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DOXORUBICIN-INDUCED IMMUNOGENIC CELL DEATH IS ABLE TO IMPAIR TUMOR PROGRESSION AND DISTANT METASTASIS IN A HIGHLY AGGRESSIVE BREAST CANCER TUMOR MODEL

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MORTE CELULAR IMUNOGÊNICA INDUZIDA POR DOXORRUBICINA É CAPAZ DE RETARDAR A PROGRESSÃO TUMORAL E METASTÁTICA EM UM MODELO DE TUMOR DE CÂNCER DE MAMA AGRESSIVO

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RESUMO

O câncer é uma doença individual, e sua formação e desenvolvimento são específicos para cada hospedeiro. Os tratamentos convencionais são ineficazes em casos complexos como metástases e apresentam efeitos adversos graves. Novas estratégias são necessárias para abordagem do problema, e o uso da Morte Celular Imunogênica como um iniciador de resposta do sistema imunológico através da exposição de padrões moleculares associados a danos (DAMPs) por células cancerígenas é apresentado como uma abordagem de vacinação terapêutica personalizada neste trabalho. Para isso, células de adenocarcinoma mamário murino foram expostas à doxorrubicina com o intuito de induzir a liberação de marcadores associados à morte celular imunogênica, como ATP e calreticulina, e enxertadas subcutaneamente em camundongos BALB/c. Como controle do protocolo, as células foram também expostas à morte por necrose. Este protocolo, aqui chamado de "vacinação", foi realizado três vezes, com um intervalo de sete dias entre cada repetição. Após o último transplante das células em estágio de morte imunogênica, o protocolo de vacinação foi desafiado com o enxerto subcutâneo das mesmas células cancerosas, sem exposição à doxorrubicina, no flanco oposto ao da vacinação. O estágio de "desafio" consiste em avaliar se há, ou não, resposta imunológica específica dos camundongos às células cancerosas. O acompanhamento clínico dos camundongos foi realizado por 6 semanas, nas quais os camundongos foram tomografados para avaliação do surgimento de metástases pulmonares e tiveram a aparição e volume tumoral monitorados. A indução de morte celular imunogênica iniciada por doxorrubicina nas células de adenocarcinoma mamário murino foi avaliada por citometria de fluxo e análise de imagem. Os resultados mostraram que os camundongos vacinados com células em estágio de morte celular imunogênica após exposição à doxorrubicina não apresentaram tumor primário durante as 6 semanas de avaliação, bem como não houve indicativos de desenvolvimento de lesões metastáticas em sítios secundários.

Palavras-chave: câncer de mama; doxorrubicina; vacina; morte celular imunogênica.

ABSTRACT

Cancer is an individual disease, and its formation and development are specific to each host. Conventional treatments are ineffective in complex cases such as metastases and have serious adverse effects. New strategies are needed to approach the problem, and the use of Immunogenic Cell Death as an initiator of the immune system response through the exposure of damage-associated molecular patterns (DAMPs) by cancer cells is presented as a personalized therapeutic vaccination approach in this work. To this purpose, murine mammary adenocarcinoma cells were exposed to doxorubicin in order to induce the release of markers associated with immunogenic cell death, such as ATP and calreticulin, and grafted subcutaneously in BALB/c mice. As a protocol control, cells were also exposed to death by necrosis. This protocol, here called “vaccination”, was performed three times, with an interval of seven days between each repetition. After the last transplantation of cells in the stage of immunogenic death, the vaccination protocol was challenged with subcutaneous grafting of the same unexposed cancer cells, without exposure to doxorubicin, on the flank opposite that of vaccination. The “challenge” stage assesses whether or not mice have a specific immune response to the cancer cells. The clinical follow-up of the mice was carried out for 6 weeks, during which the mice were scanned to assess the appearance of pulmonary metastases and had their tumor appearance and volume monitored. Flow cytometry and image analysis evaluated the induction of doxorubicin-initiated immunogenic cell death in murine mammary adenocarcinoma cells. The results showed that mice vaccinated with cells in the immunogenic cell death stage after exposure to doxorubicin did not present a primary tumor during the 6 weeks of evaluation, as well as there were no indications of the development of metastatic lesions in secondary sites.

Keywords: breast cancer; doxorubicin; vaccine; immunogenic cell death.

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INTRODUCTION

Cancer is a multifactorial, individual, heterogeneous disease and its formation and individual development are specific to each host (Hanahan & Weinberg, 2011). Although conventional treatments are effective for cases where there is no spread of the disease to secondary organs, these treatments have many limitations associated with adverse effects resulting from their use. The lack of specific targeting of chemotherapeutics, which are the standard treatment, leads to high systemic toxicity and, therefore, culminates in severe adverse effects (Tsimberidou et al., 2020). When it comes to more complex cases of the disease, where there are already secondary sites of the spread of cancer cells, these treatments are even less effective and end up becoming even more severe, since the treatment regimen with chemotherapeutics is even more intense (Steeg, 2016). Recently, approaches that promote a targeted immune response of the host to cancer cells have been used with significant success in clinical medicine.

In this scenario, several immunological approaches have been published in the literature. The first reports date from the beginning of the 19th century when medical doctors injected BCG vaccines into tumor sites aiming at the activation of immune surveillance against those tumor sites. Despite some interesting initial results, these initiatives were not followed due to the specificity of the immunological strategy. It is shown, however, that cancer immunotherapy approaches might be the future of safe and effective treatment. More recently, more assertive approaches have been proposed in the last twenty years, such as blocking cellular signaling (Lu, 2020), or therapeutical approaches (Loibl et al., 2021). Among these immune targeting examples, some authors have proposed the development of adaptive immunological response by producing anti-tumor vaccines with tumor cells undergoing cell death (Ahmed & Tait, 2020; Bezu et al., 2015; Galluzzi et al., 2017).

One specific cell death pathway explored in this strategy is immunogenic cell death (ICD). Several groups, including ours, have published original reports and review

articles presenting this strategy by using tumor cells under immunogenic cell death to produce anti-tumor vaccines (de Lima et al., 2020; Pol et al., 2015; Rodrigues et al., 2020, 2022; Vacchelli et al., 2014). This approach differs from the prophylactic vaccine model, as the therapeutic immunization aims at inducing tumor-specific adaptive immune responses to an already established tumor. Therefore, it is understood that the study of methodologies that promote the development of the host's adaptive immune response mediated by ICD death might be a key aspect in the process of developing effective approaches to the treatment of breast cancer.

It is known that Pathogen-Associated Molecular Patterns (PAMPs) are responsible for activating receptors expressed in immune system cells, such as monocytes, macrophages, and dendritic cells, and that these cells not only activate the host's primary line of defense but can also trigger antigen-specific responses (Cao, 2016). However, this activation of adaptive immunity also depends on the signaling of Pattern Recognition Receptors (PRRs), which are activated by Damage-associated molecular patterns (DAMPs). When DAMPs are produced by cells in the dying stage, they act as adjuvants in communicating the state of danger to the immune system, triggering not only a primary line of defense but also an adaptive one (Fuchs & Steller, 2015; van Kempen et al., 2015). Thus, cell death recognized by PRRs and mediated by DAMPs is considered immunogenic, as it activates an immune system response caused by the death of cells that show such patterns.

Following the combination of these immunological fundamentals, our aim in this article was to develop and evaluate a therapeutic vaccine for a pre-clinical aggressive stage IV metastatic breast cancer model. As the main results, we will present the full development and characterization of the vaccine, composed of breast cancer cells (4T1) dying via ICD after exposure to the anthracycline drug doxorubicin. Moreover, we evaluate and confirm the effectiveness of the vaccine to impair the appearance of both primary tumors, as well as indications of distant metastatic lesion development; one important novelty of this article was the transfer of immune competent cells from vaccinated mice to immunocompromised nude mice, which are presented as a model for the study of cell-mediated immunologic deficiencies since they possess a vestigial

thymus which is incapable of producing mature T-cells (Pelleitier and Monplaisir, 1975). As a highlight, the nude mice that received the immunocompetent cells showed improved survival, even after tumor cell challenges. These results together indicate that this therapeutic vaccine could be further developed for clinical conditions.

RATIONALE

The growing estimate of cases of breast cancer, which currently impacts the lives of 2.1 million women worldwide annually (WHO, 2018), highlights the need to improve available conventional treatments and develop new therapeutic approaches.

Despite being effective for cases in which there is no spread of the disease to secondary organs, conventional treatments have many limitations associated with adverse effects arising from their use. The lack of specific targeting of chemotherapy, which is the standard treatment, leads to high systemic toxicity and, therefore, culminates in severe adverse effects. When it comes to more complex cases of the disease in which there are already secondary sites of cancer cell dispersion, these treatments are even less effective and end up becoming even more severe, since the treatment regimen with chemotherapy drugs is even more intense.

