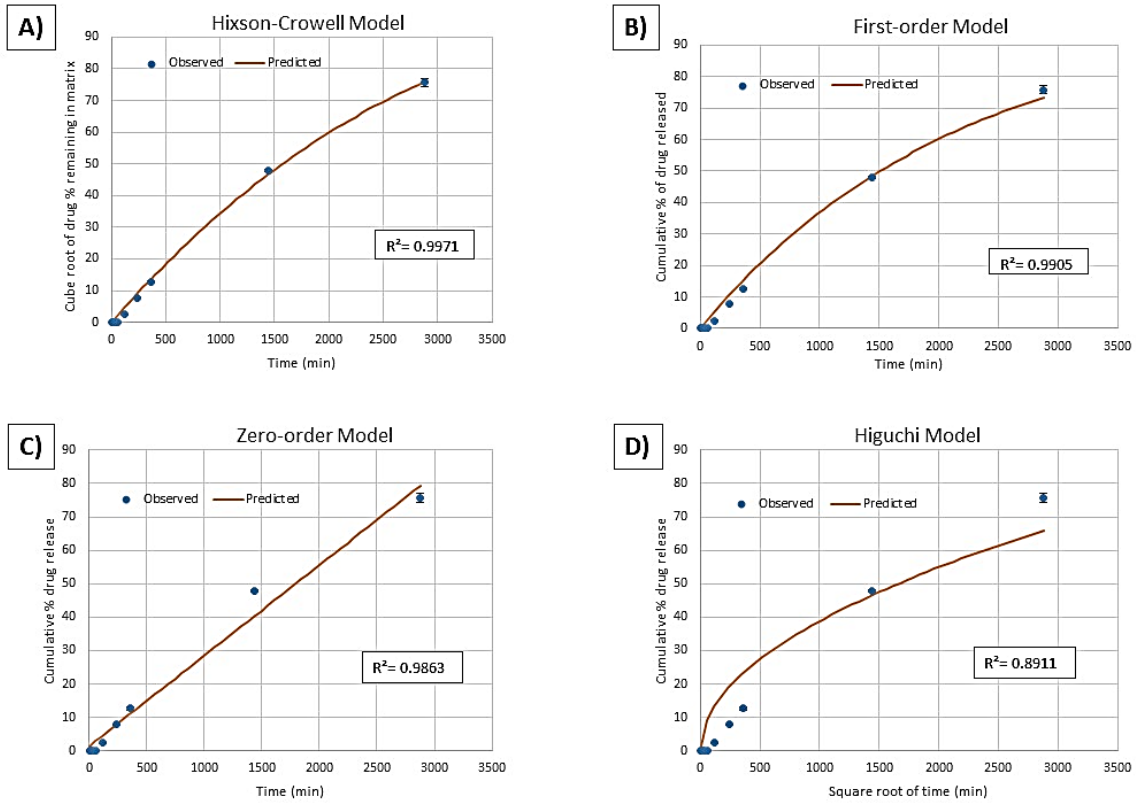

Figure 4. Hixson-Crowell Model (a), First-order Model (b), Zero-order Model (c) and Higuchi Model (d) release kinetics, predicted vs observed.

As observed in Table 1 and with the graphs grouped in figure 4, the mathematical model that best suits the release profile is Hixson-Crowell, considering its larger R^2 and smaller AIC. The Hixson-Crowell model applies to different pharmaceuticals and derives an equation for the dissolution rate, where there is a decrease in the surface area and volume of the particle while retaining its initial geometric shape [23–25]. Thus, it is suggested that the release of curcumin by SLN-C, in hydro-alcoholic medium, occurred by means of dissolution.

3.3 XPS Analysis

In figure 5 is shown the XPS spectra of C 1s and O 1s of solid lipid nanoparticles of murumuru with curcumin (SLN-C) and solid lipid nanoparticles of murumuru (SLN). The C 1s fitted spectra of the samples (Figure 5A and 5C) show the peaks associated to C−H/C−C, C−OH/C−O−C, and O−C=O bonds. In the spectra are shown the expected positions of C−H/C−C sp^2 bonds assigned at 284.8 eV, C−OH/C−O−C bonds at around 286.8 eV and

O−C=O bonds at around 289.2 eV. The Figure 6 also shown the O 1s spectra for SLN-C (Figure 5B) and SLN (Figure 5D). The expected positions of the C−O−C and O−C−O/O*−(C=O)−C bonds at around 532.4 eV and 533.3 eV, respectively. The elemental composition of SLN-C was at 88 % and 12.1 at. %, and SLNM was 88.1 % and 11.9 %, for carbon and oxygen, respectively.

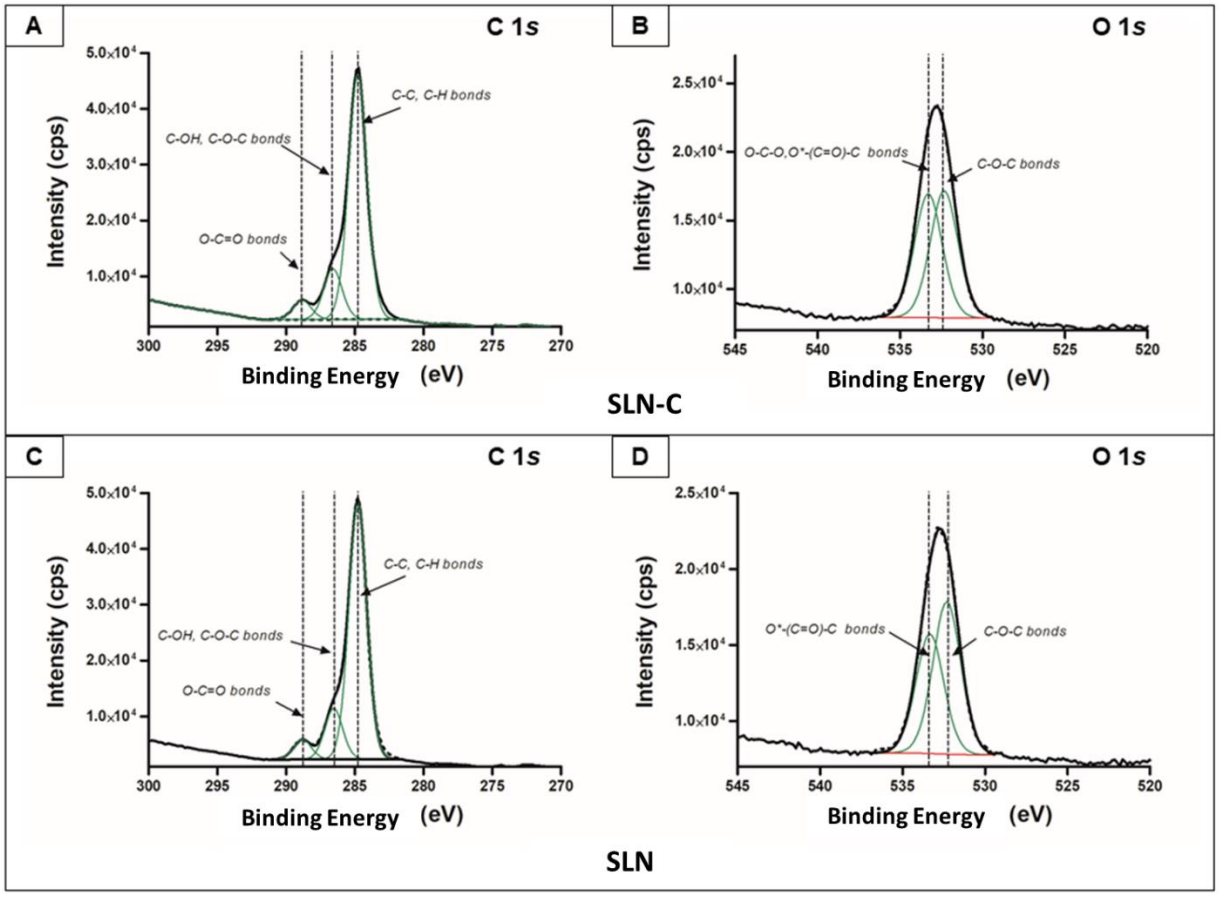

Figure 5. XPS spectra of solid lipid nanoparticles of curcumin (SLN-C) and solid lipid nanoparticles (SLN). A and C correspond to the C 1s spectra and B and D to the O 1s spectra, for both SLN-C and SLN.

XPS analysis gives information about the chemical structure and surface composition of nanoparticles. It was observed that the presence of curcumin in SLN-C did not change the surface composition of nanoparticles when compared to SLN. This is also corroborated by the elemental composition calculated, as the molar percentages of carbon and oxygen were around 88% and ~12.1%, respectively, in SLN-C and ~88.1% and 11.9 %, respectively, in SLN. In Figure 5A and 5B, a large peak in the C 1s spectrum of the SLN-C and SLN surface

is attributed to the convolution of the C−H/C−C, C−OH/C−O−C, and O−C=O species, with components centered around 284.8, 286.8 and 289.2 eV, confirming the structure of the components of the nanoparticles, as well as their homogeneous distribution in these nanostructures. Furthermore, the O−C=O component observed in the curve indicates a successful association of murumuru and curcumin. The O 1s spectra in Figure 5B and 5C are deconvoluted into two peaks of binding energies around corresponding to ~532.4 eV for C−O−C bonds and ~533.4 eV for O−C−O/O*−(C=O)−C bonds, reinforcing a homogeneous matting of curcumin and murumuru over the body of lipid nanoparticles.

3.4 Spectroscopic study (FTIR-ATR and Raman)

The FTIR spectra of Span® 20, Tween® 80, murumuru butter, Compritol® ATO 888, curcumin, SLN, physical mixture and SLN-C are presented in Figure 6, whose corresponding frequencies and assignments are shown in Table 2.

The spectrum of curcumin shows a broad peak at 3507 cm^{-1} indicating the presence of OH group. A peak corresponding to C=C and C=O is observed at 1626 cm^{-1}. The peak at 1600 cm^{-1} can be assigned to aromatic ring stretching (C=C ring). C=O, enol C-O, C-O-C peaks are found at 1509 cm^{-1}, 1273 cm^{-1} and 1027 cm^{-1}, respectively [26]. Loading curcumin into the SLN nanoparticles caused shielding, observable on its absorption peaks at 715–1700 cm^{-1}, but three characteristic absorption peaks of curcumin are still observed at 947 (νC=O + δin-plane CCH), 1240 (νCO (aromatic) and 1515 (νC=O + δCCC + δC=O in-plane) cm^{-1} in the corresponding positions of SLN-C [26].