Cancer is a multifactorial disease, individual, heterogeneous in its formation and individual development for each host. Thus, the need for treatment approaches that focus on these aspects of the pathology becomes clear. Recently, approaches that promote a targeted host immune response to cancer cells have been used with significant success in clinical medicine and, for the most part, are based on the use of drug-induced immunogenic cell death to develop the adaptive host immune response to cancer cells.

Therefore, it is understood that the study of methodologies that promote the development of the adaptive immune response of the host mediated by immunogenic cell death is a primordial aspect in the process of developing effective approaches for the treatment of breast cancer, and this is what this work is proposed.

1. PURPOSE

To establish an adaptive immune response induced by immunogenic cell death effective in controlling the progression of primary tumor growth and the establishment of metastases in secondary sites

1.1 SPECIFIC GOALS

1.1.1 Induce immunogenic cell death in a murine mammary adenocarcinoma cell line (4T1);

1.1.2 To analyze the induction of adaptive immune memory to cancer cells of the murine mammary adenocarcinoma lineage (4T1) in BALB/c mice;

1.1.3 To evaluate the effectiveness of this adaptive immunological memory in combating primary tumor development and the appearance of metastases in secondary sites.

2. LITERATURE REVIEW

2.1 Immunogenic Cell Death

The immune system does not respond to the death of billions of cells daily in order to preserve body homeostasis, since a coordinated response to a continuous process would have severe consequences. However, when dealing with the death of pathogen-infected cells, a response to a specific antigen is readily initiated in the host (Galluzzi et al., 2017). Thus, it became important to identify what differs in the activation processes of the immune system and which mediators are involved. It is known that Pathogen-Associated Molecular Patterns (PAMPs) are responsible for the activation of receptors expressed in cells of the immune system, such as monocytes, macrophages and dendritic cells, and that these cells not only activate the host's primary line of defense but can also trigger antigen-specific responses (Cao, 2016). However, this activation of adaptive immunity also depends on the signaling of Pattern Recognition Receptors (PRRs), which are activated by Damage Associated Molecular Patterns (DAMPs), which when produced by cells in the death stage act as adjuvants in communicating the state of danger to the defense system (Fuchs & Steller, 2015). In this way, not only is a primary line of defense initiated, but also an adaptive one. Thus, cell death recognized by PRRs and mediated by DAMPs is considered immunogenic, as it activates an immune system response to the death of cells that display such patterns.

Activation of PRRs by DAMPs is a crucial process in the organism's understanding of the dangerous situation. DAMPs are expressed on the cell surface as antigenic epitopes and may be encoded by microbial genes or by host genes that undergo the process of oncogenic mutation and tumor progression (van Kempen et al., 2015). Thus, it is possible that a more efficient and specific immune system response is triggered to help control tumor progression and establish metastases.

2.2 Types of Immunogenic Cell Death

Nowadays there is no longer an understanding that apoptotic death, because it is regulated, does not produce an active response from the immune system, just as death

by necrosis is no longer the only one characterized as pathological and immunogenic, since there are deaths by regulated necrosis involved in the maintenance of body homeostasis, as well as there are apoptotic deaths that trigger antigen-specific immune response (Galluzzi et al., 2015). Some of the processes that are characteristic of the ICD process can be seen in Figure 1.

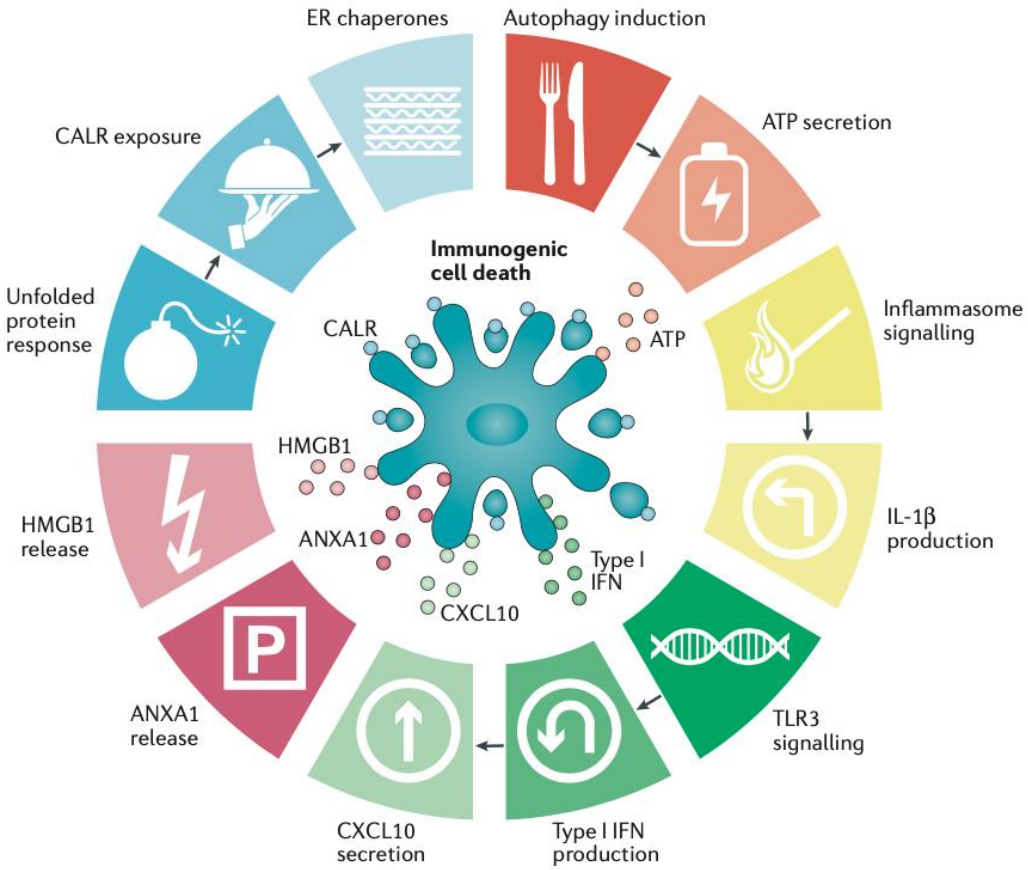

Figure 1 - Processes associated with Immunogenic Cell Death. Adapted from Gallozzi et al, 2016

Several means by which immunogenic cell death is initiated are known, such as infection by pathogens, radiation, necroptosis and the one of interest in this work: the chemotherapeutic-induced ICD. Infection by pathogens is a natural defense process that features unfolded protein response, inflammasome signaling, and ATP secretion. Radiation-induced ICD exhibits all of the responses seen in pathogen-induced ICD and

most of the responses seen in Figure 1, with the exception of ANXA1 release. Necroptosis is a newly discovered pathway of regulated necrosis that requires RIPK3 and MLKL proteins and is induced by death receptors, interferons, toll-like receptors, intracellular RNA, and DNA sensors (Pasparakis & Vandenabeele, 2015) and is accompanied by the release of HMGB-1, secretion of CXCL10 and ATP, and exposure of CALR on the cell membrane.

The ICD induced by chemotherapy will be treated in a separate topic because it is the ICD of interest in this work.

2.3 Chemotherapy-induced Immunogenic Cell Death

As explained, ICD is capable of activating an adaptive immune response against dead cell-associated antigens (Bezu et al., 2015), evidencing the existence of different approaches to use this response against cancer cells. Thus, the mechanisms responsible for this type of regulated cell death began to be studied in depth, the main associated effectors, and how it could be artificially initiated once the development of an adaptive host response to these cells means one more frontier to be crossed by the progression of the disease.

Cells respond in a specific way when exposed to different drugs, as is routinely observed in in vitro tests. In some cases, little or no response is elicited, while in other cases the process of cell death is initiated. One of the strategies considered was to use chemotherapeutic drugs already in clinical use to evaluate the initiated death process and it was observed that some of them, such as doxorubicin, paclitaxel, and mitoxantrone, are capable of initiating the process of immunogenic cell death. (Brignone et al., 2010; Casares et al., 2005).

For this characterization, it is necessary to understand what characterizes chemotherapy-induced ICD and what role each of these features plays in the development of the host's adaptive immune response to cancer cells.

According to Galluzzi et al., 2016, ICD has all the characteristics shown in Figure 1, highlighting especially the secretion of adhesin triphosphate (**ATP**), the release of protein 1 of the high mobility group (**HMGB-1**), and Calreticulin exposure (**CRT**) on the

cell membrane. Each of these three effectors plays an essential role in the development of the adaptive immune response, they are the focus of this work and are described below.

2.3.1 Immunogenic Cell Death Effectors

2.3.1.1 Calreticulin

Calreticulin is an abundant protein in tissues and is one of the main proteins involved in the process of binding Ca^{2+} to the endoplasmic reticulum, being directly involved in the process of maintaining intracellular Ca^{2+} homeostasis. As a chaperone, CRT prevents the aggregation of partially folded proteins, in addition to being involved in the process of regulating cell adhesion to the extracellular substrate (Michalak et al., 1999). In the laboratory, CRT is commonly used as a specific marker for the endoplasmic reticulum in cell sublocalization studies. (Obeid, Tesniere, et al., 2007). In more recent studies, CRT is also shown to be an integral part of the so-called peptide loading complex (PLC), a multicomponent complex that assembles in the ER membrane to ensure proper loading of cellular antigens onto Class I MHC molecules. (Fucikova et al., 2011).

In their 2007 paper, Obeid et al. presented evidence that CRT is exposed in cells undergoing ICD and absent on the surface of cells undergoing non-immunogenic cell death. Their findings are corroborated by the deepening of the group's studies, presented in their 2015 article, where Obeid et al. demonstrated that the transfer of CRT from the ER to the cell membrane is one of the steps responsible for converting silent apoptotic cell death into immunogenic cell death because CRT exposed on the membrane emits an “eat me” signal to circulating macrophages, the that helps stimulate the development of an adaptive immune response, a conclusion they reached after tests in which the CRT knockdown prevented the activation of ICD (Obeid, Panaretakis, et al., 2007). Therefore, it can be said that CRT is an essential effector in the process of ICD and in the possible development of the adaptive immune response.

2.3.1.2 HMGB-1

The HMGB-1 protein is highly conserved and present in the nuclei and cytoplasm of nearly all cell types. In a 2005 literature review, it was shown that some of its main roles include that secretion of HMGB-1 in the extracellular environment acts as a pro-inflammatory cytokine thanks to its ability to recruit macrophages, neutrophils and dendritic cells, which HMGB-1 performs functions as a nuclear protein, participating in the regulation of gene transcription, and it is a protein that also acts as an inducer of cell migration and proliferation (Yang, 2005).

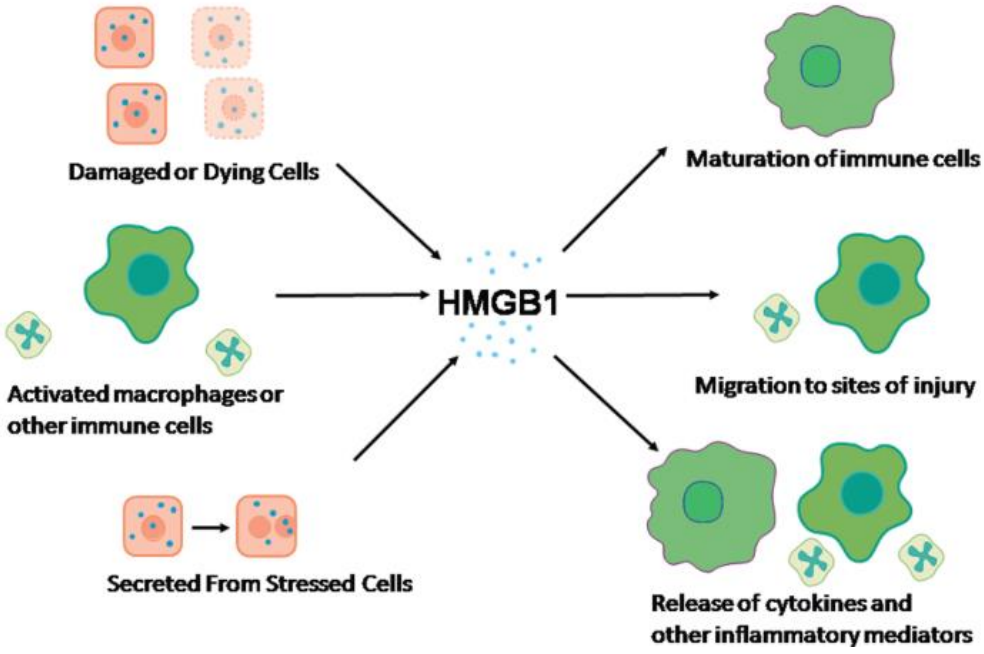

Figura 2 - How HMGB1 Elicits the Immune Response

For the present work, the role of a pro-inflammatory cytokine is of interest, since the release of HMGB-1 in the extracellular environment due to necrotic cell death results in the recruitment of mononuclear cells to the site of tissue damage, also informing the cells neighboring areas that tissue repair may be required (Dumitriu et al., 2005). Despite referring to necrotic death, the release of HMGB-1 is also observed in ICD (Galluzzi et al., 2015), and although the mechanisms are not completely elucidated, in in vivo studies, HMGB-1 increases the number of antibodies directed against exogenous antigens and induces a long-lasting protective immune response

to a highly tumorigenic lymphoma (Rovere-Querini *apud* Dumitriu *et al.,* 2005). In other studies, the maturation-inducing role that HMGB-1 plays in dendritic cells is also observed by increasing the expression of many cell surface markers, as well as by the secretion of inflammatory cytokines (Klune et al., 2008). In the study by Casares et al. 2005), doxorubicin was effective in promoting HMGB-1 secretion by CT26 cells after exposure.

2.3.1.3 ATP

ATP is well known for its role as a universal energy source, but a long series of studies have demonstrated that ATP is a critical signaling molecule that allows cells and tissues throughout the body to communicate with each other, as once secreted into the extracellular space, it acts as a primary signal involved in the control of various physiological and pathological mechanisms (Khakh & Burnstock, 2009).

Essentially, each cell in an organism can release ATP in a controlled manner, thus many physiological activities are controlled by ATP, such as neurotransmission and neuromodulation, development and aging, control of hormone secretion, and the normal function of the liver and kidney (Dou et al., 2018).

Studies have demonstrated that extracellular ATP signaling functions as an important dynamic regulatory pathway to coordinate appropriate immune responses in various pathological processes, including intracellular infection, host-tumor interaction, and pro-inflammatory vascular injury (Hart *et al.,* 2008 e Zeiser *et al.,* 2016 *apud* Dou *et al.,* 2018). ATP can mobilize an efficient innate immune response, including recruitment of innate immune cells (such as macrophages, neutrophils, and eosinophils), secretion of cytokines, and production of inflammatory mediators such as reactive oxygen species (ROS) (Dou et al., 2018).

2.4 Immunogenic Cell Death and Cancer

As previously demonstrated, ICD appears to be an important initiator of adaptive immunity in the context of infectious and cancerous diseases. In traditional approaches

to cancer treatment, the immune response stimulated by chemotherapy involves the participation of dendritic cells as well as various populations of T lymphocytes. However, cancers controlled by oncogenes often end up acquiring the ability to evoke immune escape mechanisms or are simply capable of not evoking any type of immune response (Kroemer et al., 2013).

Thus, the use of ICD as a treatment approach in cancer comprises the release of molecular patterns associated with damage to tumor cells present during cell death, such as CALR and ATP, which result in the activation of tumor-specific immune responses, promoting the response immediate and long-term immune response, combining direct killing of cancer cells and antitumor immunity (Galluzzi et al., 2017). By being able to promote long-term specific immunological memory, this approach is treated as a kind of vaccination.

3. Adaptive immune response

Host immunity is dissected into innate and adaptive immune responses. The innate responds rapidly and nonspecifically to pathogens, while the adaptative responds slowly but specifically, usually with the generation of long-term immunological memory. During an infection, innate immunity is the first to be triggered, initiating inflammation, and taking no more than minutes or hours to be fully activated. Activation of the adaptive immune response and induction of classical immune memory in lymphocytes is dependent on the innate immune system, in particular antigen-presenting cells such as dendritic cells, and can take days to weeks (Netea et al., 2019).

Two properties that distinguish between innate and adaptive immunity are specificity and the ability to build long-term immunologic memory. The innate immune response is traditionally considered nonspecific and nonadaptive, whereas the adaptive immune response accurately recognizes various pathogens and subsequently builds immunologic memory. Adaptation of immunological memory is defined as a long-term change in the immune response due to a constant change in environmental conditions or chronic injury, resulting in a new functional state. In this

case, the functional state of the immune system during adaptation does not return to the low baseline state that existed before the stimulus (Netea et al., 2019).

The adaptive immune system consists of B and T lymphocytes which express highly specific receptors for antigens, called B cell receptors and T cell receptors. convert the population of immune cells into cells that can recognize the antigen encountered. The clonal expansion of antigen-activated B and T cells not only provides a better defense during primary infection but also makes the immune response more efficient during secondary infection by the same stimulus. A high number of memory cells generated during clonal expansion increases the chances of antigen encounters and makes the secondary immune response much faster and more efficient (Cooper & Alder, 2006).

Therefore, we will now address the main effectors of the adaptive immune response that are of interest in this work.

3.1 Adaptive immune response effectors

Ideally, cancer immunotherapy initiates and sustains a long-lasting T-cell-promoted anti-tumor response that is maintained by the release of antigens from cancer cells in the immunogenic death stage presented to naive T cells in lymphoid organs.