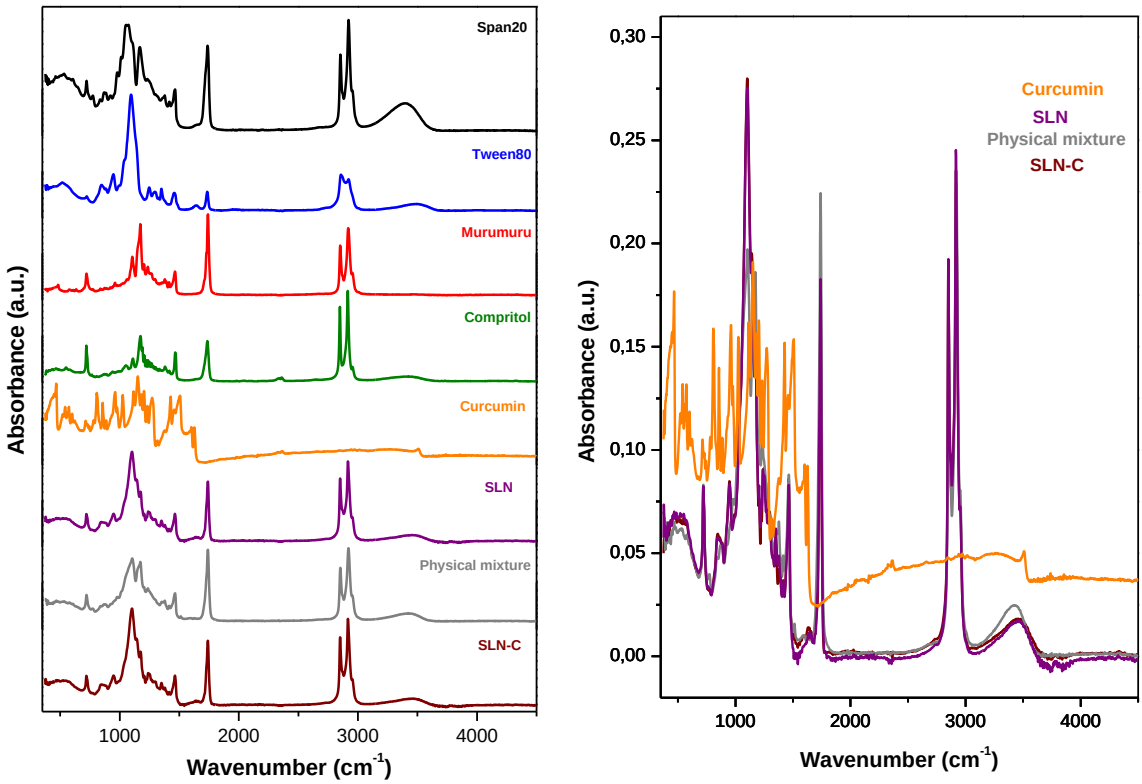

Figure 6: FTIR spectra of Span® 20, Tween® 80, Murumuru better, Compritol®, Curcumin, Solid lipid nanoparticles (SLN), Physical mixture and Solid lipid nanoparticles of curcumin (SLN-C).

Table 2: FTIR spectra of Compritol® ATO 888, Span® 20, Tween® 80, murumuru butter, curcumin, SLN, physical mixture and SLN-C								Assignment
Compritol®	**Span® 20**	**Tween® 80**	**Murumuru butter**	**Curcumin**	**SLN**	**Physical Mixture**	**SLN-C**	
3398	3398	3493	3464	3507	3450	3433	3456	vOH
2914	2922	2922	2957, 2920	2972, 2945	2916	2953, 2922	2953, 2916	v_{as}CH
2849	2852	2856	2852	2845	2851	2852	2851	v_sCH
1736	1738	1736	1742, 1728	-	1740	1740	1742	vC=O
-	-	1641	-	-	1693	-	-	vC=O
-	-	-	-	1626	-	1636	1641	vC=O + vC=C
-	-	-	-	1600	-	1600	1590	vC-C aromatic
-	-	-	-	1508	-	1514	1515	vC=O + ™CCC + ™C=O in-plane
1466	1466	1458, 1350, 1248	1466	1456	1464	1464	1464	™CH (CH_2 alkane)
-	-	-	-	1273	-	1236	1240	vCO (aromatic)
1175	1169	-	1173	-	-	1173	1175	vCO
-	-	1094	1105	1027	1101	1105	1101	vCOC
-	-	-	-	962	-	943	947	vC=O + ™in-plane CCH
719	721	723	721	729		-	-	*Rocking* CH_2
-	-	-	-	714	719	721	721	™ in-*plane skeletal*- CCH and CCH aromatic + vC=C

During the preparation of SLN, it is always possible that intermolecular, weak interactions such as the formation of hydrogen bonds are formed between nanoparticle constituents, which can be evidenced by certain frequency shifts. However, FTIR spectra of SLN-C indicated the expected peaks for the free curcumin, confirming the successful incorporation of this compound to the matrix of nanoparticles without any evidence of interactions of weak chemical interactions. Thus, interactions such as hydrogen bonds are not present in the SLN-C. Wang *et al* (2018) studied the curcumin loaded into solid lipid nanoparticles composed of stearic acid, lecithin and Myrj52®. The authors observed in the FTIR analysis that curcumin was maintained into the nanosystem only by physical interactions and not by a chemical reaction. Sharma et al also studied curcumin loaded into the solid lipid nanoparticles by FTIR analysis, and the study showed that the curcumin did not chemically interact with the carrier components, i.e., stearic acid, tristearin and soya lecithin [27].

Figure 7 shows the Raman spectra of both the SLN and the SLN-C. Analyzing the Figure 7 it is possible to observe bands around 1630 cm^{-1} in the SLN-C spectrum, but not in the SLN spectrum, which are attributed to the vibrational modes ν (C = C) and ν (C = O). The band at 1604 cm^{-1} was also observed in the Raman spectra of SLN-C which was attributed to the vibrational mode of the aromatic ring ν (C-Canel) [28]. The presence of these bands (1630 region and 1600 cm^{-1} region) are characteristic of the enolic form of curcumin, confirming the presence of curcumin in nanocarrier. Thus, based on the spectroscopy study it can be observed that the samples were stabilized as a physical mixture without the formation of chemical bonds.

Mohan *et al* (2012) studied the inclusion complexes of curcumin with three derivative cyclodextrins. The authors suggested by Raman spectrum that curcumin exists in the enol form rather than in the diketone form [26], as noticed in our study. This result was also observed by Nguyen *et al* (2016) with curcumin-loaded liposome formulation.

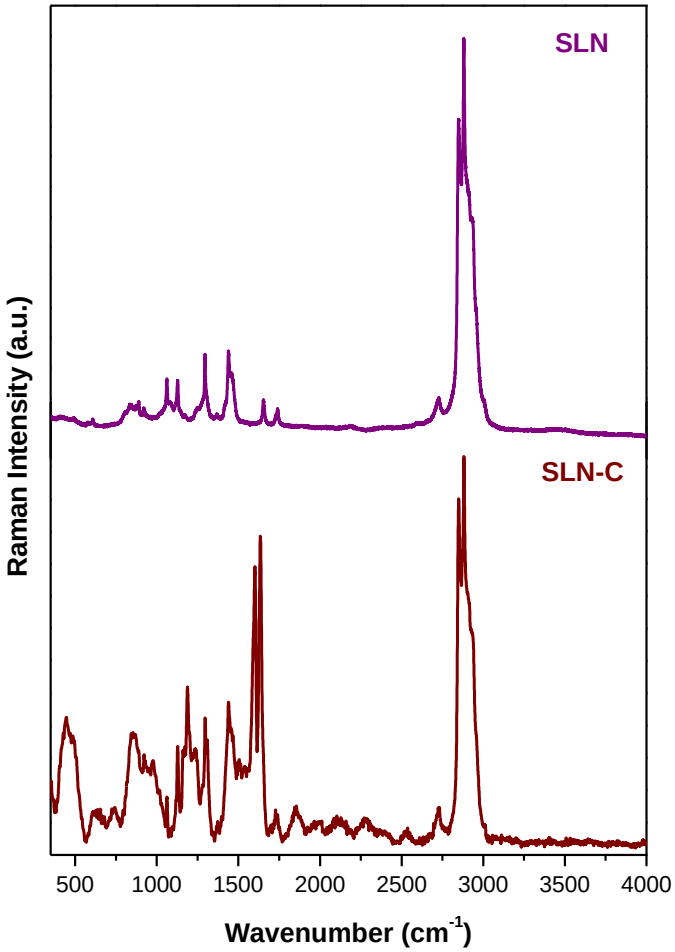

Figure 7. Raman spectra of solid lipid nanoparticles (SLN) and solid lipid nanoparticles of curcumin (SLN-C).

3.5 Curcumin internalization and cytotoxicity in CT26 cells

Images obtained by confocal scanning microscopy showed the presence of significant amounts of curcumin in the cytoplasm of CT26 cells exposed to either curcumin or SLN-C (Figure 8). The fluorescence intensity of curcumin was noticeably higher in cells exposed to free curcumin in comparison to that observed in cells exposed to SLN-C (Figures 8, C and D). This was expected, as curcumin is a highly hydrophobic compound and can thus cross the lipid bilayer by simple diffusion [29]. Moreover, the uptake of nanoparticles is usually saturable and slower than simple diffusion, as studies report that solid lipid nanoparticles are taken up by cells by means of energy-dependent processes (endocytosis) [12]. Despite this difference in interiorization, CT26 cells were more sensible to SLN-C than to free curcumin, as evidenced by the cytotoxicity assay (Figure 9). It was observed that the exposure of CT26 cells for 4 or 24 hours to SLN-C led to a significant reduction in cell viability ($p < 0.05$) at all concentrations

tested; free curcumin, however, significantly reduced cell viability only at 24 h of treatment (Figure 9).

However, it is noteworthy that in the 24 hours the free curcumin showed a decrease in viability with the increase of concentration, showing no differences in the higher concentration used compared to 10 µg/mL SLN-C. There are several studies that show an increase in cytotoxicity in tumor cells when curcumin is combined with nanostructures comparing with free curcumin, these tests being carried out on human lung carcinoma, human breast carcinoma and human neuroblastoma cells. The reason why cytotoxicity is higher when curcumin is conjugated to nanoparticles is due to the greater interaction between the components of the nanoparticles with the cells, causing greater uptake cellular [30–33]. In this work, a greater cytotoxicity of SLN-C was observed, but not a greater cell uptake. The blank SLN is a logical component of such a study to prove that the carrier itself is not toxic, however, there are reports where this control is omitted and others that only refer to blank SLN non-toxicity marginally [12]. Decrease in cell viability by more than 30% (i.e. cell viability of 70% and less) is to be considered as a cytotoxic effect [34]. The nanoparticle developed in this work did not present cytotoxicity without the presence of the drug studied here.