3.1.1 T CD4+ cells

CD4+ T cells play a central role in the function of the immune system: they help B cells produce antibodies, amplify and maintain the CD8+ T cell response, regulate macrophage function, orchestrate the immune response against a variety of pathogenic microorganisms, and regulate/suppress the immune response to control autoimmunity and regulate the magnitude and persistence of the immune response displayed (Zhu et al., 2010).

Antigen-specific contact with the CD4+ T cell allows dendritic cells to optimize antigen presentation and provide CD8+ T cells with specific cytokines and

costimulatory signals, which in turn promote their clonal expansion and differentiation into an effector T cell or a memory T cell. With the help of CD4+ T cells, a gene expression program is initiated in CD8+ T cells that enhances cytotoxic cell function by several molecular mechanisms (Borst et al., 2018).

In cancer, CD4+ T cells can target tumor cells in several ways: either directly by eliminating tumor cells through cytolytic mechanisms or indirectly by modulating the tumor microenvironment. In addition, CD4+ T cells in secondary lymphoid organs increase the extent and quality of B and CD8+ T cell responses.

3.1.2 T CD8+ cells

CD8+ T cells (CTLs) are the preferred effectors for targeting tumors and tumor cells because they recognize intracellular antigens presented by MHC class I molecules expressed by all cell types. The establishment of protective T cell immunity requires the differentiation of CD8+ T cells from an immature state to a state in which pathogen-specific memory CD8+ T cells are able to respond more rapidly and robustly to secondary infection without the need for additional specialization (Turner et al., 2021).

Immature CD8+ T cells have metabolic plasticity that allows them to settle or remain in circulation in different environments. Circulating CD8+ T cells are exposed to a variety of environments that affect their differentiation and function. These environments may be healthy tissue, infected tissue, or tissue with malignant alterations, as is the case with cancerous tissue. In these environments, immature CD8+ T cells are activated upon contact with a known antigen derived from pathogens or cancer cell antigens and activate transcriptional, translational, and metabolic programs required for rapid population expansion and differentiation into specific effector CD8+ T cells and memory cells. (Reina-Campos et al., 2021).

Effector CD8+ T cells then help eliminate pathogens and damaged cells by secreting cytokines and promoting direct cell death, while populations of long-term memory CD8+ T cells provide enhanced protection against these damaging processes.

As cancer is a chronic disease, an efficient immune response to cancer is expected to require long-lasting CD8+ T-cell immunity.

4. MATERIAL AND METHODS

To facilitate the understanding of the project, the methodology is divided into three phases, according to the experimental plan shown in the figure 3. Each of the phases is needed to evaluate which approaches will be tested and which are most appropriate for the later phase.

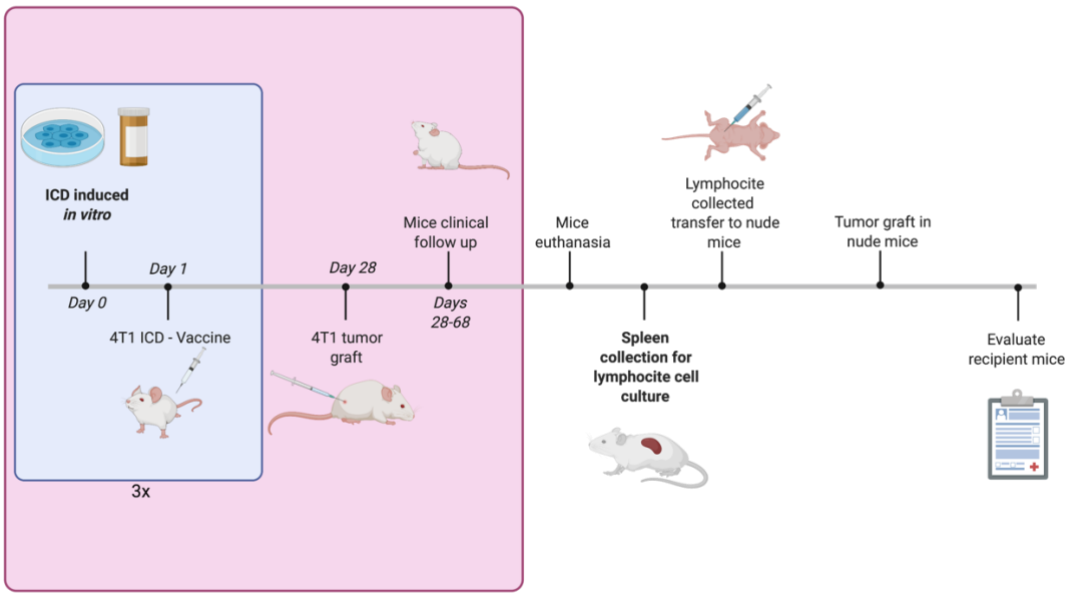

Figure 3 – **Experimental design -** To facilitate understanding of the project, an explanatory timeline has been provided. On day 0, the 4T1 cells are plated to reach 80% confluence the next day. Once this confluence is achieved, the cell culture medium is removed, and the medium containing doxorubicin is added for 24 hours. After 24 hours of exposure, the ICD-stage cells are transplanted subcutaneously into the right flank of the mice. This procedure is repeated three times, with an interval of one week between each repetition. One week after the last inoculation, the mice received subcutaneous transplantation of 4T1 cells into the left flank, the challenge. The mice were then studied over a 42-day

period before being euthanized. Their spleens were harvested, and a lymphocyte-enriching culture was performed for 24 hours. After this time, the lymphocytes were harvested and transplanted intraperitoneally into nude mice. The day after transplantation, the mice received the subcutaneous graft containing 4T1 cells and were monitored clinically until the day of euthanasia.

4.1 Stage 1: Cell culture and mice maintenance

4.1.1 Cell culture

For this work, 4T1 mouse adenocarcinoma cells from the cell collection of the Department of Genetics and Morphology at the University of Brasilia were used. To ensure the reproducibility of results, this strain was also expanded and frozen for use in later stages. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) in an incubator at 37°C and 5% CO2. After reaching 80% confluence, cells were resuspended in 1.5 ml trypsin and centrifuged. After centrifugation, the trypsin was discarded and a new DMEM was added as an environment for subcutaneous transplantation into BALB/c mice.

4.1.2 Mice maintenance

The BALB/c mice used in this work were obtained from the vivarium of the Catholic University of Brasília - UCB. The animals were between 4 and 6 weeks old and weighed between 20g-23g. The mice were kept in the vivarium of the Department of Genetics and Morphology (Institute of Biological Sciences, UnB), during the experimental period. In the vivarium, mice were housed in cages of 5 (five) animals each, lined with wood crisps and placed on ventilated shelves with an air filtration system. Environmental conditions were controlled, with a temperature of 22ºC, humidity between 50% and 100%, and an automatic 12-hour light-dark cycle. Feed and water supply ad libitum.

4.2 Stage 2: ICD-stage 4T1 cell vaccination

At this stage, 4T1 cells were exposed to the drug doxorubicin in an in vitro culture to induce immunogenic death. Twenty-four hours after exposure to the drug, the cells were prepared for subcutaneous transplantation into the right flank of the mice. This procedure, called vaccination, was repeated once a week for three weeks. Seven days after the last vaccination, a transplantation of viable 4T1 cells, called challenge, was performed into the left flank of the mice, initiating the clinical follow-up phase (Figure 4). After 6 weeks, the mice were euthanized, and their material collected for the next phase. The experimental design for phase 2 is shown in Figure 5.

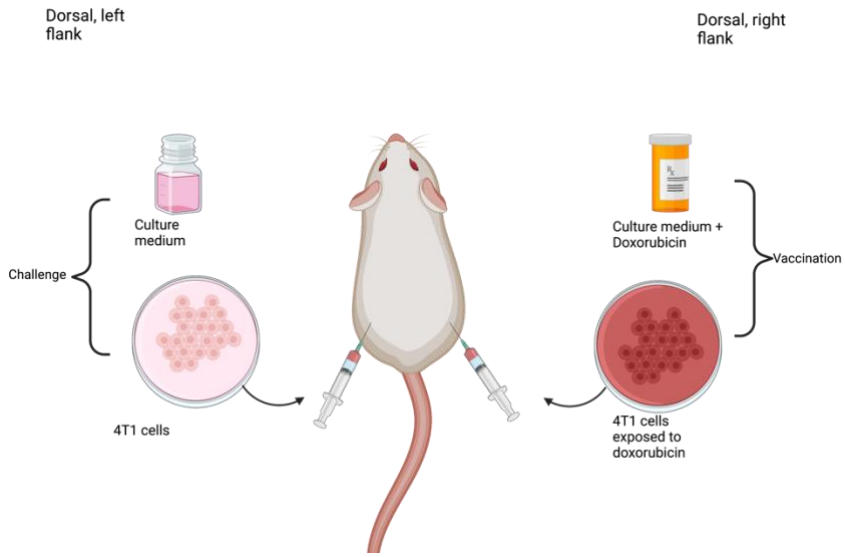

Figure 4 - **Vaccination and challenge scheme**. Representative scheme of vaccination and challenge. Mice received the 4T1 cells at the ICD stage, called the vaccination stage, on the right flank. After three weeks of vaccination, the mice received the "healthy" 4T1 cells on the left flank, called the challenge.

4.2.1 Exposure of cells to drugs for ICD inducing

Cells were plated out in flat-bottomed 12-well plates the day before exposure to treatment groups. One complete 12-well plate was used for each experimental group. On the day of exposure, the confluence of cells that had been previously 80%

plated out was checked. The conventional culture medium was removed and the medium containing the treatment was added to each plate. These cells were exposed to the treatment for 24 hours. After these 24 hours, the medium was discarded, and the cells were removed from the plates with a cell scraper to prevent the surface molecules from being altered by trypsin. For the control groups, cells in the *Thaw/Freeze* group were heated to 50ºC and cooled to -80ºC on the day of inoculation to induce death by necrosis. The saline group received subcutaneous administration of PBS.

4.2.2 Vaccination

Cells exposed to doxorubicin treatment were transplanted subcutaneously into the left flank of female BALB/c mice. For transplantation, animals were anesthetized intraperitoneally with 80μL with a total volume of 10% ketamine hydrochloride solution containing 2% xylazine hydrochloride, 80mg/kg, and 10mg/kg, respectively. This inoculation was performed once a week for three weeks.

4.2.3 Subcutaneous graft

Seven days after the last administration of cells in ICD, a subcutaneous graft of 4T1 cells was performed in the region contralateral to the vaccine, that is, in the right flank of the mice (Figure 4). For this, they were also anesthetized intraperitoneally. Thus began the period of clinical follow-up:

4.2.4 Body weight monitoring

The body weight of the mice was measured on a precision balance the day before the start of transplantation and weekly until the day of euthanasia.

4.2.5 *In vivo* tumor volume monitoring

Tumor volume measurements were started when the primary tumors resulting from the graft on the right flank became visible. Measurements with a caliper were

performed weekly until the day of euthanasia. Tumors were measured in vertical angles, length, using the animal's head as a landmark, and in horizontal angles, width, using the hind legs as a landmark. The following equation (GANASSIN et al., 2018) was used to calculate the final tumor volume:

$$TV = \frac{4\pi}{3}\left(\frac{L+W}{2}\right)$$

4.2.6 Monitoring metastasis appearance

To assess the presence of metastases in distant tissues, microtomography of the mice was performed using the PET-SPECT Albira® device. This procedure began after day 14 of tumor transplantation. The mice were also anesthetized for this procedure, which was repeated once per week until the week of euthanasia.

4.2.7 Survival curve

To generate the mouse survival curve, cages were checked daily to determine the physical/clinical condition of the animals. The day and animal identification were noted if an animal was found dead. After euthanasia, the data were pooled and presented in the form of a survival curve.

4.2.8 Euthanasia

Since the spleen had to be removed for the collection and cultivation of primary lymphocytes, the animals were euthanized uniformly and continuously in a CO gas chamber.

4.3 Stage 3: verification of the activity of primary lymphocytes after transplantation in immunocompromised mice

To determine whether lymphocytes with a specific response to 4T1 cells were formed, cells harvested from the spleens of mice and cultured in lymphocyte-

enriching medium were transplanted intraperitoneally into BALB/c nude mice. The day after transfer, the mice received a subcutaneous graft containing viable 4T1 cells, which began the clinical follow-up. The experimental design for this phase is shown in Figure 6.

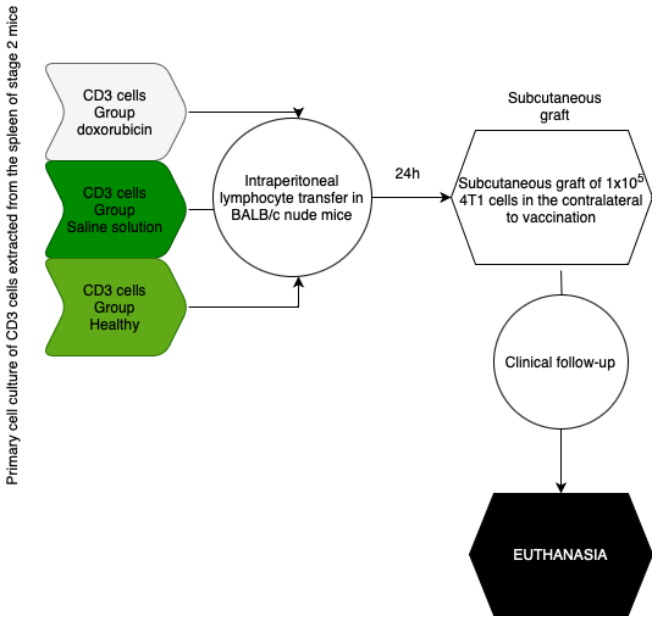

Figure 5 – **Lymphocyte transplantation scheme for verification of primary lymphocyte activity after transfer in immunocompromised mice** - After euthanasia of BALB/c mice, the harvested spleens were cultured in lymphocyte-enriched medium and later transplanted intraperitoneally into nude mice. The following day, the mice received the subcutaneous graft on the right flank. The nude mice were then clinically monitored until the day of euthanasia.

4.3.1 Lymphocyte transplantation

Lymphocytes derived from the spleens of stage 1 mice were transplanted intraperitoneally into BALB/c nude mice at a concentration of $1.4x10^7$ cells/200μL. Anesthesia was not required at this stage because the intraperitoneal route can be achieved by physical restraint of the animal.

4.3.2 Subcutaneous graft

Subcutaneous transplantation of viable 4T1 cells is performed in the same manner as described in the previous phases.

4.3.3 Clinical monitoring

The clinical follow-up of mice in this stage will be the same as in previous stages.

4.4 *In vitro* studies to validate the induction of Immunogenic Cell Death by exposure to Doxorubicin

4.4.1 Calreticulin exposure – Flow cytometry

4T1 cells were cultured in 6 flat-bottom well plates with DMEM culture medium for 24 hours. After reaching 80% cell confluence, the culture medium was replaced with treatment medium containing 5µM doxorubicin for an additional 24 hours. The culture medium was removed, and phosphate-buffered saline was added. Cells were then carefully removed from the plate using a cell scraper, transferred to Falcon tubes, and then centrifuged.

The supernatant was discarded, and zombie UV dye diluted in FACS buffer was added and incubated for twenty minutes protected from light. The cells were carefully washed with buffered saline and centrifuged again. The supernatant was discarded and the antibody against calreticulin (Abcam®, ab2907) diluted in FACS buffer was added. After one hour of light-protected incubation, cells were washed again with buffered saline. The secondary antibody (Alexa® 647), also diluted in FACS buffer, was added and incubated for 45 minutes, also light protected. Cells were washed and centrifuged twice, the supernatant was discarded and cells were resuspended in FACS buffer and analyzed using the BD FACSCanto (BD BioScience). To quantify the intensity and homogeneity of exposure to calreticulin on the cell membrane, the same flow cytometry staining protocol was used for cell analysis with ImageStream®X Mark II (Luminex, DiaSorin).

4.4.2 ATP secretion in extracellular medium

HEK293T cells transfected with pmeLUC were used as reporter cells to measure ATP release in the extracellular medium. For this purpose, HEK293T cells were propagated in a 9 cm Petri dish. Once 95% of the cells were confluent, the culture medium was replaced with the transfection medium containing DMEM, OptiMEM, PEI, and 9.4 µg of pmeLUC DNA. Cells were incubated with the transfection medium for 24 hours.

After 24 hours, the medium was removed, and the cells were carefully washed with PBS and then detached from the Petri dish using PBS/EDTA and transferred in triplicate to a 96-well plate with a white flat bottom.

The ICD induction protocol used in this work was performed in parallel in 4T1 cells. After 24 hours of exposure to doxorubicin, the supernatant of 4T1 cells was collected and added to the wells containing HEK293T reporter cells immediately after addition of the firefly substrate. Luminescence was read in FluoStar® (BMG Labtech).

5. RESULTS AND DISCUSSION

There are strong indications showing that, in the near future, personal medicine therapies will be widely used for several diseases. The improvement in the understanding of diseases, including cancer, may create a scenario where the development of personalized therapies will be increasingly feasible. For instance, following the main topic of the present article, the development of customized individual therapeutic vaccines may be a reality in the next decades. The main idea of this approach is to train the immune system with tumor antigens and develop an adaptive immune response against a specific malignant tumor.

Within this discussion, for real clinical situations, individuals with an established case of malignant cancer might be eligible for treatment with a therapeutic vaccine in

which antigens derived from the tumor are used to promote a specific immunological response. Bearing in mind the limitations of the tumor models, the present article aimed to propose the development of a therapeutic vaccine for an aggressive metastatic breast cancer model. The strategy proposed here was to use tumor cells (4T1 stage IV metastatic breast cancer) under immunogenic cell death induced by doxorubicin to vaccinate immunocompetent BALB/c mice.