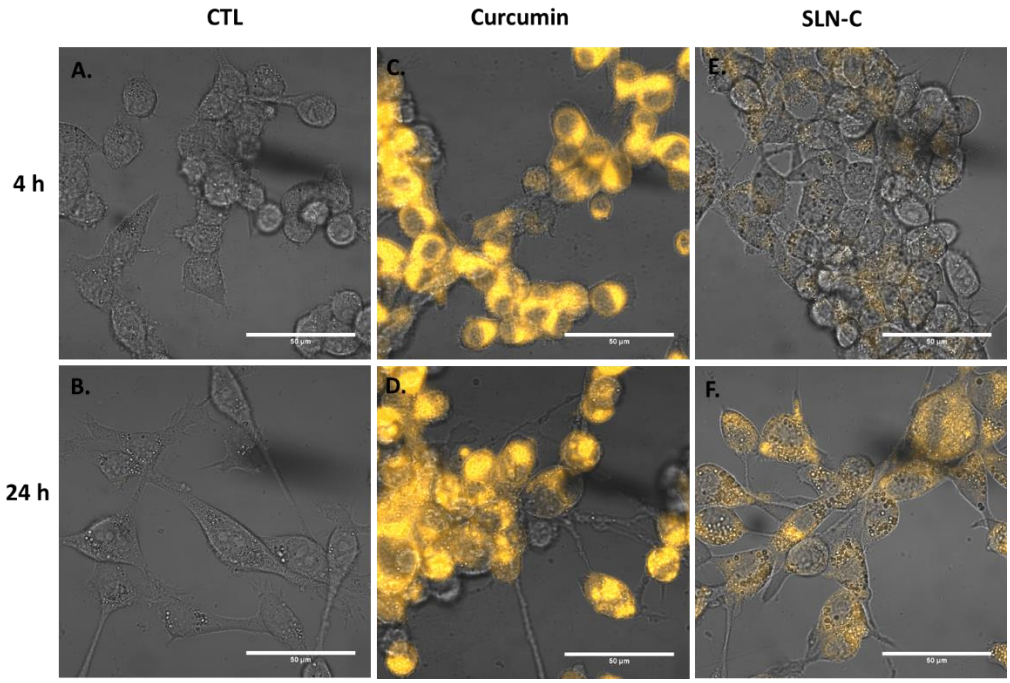

Figure 8. Internalization of free or nanostructured curcumin (SLN-C) in CT26 cells after 4 or 24 hours of exposure. To evaluate internalization, a concentration of 10 µg/mL curcumin was used. A) and B) control cells in 4 and 24 hours, respectively. C) and D) fluorescence intensity of free curcumin after 4 and 24 hours, respectively; E) and F) fluorescence intensity of nanostructured curcumin (SLN-C) after 4 and 24 hours, respectively.

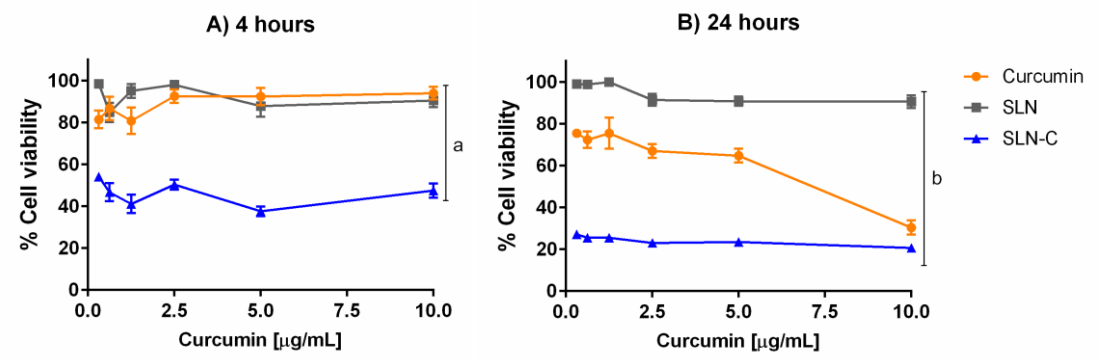

Figure 9. Effect of cytotoxicity against CT26 cells after 4 or 24 hours of exposure. A) and B) viability of CT26 cells exposed to different concentrations of free curcumin (orange line), solid lipid nanoparticles of curcumin (SLN-C) (blue line) and solid lipid nanoparticles (SLN) (gray line) treatments after 4 and 24 hours, respectively. Data are expressed as mean ± standard error of the mean. [a]$p<0.05$ for SLN-C vs control, and vs curcumin, at all concentrations. [b]$p<0.05$ for SLN-C vs control, at all concentrations, and vs curcumin, at all concentrations except 10.0 µg/mL. Statistical analysis: one-way ANOVA test of Tukey's multiple comparisons.

3.6 Intracellular calcium level dysregulation and morphological alteration

Curcumin has been shown to inhibit the endoplasmic reticulum-associated Ca^{2+} ATPase, thus increasing the cytosolic Ca^{2+} concentration [35]. This effect has been corelated to the induction of apoptosis in ovarian cancer cells [36]. In CT26 cells a statistically significant rise in calcium level was induced only by free curcumin after 4 h of treatment (Figure 10, A), as the 24-h treatment had no evident impact on this parameter (Figure 10, B). Interestingly, although SLN-C were taken up by these cells, the cytoplasmic Ca^{2+} homeostasis remained undisturbed. In this study we show that CT26 cells exposed to free curcumin for 4 hours show an increase in intracellular calcium, possibly due to the inhibition of ATPase activity, but in 24 hours of exposure the cells are able to return to normal calcium level. Calcium is a major signaling molecule involved in the regulation of numerous physiological processes, including cell proliferation and apoptosis. Elevated cytosolic Ca^{2+} concentration can promote cell survival and apoptosis, depending on the extent of the Ca^{2+} response [37, 38]. The effect on cell morphology after 24 hours of treatments was also verified (Figure 11). Thus, to verify the granularity and size changes in CT26 cells, flow cytometric analysis was performed. The results show that curcumin murumuru nanoparticles caused granularity changes. All other treatments showed no difference with the control group (Figure 11). The same is observed by light microscopy in which it is possible to observe several vesicles within the cells treated by SLN-C (Figure 11).

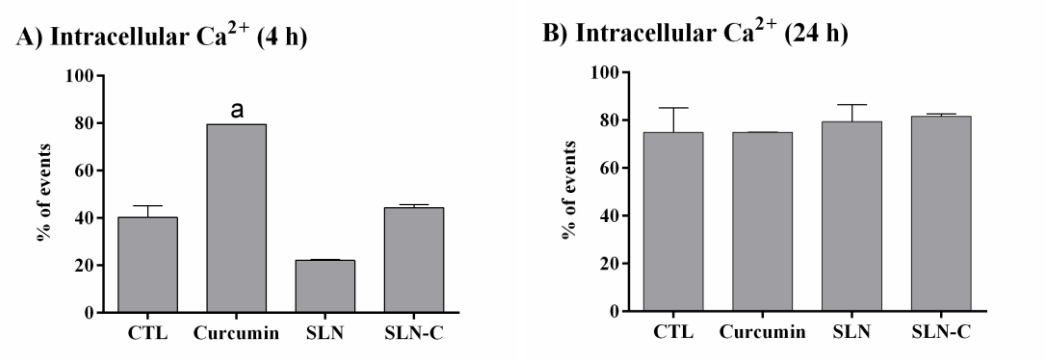

Figure 10. Intracellular calcium level and morphological alteration. A) and B) intracellular calcium after 4 and 24 hours, respectively. Control cells (CTL); curcumin, solid lipid nanoparticles (SLN) and solid lipid nanoparticles of curcumin (SLN-C). [a]$p<0.05$ for curcumin vs all treatments. Statistical analysis: one-way ANOVA test of Tukey's multiple comparisons.

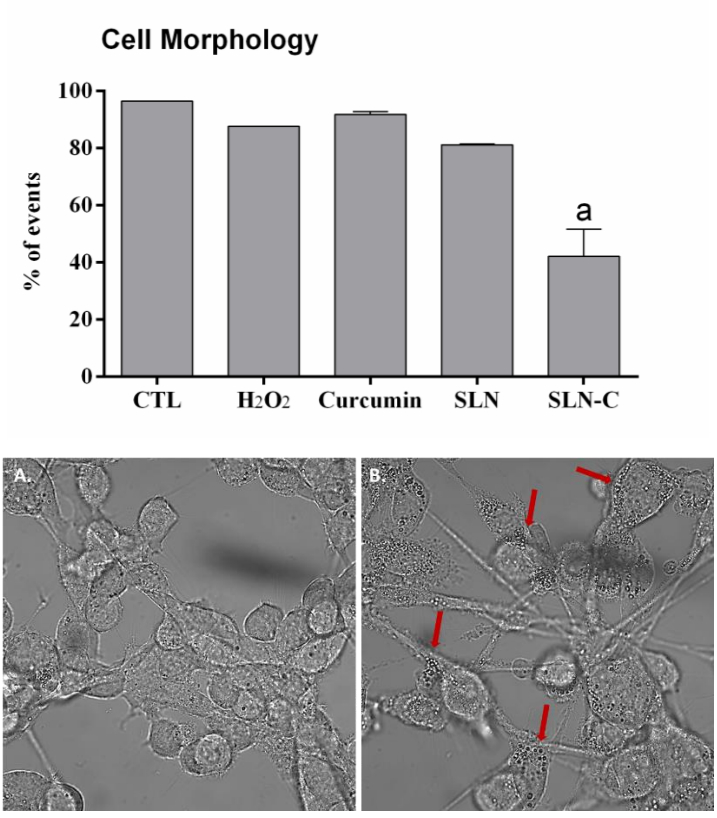

Figure 11. Graphical representation of the percentage of viable cells under the influence of curcumin treatments (free curcumin), H_2O_2 (hydrogen peroxide - positive control for cell damage/necrosis), solid lipid nanoparticles of curcumin (SLN-C), solid lipid nanoparticles (SLN) and control (culture medium) in the morphology of CT26 cells about granularity after 24 hours of exposure to treatments. Superscript "a" means difference significant $p<0.05$. Data expressed as mean ± standard error of the mean. Statistical analysis: one-way ANOVA test of Tukey's multiple comparisons. The light microscopy images show in "A" control cells and in "B" cells exposed to SLN-C for 24 hours, where the red arrows point to granules in the cells.

3.7 3D cultive (spheroids)

Both curcumin and SLN-C were interiorized by spheroids, with a higher curcumin-specific fluorescence intensity in the periphery of these cells' agglomerates (Figure 12). This pattern of curcumin distribution was expected, as a similar study using curcumin also found an accumulation of free curcumin and curcumin combined with ruthenium and cobalt metals in the periphery of colorectal adenocarcinoma cell spheroids after 24 h of the exposition [39]. Spheroids (three-dimensional aggregates of tumor cells) are useful *in vitro* models of solid, avascular tumors. This model has many of the same characteristics of a microtumor, including concentration gradients of both oxygen and biochemical waste products, pH gradient, areas of hypoxia and necrosis, and similar drug diffusion profiles [39]. The diffusion of SLN-C into spheroids, a compact cell agglomerate, evidences that nanostructures may indeed be used to deliver their cargo to deeper layers of tumors, as suggested previously by the literature [40]. The nanostructure might penetrate into the tumor parenchima and into the tumor cells, or even reach the tumor parenchima and release the drug, which could next be taken up by target cells [41].

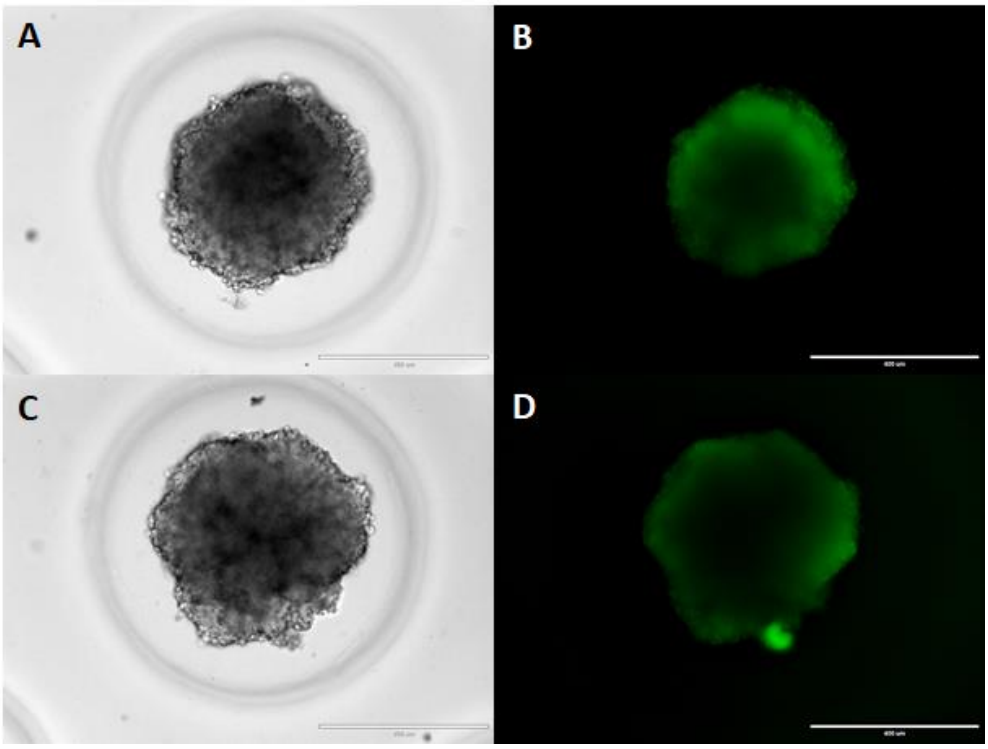

Figure 12: Fluorescence microscopy showing internalization in spheroids of CT26 cells after exposition to free curcumin or SLN-C for 1 h. A and B spheroid exposure to free curcumin. C and D spheroid exposure to SLN-C. The green color indicates the curcumin-specific fluorescence.

4. Conclusion

Curcumin has many *in vitro* bioactivities, as shown by a number of investigations, but pharmacotechnical and pharmacokinetics issues, such as its low solubility in aqueous media and its rapid metabolization, for instance, have limited its clinical application. The formulation described in the present work, SLN-C, is a candidate to circumvent these drawbacks. Moreover, differently from most of the works already published, the method for obtaining the SLN-C is easy to perform, demands only basic equipment and is readily scalable. Further experiments are now necessary to verify the bioavailability and biodistribution of curcumin administered *in vivo*.

Conflicts of interest

The authors have no conflicts of interest to declare.

Funding information

This study was funded by the Brazilian government agencies FAP-DF/Brazil (0193.001020/2015, and 0193.001626/2017), PRONEX FAP-DF, CNPq (0447628/2014-3, 193.001.200/2016) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

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CHAPTER 3: CURCUMIN INDUCES IMMUNOGENIC CELL DEATH IN MURINE COLORECTAL CT26 CANCER CELLS

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Future publication in: Biochemical Pharmacology

ABSTRACT

Immunogenic cell death (ICD) is a modality of apoptosis with a very specific temporospatial exposure of immune adjutants, the so-called damage-associated molecular patterns (DAMPs). Some chemotherapeutic drugs induce have been shown to induce ICD in cancer cells, an effect that can be explored in the immunotherapy of cancer. In this context, curcumin has been shown to be an inhibitor of cancer cell proliferation, but its potential activity as an ICD inducer has not been investigated. In this context, the goal of this work was to investigate whether curcumin has the capacity induce ICD in the murine colorectal carcinoma CT26 cells. The results show that curcumin induced apoptosis and DNA fragmentation in CT26 cells. Moreover, curcumin induced the exposure of the DAMPs calreticulin and HSP90 on the outer leaflet of the plasma membrane and the release of the DAMPs HMGB1, ATP and IL-1β. Curcumin-treated CT26 cells were immunogenic *in vivo*, as 80 to 100% of the BALB/c mice vaccinated with these cells were resistant to a subsequent challenge with viable CT26 cells. Taken together, these results suggest that curcumin is an ICD inducer. Further studies may focus on this activity in other cell lines, and to investigate the possible activation of the immune system by curcumin-treated tumors in *in vivo* established cancer models.

Keywords: Immunotherapy, cancer, DAMPs, immunogenic cell death, curcumin

ABBREVIATIONS

AO - Acridine orange

ATF4 - Activating transcription factor-4

ATF6 - Activating transcription factor-6

DAMPs - Damage-associated molecular patterns

DCs - Dendritic cells

ELISA - Enzyme Linked Immuno Sorbent Assay

ER – Endoplasmic reticulum

F/T - freezing/thawing

HMGB-1 - High mobility group box 1 protein

ICD - Immunogenic cell death

IL-1β - Interleukin 1 beta

MTX - Mitoxantrone

PI - Propidium iodide

THA - Thapsigargin

TU - Tunicamycin

XBP1 - Factor X-box-binding protein 1

1. Introduction

Targeting the immune system is one of the most promising approaches to treat cancer. The immunotherapies already approved for the clinical practice include cancer vaccines, adoptive transfer of *ex vivo*-activated T and natural killer cells, oncolytic viruses and administration of antibodies or recombinant proteins that either costimulatory cells or block the so-called immune checkpoint pathways [1]. Despite the limited efficacy of the clinically available immunotherapies, recent advances in the field of tumor immunology can be used to give improved tools to activate the immune system against tumors. In this context, preclinical and clinical investigations suggest that some classical anticancer therapies, such as radiotherapy and anthracycline-based chemotherapy , to induce immunogenic cell death (ICD) in cancer cells [2, 3].

ICD occurs sequentially involving changes in the cell surface and release of mediators called damage-associated molecular patterns (DAMPs). It is a prominent pathway for the activation of the immune system against cancer cells, which may result in the success of long-term anticancer therapies due to immune responses, thus increasing the efficacy of the therapy compared to the conventional anticancer agents [4]. Thus, the efficacy of certain anticancer agents also rely on their ability to induce a robust immune response against cancer cells [3].

Curcumin, a compound found in the rhizome of the *Curcuma longa*, inhibits the proliferation of cancer cells [5–7]. It is known that curcumin imbalances the intracellular homeostasis of Ca^{2+}, inducing impairments in the function of the endoplasmic reticulum (ER) [5], potentially leading to ER stress and apoptosis [6]. As the ER stress responses have important roles in the induction of ICD, it is reasonable to suggest curcumin to be an ICD inducer.

Thus, the goal of the present work was to investigate whether curcumin can induce ICD in murine colorectal carcinoma cells *in vitro*. As shown by a series of experiments, curcumin induced apoptosis and the exposure of different DAMPs (calreticulin, HSP90, HMGB1 and IL-1β) *in vitro* in CT26 cells. The immunogenicity of the curcumin treated CT26 cells was further confirmed by vaccination of BALB/c mice tests.

2. Experimental design

2.1 Materials

The reagents employed in this work are as follows: 3- (4,5-dimethylthiazol-2-yl) -2,5-bromide diphenyltetrazolium (MTT, Invitrogen, USA); curcumin (Sigma Aldrich, São Paulo, Brazil), dimethyl sulfoxide (Sigma Aldrich, São Paulo, Brazil); streptomycin (Gibco, Grand Island, NY, USA); Fluo 4-AM (Invitrogen, USA); Roswell Park Memorial Institute medium (RPMI-1640, Gibco, Grand Island, NY, USA); penicillin (Gibco, Grand Island, NY, USA); fetal bovine serum (Gibco, Grand Island, NY, USA); Phosphate-buffered saline (Laborclin, Pinhais, Paraná, Brazil); Milli-Q water (Barnstead EASYpure II Thermo Scientific, San Jose, CA, USA); sodium chloride (Sigma Aldrich, São Paulo, Brazil); tris (Sigma Aldrich, São Paulo, Brazil); triton X-100 (Sigma Aldrich, São Paulo, Brazil); trypsin (Gibco, Grand Island, NY, USA). Cell lines: Murine colorectal carcinoma cells (CT26) were obtained from the American Type Culture Collection (ATCC – USA), and murine NIH-3T3fibroblasts were obtained from the Rio de Janeiro cell bank (Brazil). All other materials were purchased from Sigma (São Paulo, Brazil).

2.2 Cell culture

CT26 cells were cultured in RPMI-1640 medium (Roswell Park Memorial Institute medium) supplemented with 10% (v: v) fetal bovine serum and 1% (v: v) antibiotic solution (100 units/mL penicillin and 100 mg/mL streptomycin). NIH-3T3 cells were cultured in DMEM medium (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum and 1% antibiotic solution. The cells were kept in an incubator with a humid atmosphere with 5% CO_2 at 37 ºC.

2.3 Cell treatment

CT26 cells were seeded at a concentration of 5 x 10^4 cells/well in 12-well plates and were cultured for 24 hours. Cells were exposed to curcumin for 4 or 24 hours. The concentration of curcumin was 2.5 µg/mL. Tunicamycin (2.0 µg/mL) and thapsigargin (2.0 µM) were used as positive controls for ER stress. For cytometry, after the treatments, the cells were washed with PBS and treated with trypsin. Culture medium was then added, and the cell suspension was centrifuged at 500 G for 5 minutes. Then the supernatant was discarded, and the cells were resuspended with PBS. After these processes, the cells were marked with the respective markers

for each assay. Cells were analyzed by cytometer (FACS Verse, BD, USA) and 10,000 events were counted per sample.

2.4 Cell viability analysis by MTT method

The MTT (3- (4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) assay was used to verify cell viability [8]. The test was performed with 1×10^4 cells per well in 96-well plates - exposed for 4 or 24 h to curcumin (Curc). Mitoxantrone was used as a positive control in this work for apoptosis and therefore also tested in the cell viability assay. Each treatment was performed in triplicate and six different concentrations of curcumin (10.0; 5.0; 2.5; 1.25; 0.625; 0.312 µg/mL curcumin) were tested.

After 24 hours, cells were incubated with 0.5 mg/mL MTT in culture medium for 2.5 hours at 37 °C in a humid atmosphere and 5% CO_2. Then, the MTT solution was discarded and the formazan was extracted from the cells with 200 µl DMSO per well. The plates were analyzed by spectrophotometer (Spectramax M2, Molecular Devices, USA) and absorbance was measured at a wavelength of 595 nm. Three replicates of the experiment were performed.

2.5 Identification of necrosis and apoptosis

Cells were incubated with acridine orange (AO) (1 µg/mL) and propidium iodide (PI) (20 µg/mL) for 5 min and analyzed in a fluorescence microscope (EVOS Cell Imaging Systems, ThermoFisher). To detect and quantify the number of cells undergoing apoptosis or necrosis, the OA/PI labeling profile described by [9]. This verification was carried out by means of a double blind so that there were no biased results - by four people. All cells of 10 fluorescence images in each group were counted by 4 people, people were not aware knowledge of each group. The AO marks both viable cells and dead cells, whereas the PI marker stains only dead cells, that is, it has lost the integrity of the membrane. The forms of identification were divided into three stages represented by: Viable cell - uniform green marking both the nucleus and cytoplasm; Apoptosis cell - partially degraded plasma membrane, which allows entry of the PI. Formation of apoptotic bodies with fragmentation and chromatin condensation. The cells have orange coloring and Necrotic cell - the cell is uniformly marked with red; this is due to the change in the membrane that allows the entry of the PI.