As background, our research group recently published a report showing that doxorubicin is a chemotherapeutical drug able to induce ICD in the same breast cancer lineage (de Lima et al., 2020). Moreover, we observed that when these ICD inducers were delivered to tumor tissues in nanostructured liposomes, it was possible to observe the reduction of tumor progression, including the prevention of distant lung metastasis. It was an interesting finding, and this approach was followed by some important immunological events, including the reduction of extramedullary hematopoiesis in the liver, which is an important immunosuppression hallmark of this tumor model. The main hypothesis discussed was that the ICD inducers created an *in-situ* vaccination during drug administration.

A natural question following this previous report was to ask if this *in situ* vaccination could be replicated artificially *in vitro*. If possible, we could create vaccine systems in the lab, and immunize the animals before tumor development to evaluate its efficiency. The next steps in this direction were to develop and evaluate the vaccines produced with 4T1 cells undergoing ICD. To test this hypothesis, we prepared an experimental design with different goals that should be evaluated to create a proof-of-concept vaccine for this breast cancer vaccine.

The first point was we wanted to investigate if the 4T1 cells were indeed undergoing immunogenic cell death after exposure to 5 µM of DOX and if the ICD was the main cause of the results presented in this work. Following the consensus guidelines for the detection of immunogenic cell death, ATP release, calreticulin (CALR) exposure, and HMGB1 secretion can prompt the immune system and elicit an adaptive immune response specific to stimuli from cell-associated antigens (Kepp et al., 2014). Therefore, we aimed to show that these damage-associated molecular patterns

(DAMPs) were present. For this purpose, the protocol of exposure of the 4T1 cells to the DOX for the vaccination was replicated and the cells were then analyzed. As presented in the methods, the CALR exposure was measured by flow cytometry, and the HEK293T-pmeLUC cells were used to sample the ATP concentration in the culture medium of 4T1 cells after exposure to DOX.

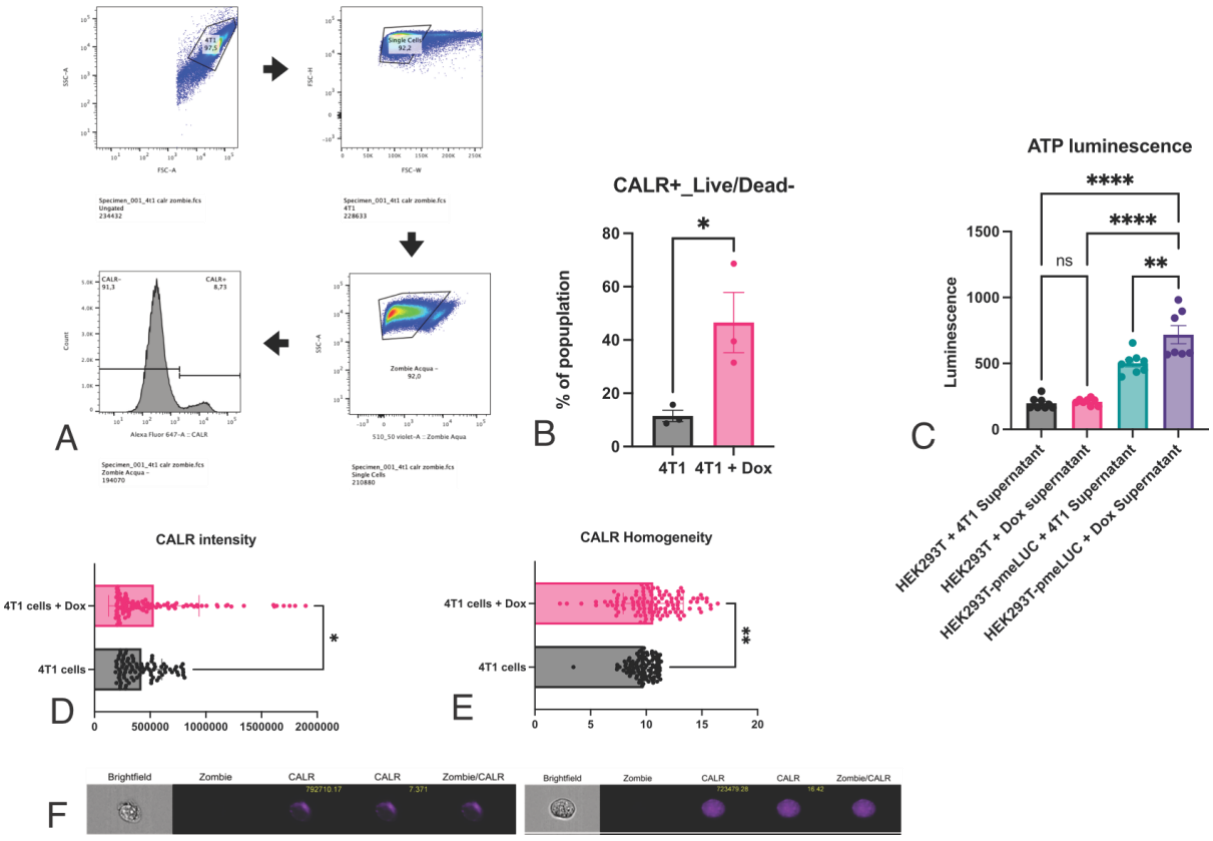

Figure 6 - **In vitro evaluation of immunogenic cell death markers after 4T1 cells were exposed to doxorubicin (DOX).** (A) *Gating strategy for flow cytometry. First, the 4t1 cells are selected, followed by the single-cell selection to ensure that a single signal from each cell is presented. After that, the population chosen for quantification of CALR in the cell membrane is chosen from the negative population of the Zombie Acqua (Live/dead) marker, meaning only the live cells signal is presented. (B) CALR positive signal presented in percentage. A significant difference can be observed between the non-exposed and exposed to DOX signals, p = 0.0379. This difference could also be observed in the intensity (D), p = 0.0170, and homogeneity (E), p = 0.0014. In (F) we can observe the higher intensity and homogeneity of the CALR signal in the cells exposed to DOX for 24h (presented right).*

As CALR is an endogenous protein with three different domains it is important to highlight that the C-domain of the protein has a high capacity for binding to calcium (Ca^{2+}) and to the cell surface; therefore, it is already localized, at some level, on the surface of the cell membrane (Michalak et al., 1999). Thus, the first aim was to show a significant increase in the CALR localization on the cell membrane post-exposure to DOX. As presented in Figure 7 (B), a significantly higher percentage of CALR was localized on the cell membrane when in comparison to the 4T1 cells non-exposed to DOX. This can be confirmed by the protocol of staining used for flow cytometry, an adaptation of the one published by (Liu et al., 2020), since the BSA present in the FACS buffer, used for dilution of the anti-CALR antibody, live/dead marker, and secondary antibody prevented unspecific binding of the anti-CALR. Another crucial step of this staining protocol is that in the gating strategy, only the live cells ($CALR^+$_$Live/Dead^-$) were selected for quantification, Figure 7 (A), ensuring an intact membrane, meaning that only the CALR available in the cell membrane is represented.

Aiming to better characterize the CALR exposure, we also used the ImageStream®X Mark II to quantify the intensity and homogeneity of CALR in the cell membrane. The gating strategy and protocol for staining were the same as the one used for flow cytometry and, as shown in Figures 7 (D) and (E), a significantly higher intensity of CALR signal and homogeneity distribution on the cell membrane could be observed. A figurative example of the quantitative results can be observed in Figure 7 (F). Therefore, we conclude satisfactorily that the DOX treatment did indeed increase the CALR exposure on the cell membrane.

Our next step in the characterization of immunogenic cell death was the quantification of ATP released into the culture medium post-exposure to DOX. As previously mentioned in the methods, HEK293T-pmeLUC cells were used as probes for quantification of the ATP released into the extracellular medium, since the pmeLUC construct was originally developed as a chimeric protein that targets locations on the outside of the plasma membrane facing the extracellular environment. It is thus able to measure the ATP content of the pericellular space (Pellegatti et al., 2005).

The pmeLUC construct uses firefly luciferase substrate, so once it was added to the cell culture medium collected from the 4T1 cells post-exposed to DOX we were able to quantify the ATP present in the medium. A significantly higher luminescence was observed in comparison to the medium of 4T1 cells non-exposed, as shown in Figure 1 (C). In order to ensure that the luminescence was indeed from the ATP and not a random one, the culture medium of 4T1 cells non-exposed and 4T1 cells post-exposed to DOX was also added to non-transfected cells in the presence of firefly substrate. As shown in Figure 1 (E), there is no significant difference between the non-transfected cell luminescence measurements, but a highly significant difference between the luminescence of the HEK293T cells and the HEK293T-pmeLUC cells can be observed, corroborating the notion that the 4T1 cells were undergoing immunogenic cell death.