2.6 DNA fragmentation analysis

The supernatants were removed, and the cells were fixed with ethanol 70%. After 24 hours the cells were analyzed by cytometer (FACS Verse, BD, USA). 10,000 events were counted per sample.

2.7 Immunofluorescence analysis

Four hours after the treatment, the cells were blocked with BSA 2% (bovine albumin) for 30 minutes, washed with PBS twice and the primary anti-calreticulin, HSP90 or HSP70 (abcam) antibody were added. The cells were then incubated for 1 h at 37 ^{o}C, and the secondary antibody (anti-IgG 488, Abcam) was added, with a further 30-min incubation in the dark at 37 ^{o}C. Cells were next labeled with DAPI and analyzed in fluorescence microscope (EVOS Cell Imaging Systems, ThermoFisher).

2.8 DAMPs analysis by ELISA test

HMGB1 and IL-1β concentrations in cell culture supernatants were assessed with ELISA kits used (IBL international and Invitrogen, respectively) according to the fabricant instructions. The plates were analyzed by spectrophotometer (Spectramax M2, Molecular Devices, USA) and measure the optical density with a photometer at 450 nm.

2.9 ATP analysis by bioluminescence

ATP concentrations in cell culture supernatants were assessed with Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Sigma Aldrich, USA) according to the fabricant instructions. The plates were analyzed by spectrophotometer (Spectramax M2, Molecular Devices, USA).

2.10 *In vivo* analysis of immunogenicity

For *in vivo* assays, 8-week old, female BALB/c mice were vaccinated on the left lower flank with treated CT26 cells (see "cells treatment" section), twice, with an interval of 10 days. Seven days after the last vaccination, mice were challenged with viable CT26 cells inoculated

on the right lower flank. Mice were then observed for tumor development for 28 days. This project was approved by the animal use ethics committee of the University of Brasilia - Protocol number: 70/2018 (Annex 1).

2.11 Laser scanning confocal microscopy interiorization analysis

After treatment, the cells were washed twice with PBS. The plate was immediately analyzed by confocal laser scanning microscope (LSM 880, Zeiss, Germany). To curcumin fluorescence emission from the treatments was used the 488 nm laser and to aluminum phthalocyanine chloride 633 nm laser.

2.12 Curcumin subcellular location

After treatment, the cells were washed twice with PBS and incubated for 30 min with marker with ER-Tracker Blue-White DPX, specific for the endoplasmic reticulum (ER) (ThermoFisher). After 30 minutes at 37 ºC the cells were washed and were immediately analyzed by fluorescence microscopy (EVOS Cell Imaging Systems, ThermoFisher).

2.13 Intracellular calcium release analysis by flow cytometry

After the treatments, the cells were washed with PBS and treated with trypsin. Culture medium was then added, and the cell suspension was centrifuged at 500 G for 5 minutes. Then the supernatant was discarded, and the cells were incubated for 1 hour with the intracellular calcium marker Fluo-4 (Invitrogen, USA). Cells were analyzed by cytometer (FACS Verse, BD, USA) for marker fluorescence (PerCP-Cy5.5 marker) and 10,000 events were counted per sample.

2.14 Western Blotting Analysis

Aliquots of 6 x 10^5 CT26 cells were lysed with NP-40 extraction buffer (150 mM sodium chloride, 10% triton X-100 and 50 mM tris pH 8.0) diluted with running buffer to 20 µg protein/mL, applied on 10% polyacrylamide gel and subjected to conventional SDS-PAGE. The proteins were transferred to a nitrocellulose membrane, subsequently blocked with 1% of

BSA solution in tris-buffered saline. The membrane was first incubated with specific primary antibodies for 3 h, then with peroxidase-conjugated secondary antibodies for 1 h. Signals were visualized by a chemiluminescence detection kit (ECL Prime Western Blotting Detection Reagents). The band intensity was measured by using Image J software, and the expression of proteins was normalized to the loading control (GAPDH) was calculated.

2.15 Statistical analysis

Statistical differences were assessed by one-way or two-way ANOVA tests, with post-tests of Tukey or Sidak (α=0.05). The quantitative results were described as mean ± standard error of the mean. The results were analyzed using GraphPad Prism® 6.0 software.

3. Results

3.1 Curcumin reduces the viability of both CT26 and NIH-3T3 cells *in vitro*

Curcumin has been shown to inhibit cell proliferation, invasion, metastasis, and angiogenesis [10–12]. The potential anticancer action of curcumin might be due to its cytotoxicity [7], but the mechanism behind this suggested activity is not yet clear [13]. In the present work, the potential of curcumin to induce ICD in a murine colorectal carcinoma cell line, CT26, was investigated. MTX was used as a positive control, while F/T was used as a negative control for ICD.

As ICD is an immunogenic type of apoptosis, the cytotoxicity of curcumin was first tested *in vitro*. It was found that curcumin significantly reduced the *in vitro* viability of both investigated cell lines, with a concentration-dependent, directly proportional effect observed at 24 h of treatment. At the concentration of 10.00 µg/mL, curcumin reduced the viability of CT26 cells to almost 16%, and to around 50% in NIH-3T3 cells in comparison to the non-treated control (figure 1). The curcumin was cytotoxic to CT26 cells at concentrations starting from 1.25 µg/mL and to NIH-3T3 from 2.5 µg/mL. Therefore, as previously reported in the literature, curcumin shows inhibitory/cytotoxic activity not only against cancer cells, but also against normal cells [14]. This effect was also dependent on the time of treatment, as expected.

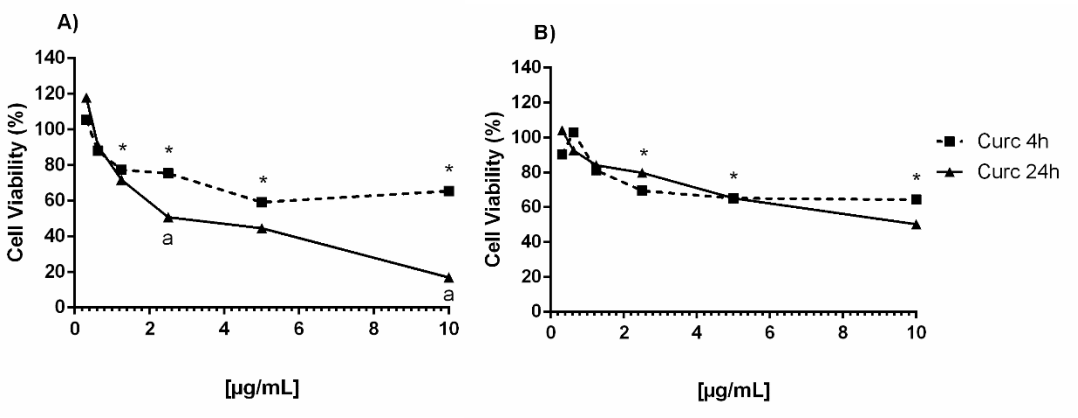

Figure 1: Viability of (A) CT26 and (B) NIH-3T3 cells upon exposure to curcumin for 4 and 24h. Data expressed as mean ± standard error of the mean. ***** Groups "Curc 4h" and "Curc 24h" statistically different from the control group. **a** $p<0.05$ for Curc 4 h vs Curc 24h. Statistical analysis: Two-way ANOVA and Tukey's multiple comparison test.

3.2 Apoptosis is the main death mechanism elicited by curcumin in CT26 cells

Apoptosis was the main death mechanism induced in CT26 cells by curcumin, as confirmed fluorescence microscopy (figure 2). Fluorescence microscopy was used to identify the type of death, apoptosis or necrosis, by means of the staining with the dyes acridine orange and propidium iodide. Previous works already showed curcumin to induce apoptosis [10–12, 15] which is a prerequisite for a drug to be an ICD inducer [16, 17]. The percentage of apoptotic cells observed by fluorescence microscopy was 92% and 98% after treatment with curcumin for 4 h and 24 h, respectively. Moreover, curcumin is shown in figures 2 to have an apoptosis-inducing ability comparable to that observed with MTX, a potent inducer of ICD [4]. As expected, F/T treatment induced almost exclusively necrosis, this process causes cell lysis/disruption of cellular membranes and thus exposure of all intracellular content [18, 19]; the cells stained for apoptosis, 4%, are probably due to artifacts of this technique.

In vitro studies suggest that curcumin inhibits cancer cell growth by activating apoptosis, but the mechanism underlying these effects is still unclear [15]. Moustapha et. Al., 2015 investigated the mechanisms leading to apoptosis in curcumin-treated cells and concluded that curcumin induced ER stress causing calcium release, with a destabilization of mitochondria, resulting in apoptosis. Both thapsigargin and tunicamycin, classically known as ER stress inducers, used as positive controls in the present work, induced apoptosis in CT26 cells (figures 2). Thapsigargin is a specific inhibitor of the Ca^{2+}-ATPase of the ER [20].

Tunicamycin, a naturally occurring antibiotic, induces ER stress in cells by inhibiting the first step in the biosynthesis of N-linked glycans in the proteins resulting many misfolded proteins [21]. It was also possible to observe by flow cytometry that curcumin and the other treatments were able to cause DNA fragmentation, which was probably a consequence of apoptosis (figure 3).

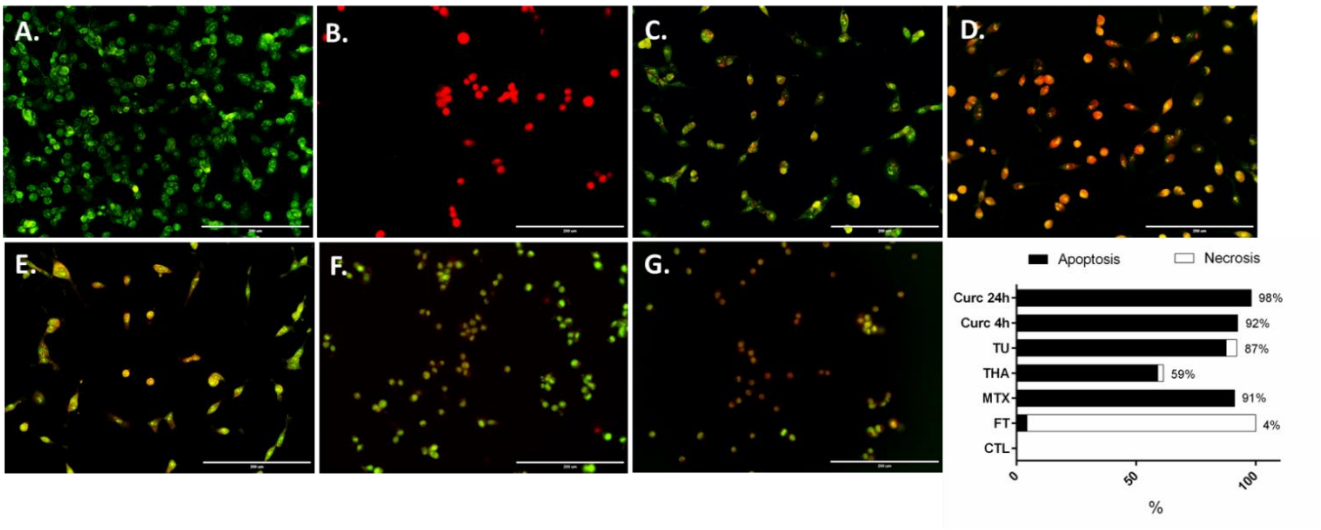

Figure 2: AO/PI labeling in CT26 cells after application of different treatments: A. non-treated control; B. freezing/thawing; C. curcumin for 4 h; D. curcumin for 24 h; E. mitoxantrone; F. thapsigargin; G. tunicamycin. Magnification 20X. Graph representing percent of fluorescence intensity AO/PI labeling in CT26 cells after application of different treatments. Control (CTL), freezing/thawing (FT), mitoxantrone (MTX), thapsigargin (THA), tunicamycin (TU), curcumin for 4 and 24 hours. Statistical analysis: Two-way ANOVA and Tukey's multiple comparison test.

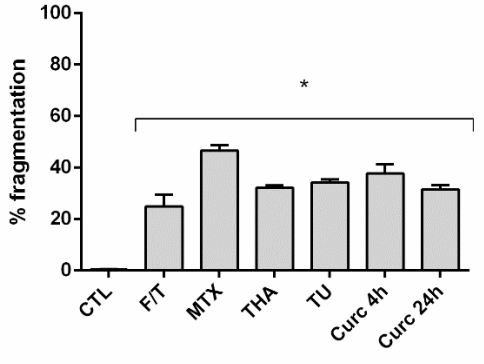

Figure 3: DNA fragmentation by flow cytometry. CT26 cells were exposed to mitoxantrone (MTX), curcumin for 4 and 24 hours (Curc 4h and Curc 24h), thapsigargin (THA), tunicamycin (TU) and freezing/thawing (F/T) as a control for necrosis. ***** Statistically different from the control group. Data expressed as mean ± standard error of the mean. Statistical analysis: One-way ANOVA and Dunnett's comparisons test.

3.3 Curcumin-treated CT26 cells release DAMPs

The treatment with curcumin induced the release and exposure of different DAMPs, which are important hallmarks of the ICD. Some DAMPs are exposed on the plasma membrane, such as heat shock proteins (HSP70 and HSP90) and calreticulin, while others, such as HMGB1 proteins, ATP, uric acid and some cytokines, are secreted into the extracellular environment. These molecules can activate cells of the immune system, such as antigen-presenting cells, potentially leading to an antigen-specific T cell-response [22]. ICD is an immunogenic type of apoptosis characterized by the release of damage-associated molecular patterns (DAMPs) [17]. DAMPs attract and activate a series of immune cells, such as the DCs, to initiate antigen presentation and T cell activation, and hence to prime an adaptive antitumor response that targets malignant cells in both the primary tumor and in metastasis. Ultimately ICD culminates in a cascade of events that may eliminate the tumor cells and set immune memory against tumor-associated and/or tumor-specific antigens [17].

One of the main DAMPs is calreticulin, a 46-kDa multifunctional protein predominantly located in ER, which is found exposed on the plasma membrane of cells undergoing ICD [23, 24]. Calreticulin is a chaperone protein that participates in maintenance of calcium levels, protein folding and trafficking, apoptosis and dead cell clearance and others cellular processes [23]. In this study, CT26 cells exposed to curcumin exposed a significant amount of calreticulin, comparable to the cells treated with MTX, onto the extracellular leaflet of their plasma membrane. As expected, for non-treated CT26 cells (control), no calreticulin was detected on the plasma membrane, as expected (figure 4).

Moreover, non-treated cells permeabilized with surfactant showed an intense intracellular fluorescence signal for calreticulin. This result was expected, as calreticulin is present in the lumen of the ER, an organelle involved in a series of cellular processes, particularly in the synthesis and folding of certain proteins. Calreticulin functions as a chaperon for many proteins, such as integrins, surface receptors, and transporters [25]. Furthermore, the other major function of the calreticulin is regulation of Ca^{2+} homeostasis. More than 50% of Ca^{2+} stored in ER lumen associates with calreticulin. Therefore, higher levels of calreticulin may lead to increase intracellular Ca^{2+} storage and calreticulin deficient cells have a lower capacity for Ca^{2+} storage in the ER lumen [26].

Cell surface calreticulin is considered as a strong “eat-me” signal and promotes phagocytic uptake of cancer cells by antigen-presenting cells [24]. Surface calreticulin expression on dying cancer cells is critical in ICD [16].

Another DAMP is HSP90, a highly conserved chaperone that plays an important role in the folding of newly synthesized proteins and in the refolding of certain proteins affected by stressing conditions [27]. Various kinds of cellular stresses, e.g. oxidative stress, irradiation and chemotherapeutic drugs, might lead to transcriptional and translational activation of HSPs. Some HSPs remain in organelles or in the cytoplasm, while HSP90 can be translocated to the plasma membrane under cellular stress [28]. The results are similar to those found for calreticulin exposure. For non-treated CT26 cells (control), no HSP90 was detected on the plasma membrane, for surfactant-permeabilized cells, an intense, intracellular green fluorescence was observed and cells treated with curcumin showed higher fluorescence intensity, indicating that the cells exposed HSP90 on the outer leaflet of the plasma membrane (figure 5). In figure 6 the fluorescence intensities of the DAMPs are represented in a graph.

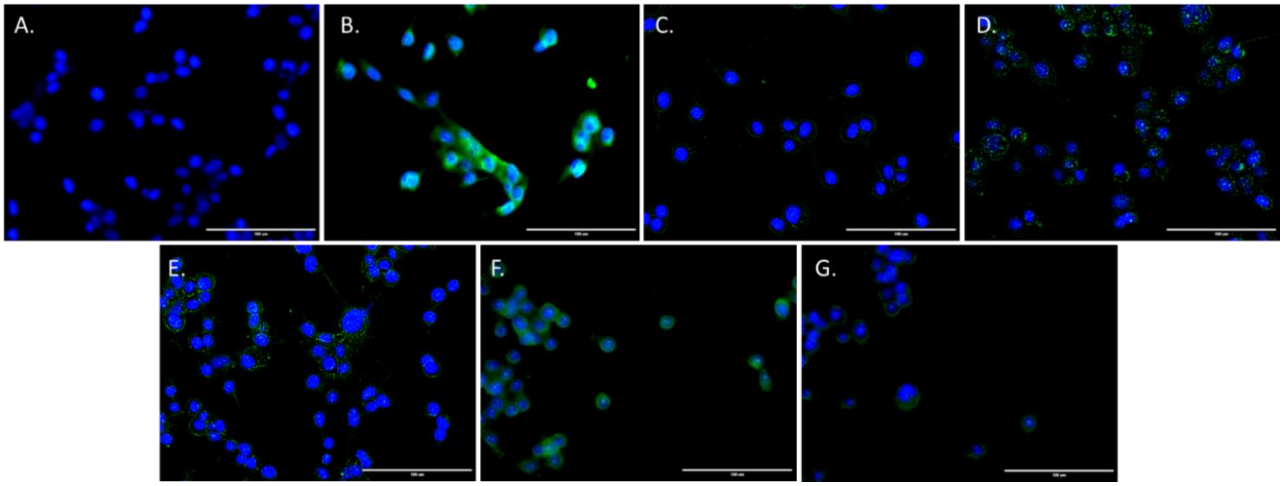

Figure 4: Calreticulin labeling in CT26 cells after application of different treatments. A. Control; B. Permeabilized; C. Curcumin for 4 hours; D. Curcumin for 24 hours; E. Mitoxantrone; F. Thapsigargin and G. Tunicamycin (TU). The blue color refers to the marking of the nucleus and the green color of calreticulin. Magnification 40X.

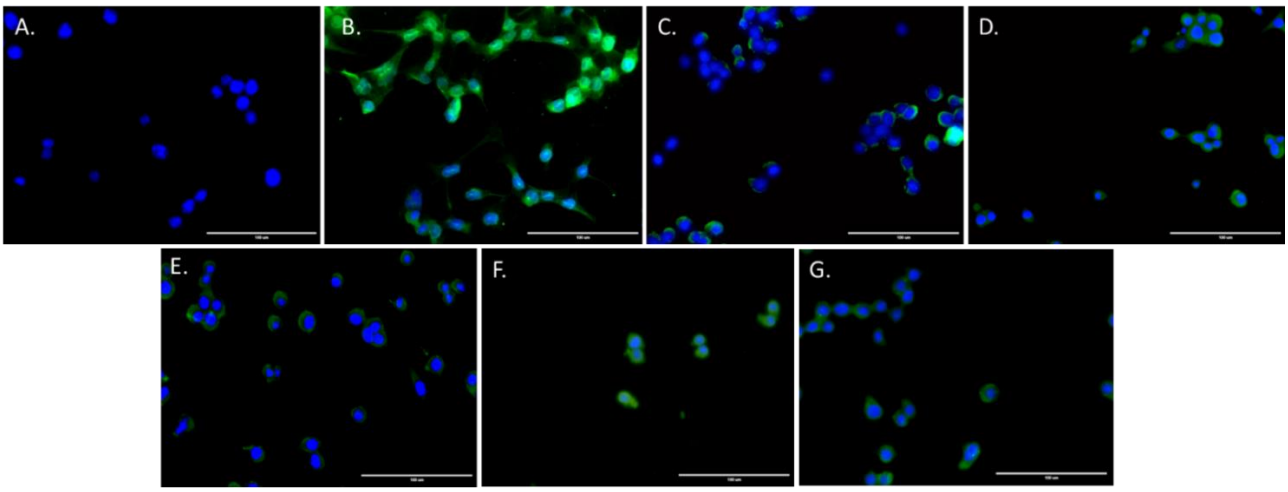

Figure 5: HSP90 labeling in CT26 cells after application of different treatments. A. Control; B. Permeabilized; C. Curcumin for 4 hours; D. Curcumin for 24 hours; E. Mitoxantrone; F. Thapsigargin and G. Tunicamycin (TU). The blue color refers to the marking of the nucleus and the green color of HSP90. Magnification 40X.

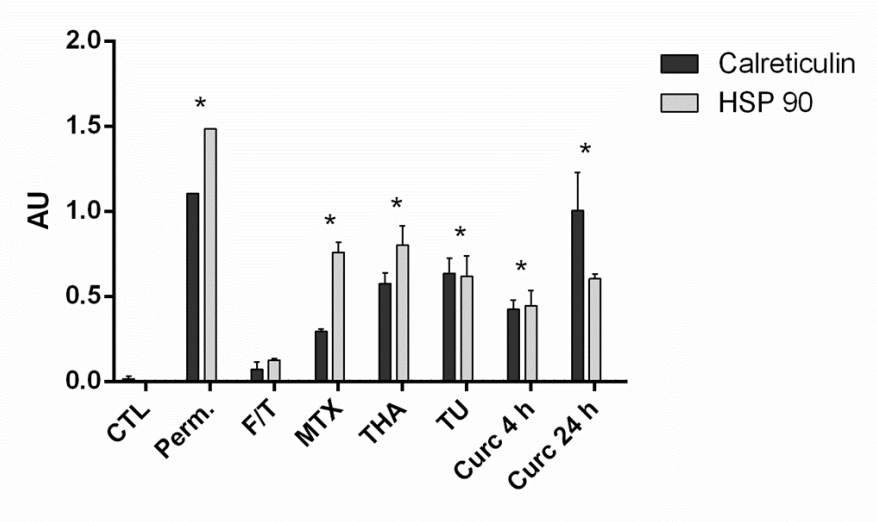

Figure 6: Graph representing DAMPs labeling in CT26 cells after application of different treatments. Control (CTL), permeabilized (Perm.), freezing/thawing (F/T), mitoxantrone (MTX), thapsigargin (THA), tunicamycin (TU), curcumin for 4 and 24 hours. ***** Statistically different from the control group. Data expressed as mean ± standard error of the mean. Statistical analysis: Two-way ANOVA and Tukey's multiple comparison test.