Once we were able to show that the presence of two of the main markers of ICD were enhanced after the exposure to DOX, we proceeded to evaluate the *in vivo* efficiency of using the 4T1 cells under ICD as a therapeutic vaccine. The first step in this evaluation was to evaluate the immune cell chemotaxis in the tissues where the vaccine was administered. This is a key point for the immune response since the presence of effective antigen-presenting cells is a crucial step for the adaptive immune response. Following this question, we prepared an *in vivo* imaging experiment set up to analyze the approach of immune cells to the vaccination site. For cell tracking analysis, we used two chemiluminescent probes luminol and lucigenin, which can track and label different immune cell populations, as shown in Figure 7 (A-C).

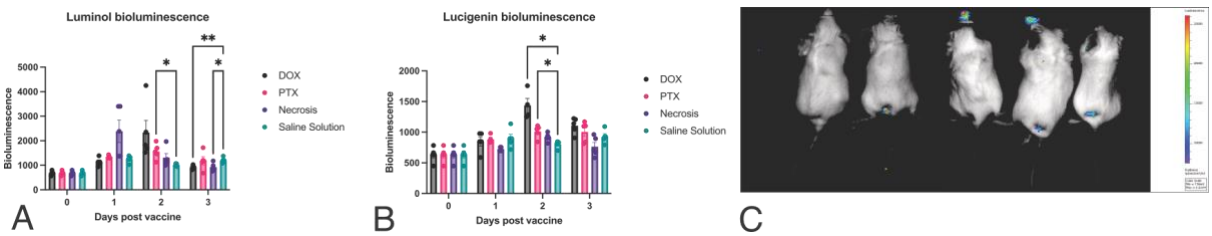

Figure 7 - **Chemotaxis in mice tissues.** Luminol and lucigenin are markers for acute and late/chronic inflammation, respectively. (A) Luminol bioluminescence, the saline solution group presented higher bioluminescence in comparison to the doxorubicin (DOX), p = 0.0057. (B) Lucigenin bioluminescence observed on the second day was significantly higher for the group vaccinated with ICD induced by DOX,

p=0.0223, in comparison to the saline solution group. (C) Graphic representation of chemotaxis happening.

Regarding these two probes, luminol and lucigenin can be used for the imaging of tissue inflammation *in vivo*. While luminol can mark acute inflammation through myeloperoxidase, bioluminescence derived from lucigenin can be closely correlated to late-phase and chronic inflammation (Tseng & Kung, 2012). Both mechanisms rely on different substrates; therefore, they can be specifically imaged. In our study, both bioluminescence from lucigenin and luminol were evaluated up to three days post-vaccine.

A basal line was drawn on day zero before the vaccination, and the readings were performed for three days after that. Another unexpected situation was that both 4T1 exposed to the necrosis protocol and 4T1 cells undergoing ICD induced by doxorubicin presented significantly less bioluminescence than that observed in the phosphate-buffered saline solution group.

As luminol bioluminescence indicates an acute inflammation, it is possible that on the third day post-vaccine, the acute inflammation in the site was already over. In contrast, the lucigenin bioluminescence observed on the second day was significantly higher for the group vaccinated with ICD induced by doxorubicin. However, as observed in the luminol analysis, this difference was not sustained on the third day of evaluation.

These scenarios can be explained by different theories; for instance, regarding acute inflammation, it is natural that neutrophils and immune defense primary cells remain at the site to promote healing, which can be observed in the maintenance of the bioluminescence in the area. However, once the APC have done their recognition duty, they migrate to promote the maturation of the adaptive immune system in different sites, mostly in regional lymph nodes, which would explain the decrease in luminescence in the vaccination region. Even though the expression of bioluminescence varies over the days, the luminol and lucigenin experiment shows

that the vaccination with the 4T1 cells undergoing ICD promotes the recruitment of immune cells to the site.

Following the chemotaxis analysis provided by the luminol and lucigenin bioluminescence, we aimed to investigate whether the vaccination with the 4T1 cells undergoing immunogenic cell death would be able either to prevent or control primary tumor progression. As previously published, 4T1 cells spontaneously form primary tumors and, after 21 days, metastases can be found in distant organs such as lungs, brains, and bones. In our study, as shown in Figure 8 (A), the group which received the 4T1 cells undergoing ICD by DOX did not present a primary tumor during the 42 days of analysis, in contrast with what was observed among the other groups. In the necrosis and positive control groups, all the mice presented tumors. In the groups in which a primary tumor was present, there was no significant difference in their volume in any of the weeks of measurement, Figure 8 (B). As the mice vaccinated with 4T1 undergoing ICD induced by DOX did not present tumors, they are represented symbolically in Figure 8 (B) for effects of comparison. As observed in Figure 8 (C), there was no statistical difference between the mice's weight in different groups, indicating that the vaccination did not affect their food uptake.

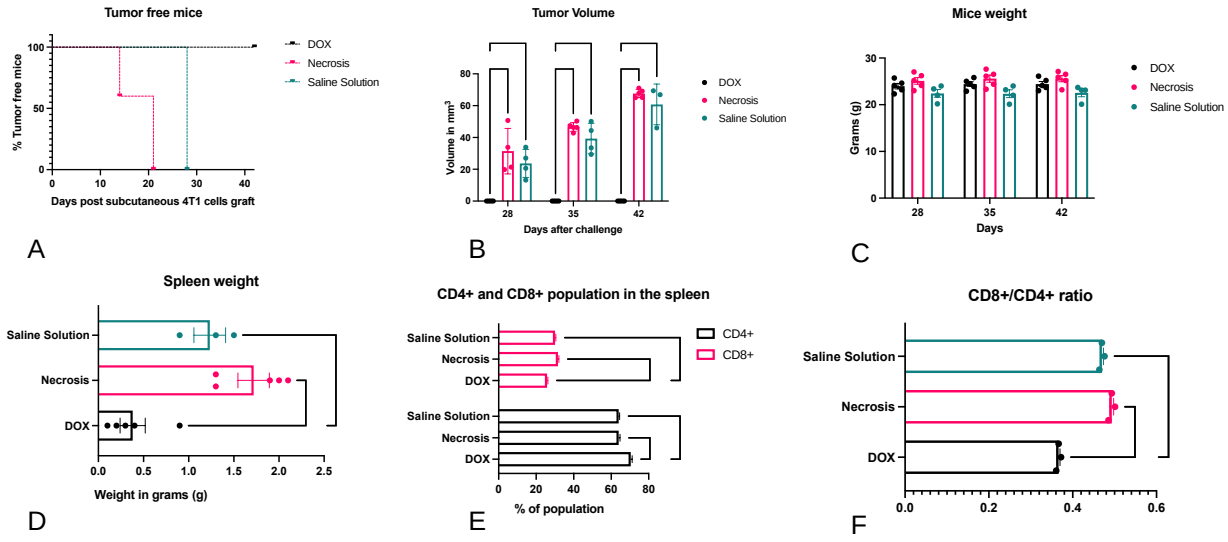

Figure 8 - **Vaccine efficiency evaluation.** Percentage of tumor-free mice represented as a survival curve. No primary tumor could be observed in the mice vaccinated with 4T1 cells undergoing ICD by DOX. (B) Tumor volume in the last three weeks of follow up. As the DOX group did not present any tumor, they are showed in the graph for effects of comparison. No statistical difference could be observed between the control groups. (C) No difference was observed in the mice weight following the weeks. (D) A significant difference in the spleen weight of the mice can be observed between the mice vaccinated with DOX, p= 0.0156. (E) A significantly higher percentage of the CD8+ population could be found in saline solution group in comparison with DOX, p= 0.0127. However, the CD4+ population was the opposite. DOX vaccinated mice presented higher percentage of CD4+ population, p= 0.0005. Finally, (F) a significantly smaller ratio of CD8+ population in comparison to the CD4+ population between the saline solution and vaccinated was found, p= 0.0084.

These results corroborate other reports from our group and the literature. The ability of doxorubicin to induce ICD has been described in other papers, but the complete growth inhibition of this aggressive tumor type is not common in the literature. As an aggressive and metastatic stage IV breast cancer tumor, the 4T1 cells can grow and spread very fast. Moreover, the 4T1 tumor model is also characterized by other pathophysiological issues, mainly related to disrupted hematopoiesis that affects different organs, including the liver, bone marrow, and spleen (de Lima et al., 2020; dos Santos Câmara et al., 2017; Longo et al., 2016). This aberrant hematopoiesis is triggered by the 4T1 cells that produce excessive growth factors, such as GM-CSF, which stimulate the differentiation of myeloid-derived cells. The key point of this process is that due to an excessive growth factor stimulus, these myeloid cells are produced in an immature form, with an immunosuppressive phenotype.