Some DAMPs, such as HMGB1 and IL-1β, are released into the extracellular medium during ICD. The high mobility group box 1 protein (HMGB1), is a very abundant nucleus-localizing non-histone chromatin-binding protein that affects various nuclear functions, such as transcription [22]. Extracellular HMGB1 can be sensed by DCs through interaction with

different receptors, including TLR4, leading to DC maturation [29]. IL-1β can be detected during or after ICD, generally being pro-inflammatory, promoting T cell differentiation and NK cell activation, for instance [30]. These two proteins were detected by ELISA after CT26 cells were exposed to different treatments, including after the treatment with curcumin (figure 7).

Cells that received tunicamycin, thapsigargin and curcumin 24 h released higher amounts of HMGB1. For IL-1β, cells that received the different treatments released this protein in the extracellular medium without presenting statistical difference (figure 7). As expected, cells subjected to F/T did not release these DAMPs, as this process causes necrosis, and not ICD (figure 14).

The nucleotide ATP is also acts as a DAMP when released to the extracellular medium by an autophagy-dependent, active process [31]. Extracellular ATP acts as a "find me" signal, guiding DC chemotaxis, for example. This interaction also activates the NLRP3-containing inflammasome leading to the production of the lymphocyte-activating cytokine IL-1β [32]. Interestingly, curcumin induced the release of ATP only at the 24-hour treatment (figure 7), with an intensity comparable to that observed for cells treated with mitoxantrone, the positive control for ICD. The stressors of the ER, thapsigargin and tunicamicyn, induced an even higher release of ATP in CT26 cells. Furthermore, frozen/thawed CT26 cells released high amounts of ATP, which is probably related to a disruption in plasma membrane barrier function.

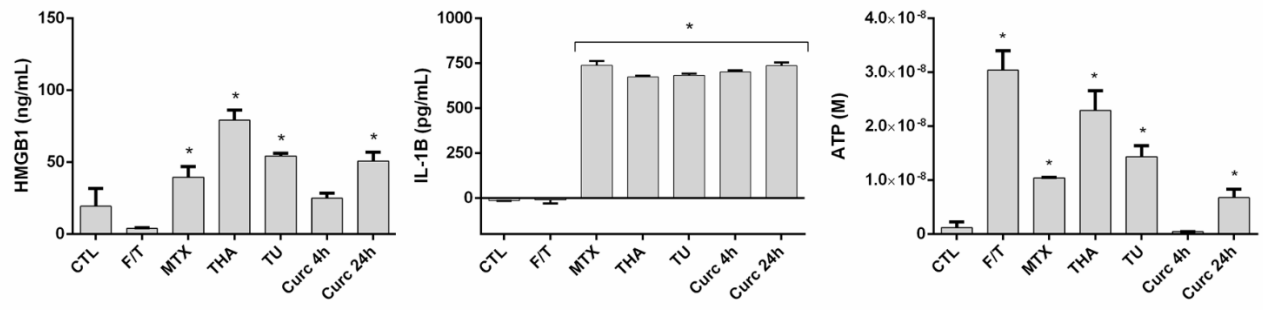

Figure 7: Extracellular concentrations of (A) HMGB1, (B) IL-1β and (C) ATP in CT26 cells culture after application of different treatments. Control (CTL), freezing/thawing (F/T), mitoxantrone (MTX), thapsigargin (THA), tunicamycin (TU), curcumin for 4 (Curc 4h) and 24 hours (Curc 24h). ***** Statistically different from the control group. ** Statistically different from the other groups. Data expressed as mean ± standard error of the mean. Statistical analysis: One-way ANOVA and Dunnett's comparisons test.

3.4 Curcumin-treated CT26 cells are immunogenic

The induction of apoptosis with the concomitant release of important DAMPs suggests that curcumin can induce ICD. However, the gold standard for assessing the immunogenicity of cancer cells treated *in vitro* with cytotoxic compounds is the vaccination and challenge of syngeneic mice [16, 33]. In this method, the cancer cells are treated *in vitro* with a cytotoxicant and then injected subcutaneously into immunocompetent mice. After a latency of one week, mice are challenged with living cancer cells of the same line, injected into the opposite flank, and monitored for tumor appearance. The absence of tumor growth is interpreted as a sign of an immune response elicited by cancer cells succumbed by ICD [33].

Mice vaccinated with CT26 cells treated with curcumin showed resistance against a subsequent challenge with viable CT26 cells (figure 8). The time of *in vitro* treatment of the cells with curcumin affected the efficiency of immunization, since none of the mice vaccinated with cells treated for 24 h with curcumin developed tumors, while 30% of mice vaccinated with cells treated for 4 h with curcumin presented tumors at the end of the experiment. This result suggests that curcumin is indeed an ICD inducer. As for the controls, 60% of mice vaccinated with MTX-treated cells were resistant to the challenge with viable CT26 cells, while only 20% of mice vaccinated with F/T CT26 cells showed no tumor by the end of the experiment, as expected.

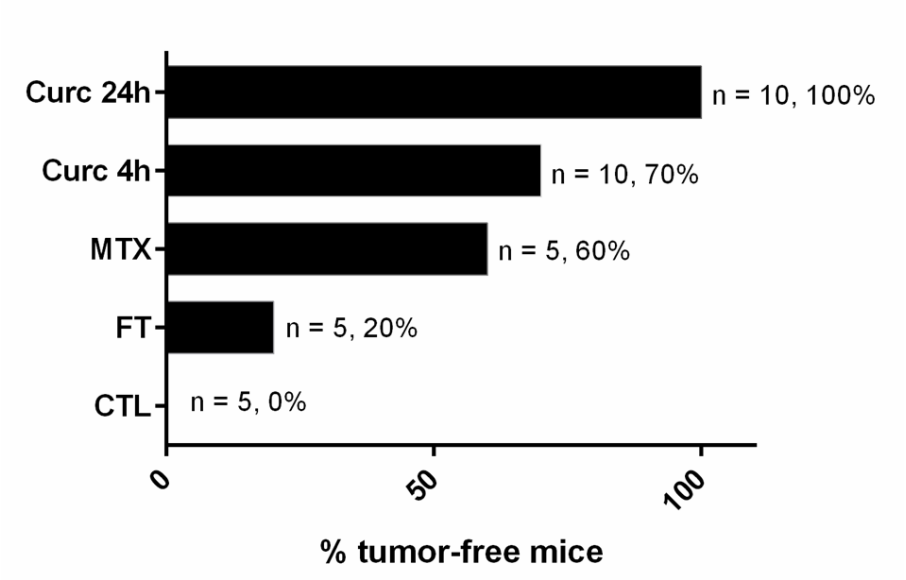

Figure 8: Percentage of tumor-free mice after inoculation of viable CT26 cells on the right flank. Control (CTL), freezing/thawing (F/T), mitoxantrone (MTX), curcumin for 4 and 24 hours (Curc 4h and Curc 24h). **n**, number of animals. Statistical analysis: Two-way ANOVA and Tukey's multiple comparison test.

3.5 Curcumin colocalizes with the endoplasmic reticulum of CT26 cells *in vitro*

It has been previously shown that curcumin is an ER stressor [6, 34]. As the induction of ICD is one of the possible results of ER stress responses [35–37], it is reasonable to suggest that the curcumin-induced ICD observed in this work is a result of ER stress. Thus, further experiments were performed to check this hypothesis.

Confocal microscopy images evidenced curcumin in the cytoplasm of CT26 cells after 15 min of exposure to curcumin *in vitro* (figure 9). A fast accumulation of curcumin inside cells has been already described in the literature [15]. This result was indeed expected, since curcumin is highly hydrophobic and probably diffuses passively through the lipid bilayer of the plasma membrane. Regarding its subcellular location, a certain amount of the interiorized curcumin was found colocalized with the ER (figure 10).

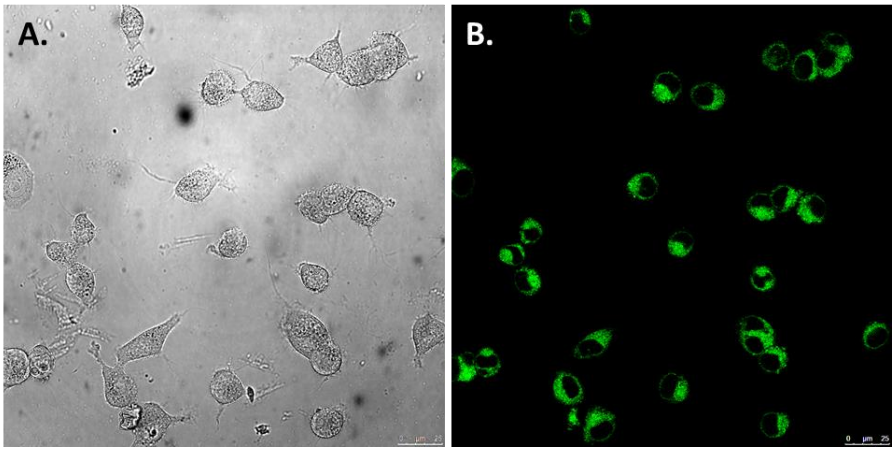

Figure 9: Interiorization of curcumin (green) by CT26 cells visualized by confocal fluorescence microscopy after 15 min of exposure to curcumin *in vitro*. Excitation length in 488 nm.

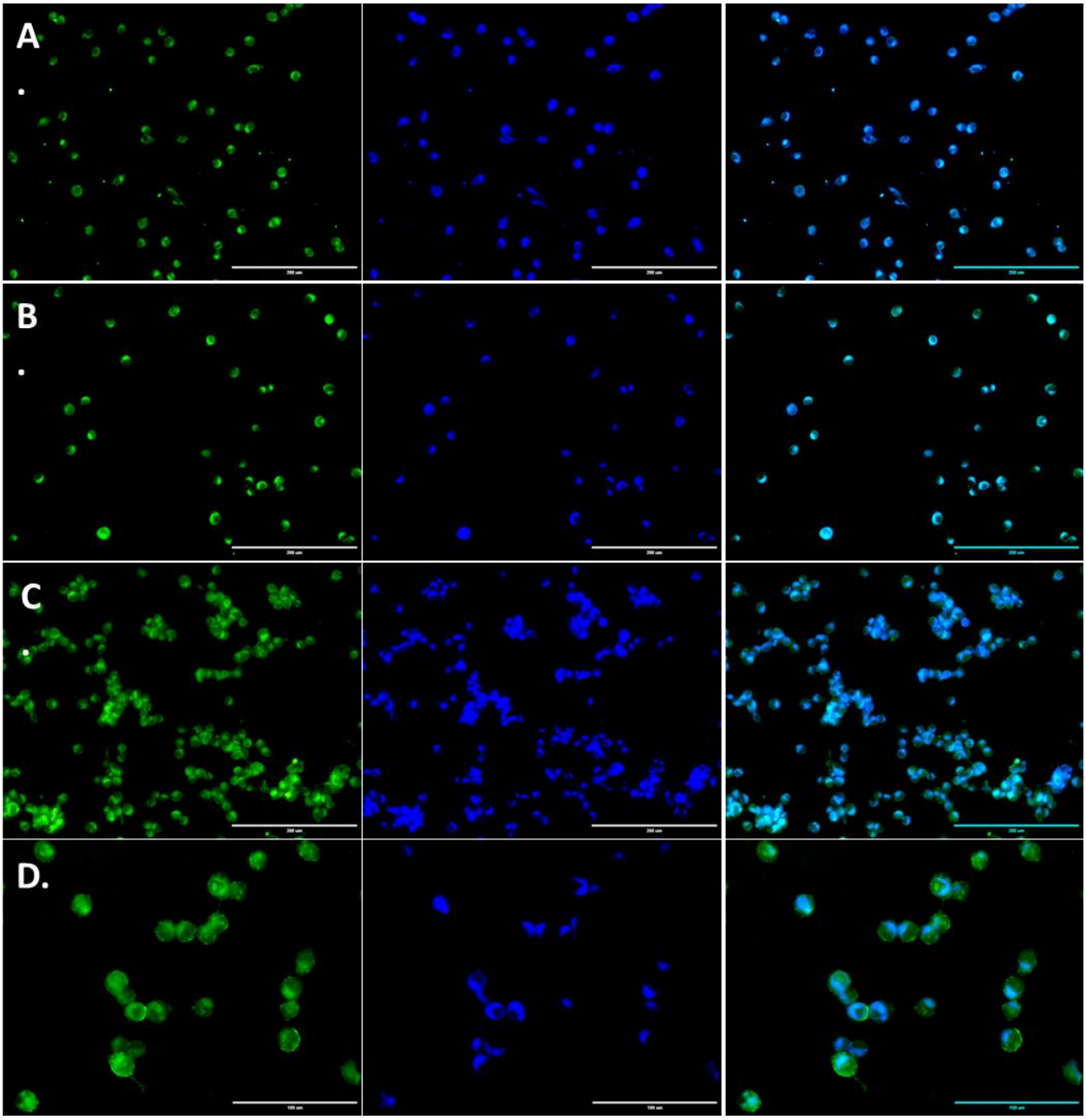

Figure 10: Curcumin colocalization profile in CT26 cells at A. 0 h; B. 4 h and C. 24 h by fluorescence microscopy. The first column represents the fluorescence of curcumin, the middle column of the marker for ER and the third column the overlap. Magnification 20X to A, B and C. Magnification 40X to D.

3.6 Cytoplasmic calcium levels

As previously mentioned, the ER is an important organelle for protein folding, translocation, and post-translational modification, but also for the intracellular calcium homeostasis. Under stressing conditions, which can include oxidative stress, hypoxia, hyperthermia, and others, the function of the ER may be impaired [37–39]. The figure 13 shows

that cells treated with curcumin presented significantly increased cytoplasmic calcium levels (figure 11), an event suggestive of ER stress, as reported in the literature [13, 34].

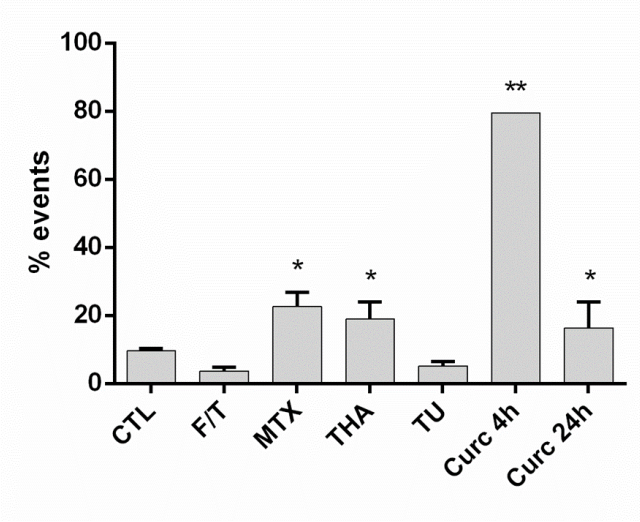

Figure 11: Intracellular calcium by flow cytometry in CT26 cells after application of different treatments. Control (CTL), freezing/thawing (F/T), mitoxantrone (MTX), thapsigargin (THA), tunicamycin (TU), curcumin for 4 and 24 hours (Curc 4h and Curc 24h). *****Statistically different from the control group. **Statistically different from the other groups. Data expressed as mean ± standard error of the mean. Statistical analysis: Statistical analysis: One-way ANOVA and Dunnett's comparisons test.

3.7 Curcumin activates cell signaling pathways involved in ER stress responses

Stressors of the ER can lead to the accumulation of unfolded and misfolded proteins in this organelle [40], triggering cellular responses such as the unfolded protein response (UPR). The UPR render the cell adapted to the unfavorable environment or it initiates ICD [41]. Curcumin has been shown to activate ER-resident UPR sensors, such as PERK (protein kinase RNA (PKR)-like ER kinase), IRE-1 (inositol requiring protein-1), and ATF6 (activating transcription factor-6), and their downstream-signaling proteins, in cervical cancer cells, but not in normal epithelial cells and peripheral blood mononuclear cells (PBMCs). The proteins IRE1, ATF6, and PERK stimulate expression of pro-apoptotic genes in response to ER stress [42]. CHOP, a key factor involved in ER stress-mediated apoptosis, has also been shown in cells treated by curcumin [34]. CHOP decreased the ratio of anti-apoptotic protein Bcl-2 to pro-apoptotic protein Bax expression, and subsequently increased the apoptotic population of cervical cancer cells. [41]. Stress is detectable in cancer cells at the level of the ER through the induction of a so-called "unfolded protein response" (UPR) [43]. UPR stimulates the phosphorylation of eIF2α, a cytoplasmic regulator of translation initiation, eventually leading

to the exposure of DAMPs such as calreticulin [36, 44, 45]. Upon ER stress the transcriptional factor X-box-binding protein 1 (XBP1) mRNA is spliced and regulates a subset of ER-resident chaperone genes in the UPR to protects cells from ER stress. Thus, XBP1 knockdown can induces resistance to several ER stresses [46]. The detection of this protein by western blotting is important to correlate the curcumin function and ER stress.

The cells treated with curcumin for 24 h showed the highest amount of XBP1 protein when compared to the control and curcumin 4 h (figure 12). The transcriptional factor XBP1 is activated by accumulating unfolded proteins and ER stress factors [46]. It is possible that the stress in the ER was caused by the increase in intracellular calcium, due to pre-exposure to curcumin, and that this culminated in the greater expression of XBP1. Methods and technologies have been developed to measure biomarkers associated with ICD and cancer immunosurveillance. Their application to clinical research could help identifying factors of prognostic and predictive relevance in treated cancer patients. Biomarkers can contribute to identify novel compounds with immunotherapeutic potential and to design innovative combinatorial treatment regimens with superior efficacy [33].

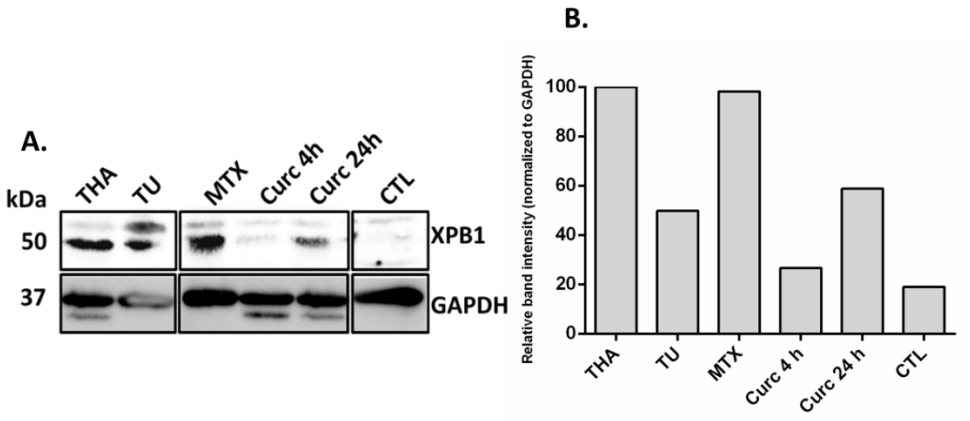

Figure 12: A. XBP1 protein expression; B. graph representing relative band intensity normalized to GAPDH. Shown are western blots for Control (CTL), Mitoxantrone (MTX), Thapsigargin (THA), Tunicamycin (TU), curcumin for 4 and 24 hours (Curc 4h and Curc 24h).

4. Conclusions

The present work shows that curcumin is not only cytotoxic, but also an inducer of ICD. This observation brings more insights into the mechanism of action of curcumin. This finding is of great relevance considering that the efficacy of certain cancer therapies, such as anthracycline-based chemotherapy, photodynamic therapy and radiotherapy, can also rely on

their ability to reinstate cancer immunosurveillance. Moreover, immunotherapy has profited of tools developed over the last years, which can circumvent immune evasion strategies deployed by cancers and reeducate the host immune system to detect and clear tumor cells. Thus, the use of curcumin not only to directly kill cancer cells, but also to help inducing an immune response against tumor antigens, is an alternative that can be explored in future studies.

Conflicts of interest

The authors have no conflicts of interest to declare.

Funding information

This study was funded by the Brazilian government agencies FAP-DF/Brazil (0193.001020/2015, and 0193.001626/2017), PRONEX FAP-DF, CNPq (0447628/2014-3, 193.001.200/2016) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

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ANNEX 1

Universidade de Brasília
Instituto de Ciências Biológicas
Comissão de Ética no Uso Animal

Brasília, 15 de fevereiro de 2019.

DECLARAÇÃO

Declaramos que o projeto intitulado "**INDUÇÃO DE MORTE CELULAR IMUNOGÊNICA EM CÉLULAS TUMORAIS SENSIBILIZADAS POR CURCUMINA E TRATADAS COM TERAPIA FOTODINÂMICA MEDIADA POR NANOEMULSÃO DE CLORETO DE ALUMÍNIOFTALOCIANINA.**" Protocolo n.º 70/2018, sob responsabilidade do Professor Luis Alexandre Muehlmann foi avaliado e aprovado pela Comissão de Ética no Uso Animal (CEUA) da Universidade de Brasília. Este projeto foi aprovado para utilização de: *Mus musculus (100)*. A presente aprovação é válida pelo período de: 08/2018 a 08/2020.

Universidade de Brasília UnB

Prof. Dr. Cássio José da Silva
Coordenador da CEUA – UnB

Comissão de Ética no Uso de Animais CEUA

*Este documento se restringe à avaliação ética do projeto supracitado e não substitui outras licenças e permissões que porventura se façam necessárias.

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