Therefore, the 4T1 cells induce the production of these aberrant lymphocytes that will protect the tumor cells, rather than inhibit them. This is quite an interesting paradox and a key point for cancer immunology. Thus, the next step in the vaccine investigation was to assess the spleen lymphocyte phenotype after vaccination, and after vaccination and tumor challenge. After the 42-day post-challenge follow-up time, the mice were euthanized, and their spleen was collected. A pool of the spleens of each group was made and the pool was stained to analyze the CD3+ (CD4+ and CD8+) population. It is important to note that a significantly higher difference between the spleen's weight in the necrosis and saline group compared to the ones in both vaccinated groups was observed, as shown in Figure 8 (D). As described in the literature, the enlarged spleen can be indicative of complications and the presence of long-distant metastasis in patients (Lees et al., 2020). And, for 4T1 tumors, splenomegaly is also related to the hyperproduction of myeloid-derived suppressor cells (dos Santos Câmara et al., 2017; Longo et al., 2016).

From the flow cytometry analysis, it was shown that a significantly higher percentage of the CD8+ population could be found in the necrosis and saline solution group. It must be taken into consideration that this population was collected from the spleen, while the bone marrow is a preferred site for the proliferation and maintenance

of CD8+ cells (Parretta et al., 2005, p. 8). Meanwhile, the CD4+ population was significantly higher in the pool from the mice vaccinated with the 4T1 undergoing ICD induced by DOX when in comparison to the necrosis and saline solution groups. This result is interesting because the CD4+ population can orchestrate a broad range of immune responses and, unlike the CD8+ population, CD4+ cells can recognize and bind to MHC class II expressed by the tumors, presenting a cytotoxic effect, which can be observed in the primary tumor control and progression observed in these groups (Kim & Cantor, 2014). Furthermore, CD4+ cells have been identified as possessing cytotoxic functions and can kill cancer cells (Oh & Fong, 2021). It is also important to bear in mind that even though a smaller ratio of CD8+/CD4+ was found, Figure 9 (F), it does not necessarily mean an impairment in the immune response against the tumor, as the population of specific anti-tumor T helper lymphocytes (CD4+) may have expanded, and further investigation of cell phenotype would be necessary. Interestingly, a similar splenocyte phenotype was detected in mice just vaccinated, not submitted to tumor challenge. As shown in Figure S1, the results for just vaccinated and vaccinated and submitted to challenge were quite similar. These data indicate that, at least for the spleen, the phenotypic population selected during vaccination was kept constant, even after tumor challenge. Beyond this phenotype discussion, by far the most significant result observed in our experiments was the reduction of distant lung metastasis in vaccinated mice. The spreading of cancerous cells from the primary site to long-distance tissues is the main complication associated with neoplastic diseases (Loibl et al., 2021), and the 4T1 model spontaneously metastasizes to the lungs, so we aimed to analyze whether the vaccination with the 4T1 cells undergoing ICD was efficient in preventing the colonization of the lung tissue by metastatic cells. For this purpose, we analyzed lung microtomography to assess the presence of metastatic foci in vaccinated and control animals.

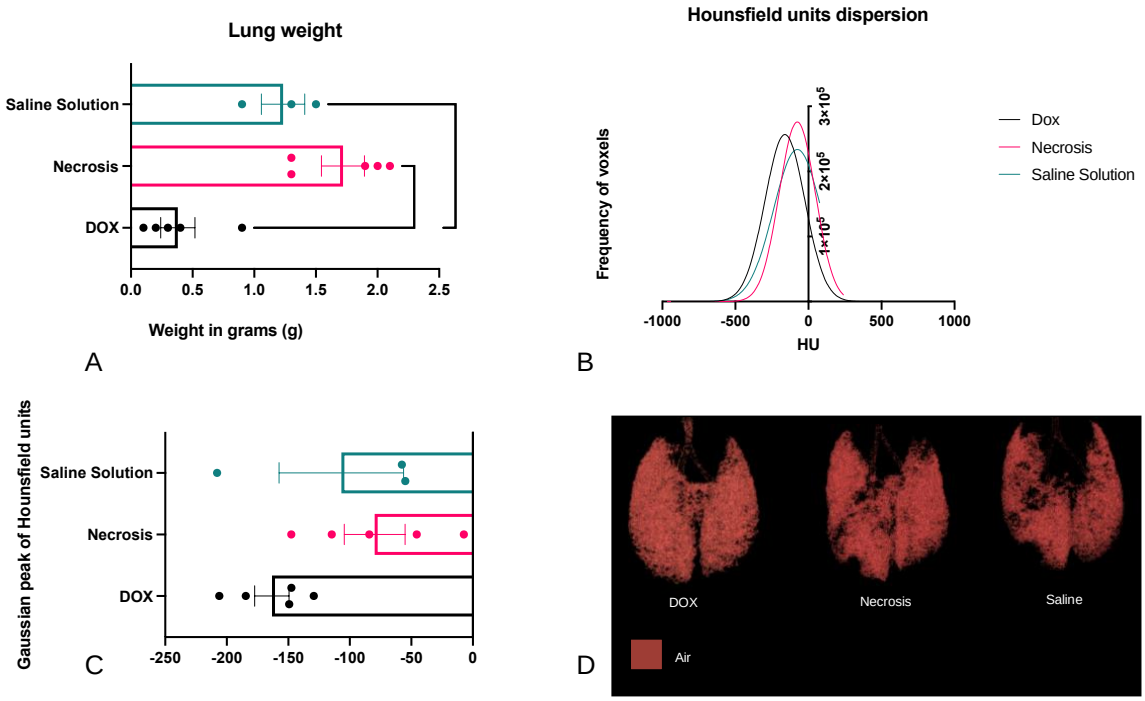

Figure 9 - **Lung metastasis evaluation.** – (A) A significant difference in lung weight can be observed between the vaccinated and non-vaccinated mice groups. DOX presented the smaller weight among the groups. This significant difference could be observed in the Hounsfield unit dispersion (B), where the vaccinated units are dislocated to the left, even though no significant difference could be observed in their Gaussian peak analysis (C). (E) Qualitative representations of the lungs through a three-dimensional reconstruction of the lung tomography. Air is represented in red, while the black parts in the lungs are dense masses.

The non-vaccinated mice groups presented a significantly higher lung weight. Moreover, the same lung microtomography showed some important density alterations that can be observed in Figures 10 (B) and (D). These two figures represent the quantitative and qualitative analysis of lung tomography, respectively. For the qualitative analysis, we can observe that the vaccinated mice had the alveolar space preserved, with a continuous aerial space within the lung structure. On the other hand, the control animals presented several radio-dense spots, which can be identified as discontinuous aerial spaces in the lung's structures. This first analysis indicates that the vaccinated mice had preservation of lung structure, with fewer metastatic foci in their inner compartments.

Moreover, in addition to these qualitative analyses, we also quantified the average lung density, by producing an individual voxel quantification. For this analysis, each voxel, which represents a volumetric pixel in a tri-dimensional image, is quantified in

the Hounsfield units (HU), which define the radio density of each of these voxels. The HU scale varies from -1000 (less dense/air) to +1000 (denser/metals) (Schreiber et al., 2011). Thus, the Gaussian dispersion to the left indicates that the lungs are less dense, suggesting that they are healthier. On the other side, the displacement to the right indicates the presence of denser voxels, which are related to the presence of metastatic foci.

In Figure 9 (B), it can be observed that the curves of lung tomographies of mice from the vaccinated group is dislocated to the left on the x-axis, indicating a higher number of negative values when in comparison with the necrosis and saline solution, meaning a lighter tissue density. To clarify this graph, we produced a column graph with the peak of each animal dispersion. In Figure 9 (C), it is possible to observe that the vaccinated mice have peaks with lower values, corroborating the notion that these lungs are healthier. In our statistical analyses, no significant difference could be observed in the peak of the Gaussian curves derived from the dispersion of the voxels on the Hounsfield unit. However, a trend can be observed: the lighter the mice's lungs, the smaller their Hounsfield unit peak, which is expected and corroborates previous results.

Following the encouraging results of the vaccination protocol presented by the groups vaccinated with 4T1 cells undergoing ICD induced by DOX on BALB/c mice, we decided to test the performance of the spleen lymphocytes collected from these specific groups in BALB/c nude mice, which are inbred spontaneous mutant immunodeficient mice, lacking T-cells. The idea was to transfer the “vaccinated” lymphocyte population to the nude mice and evaluate the immune response against tumor challenges.

As observed in Figure 9 (E), the CD4+ population collected from the spleen in the BALB/c mice was higher than 60% in some groups, while the CD8+ population reached more than 30%. Once the spleens from the BALB/c mice were collected and prepared for flow cytometry, the remaining cells were cultivated in a lymphocyte enrichment medium, aiming to encourage the proliferation of these lymphocyte populations. After 24 hours of culture in the enriched medium, the lymphocytes were transplanted

intraperitoneally into the BALB/c nude mice. The challenge with the 4T1 cells was performed 24 hours later.

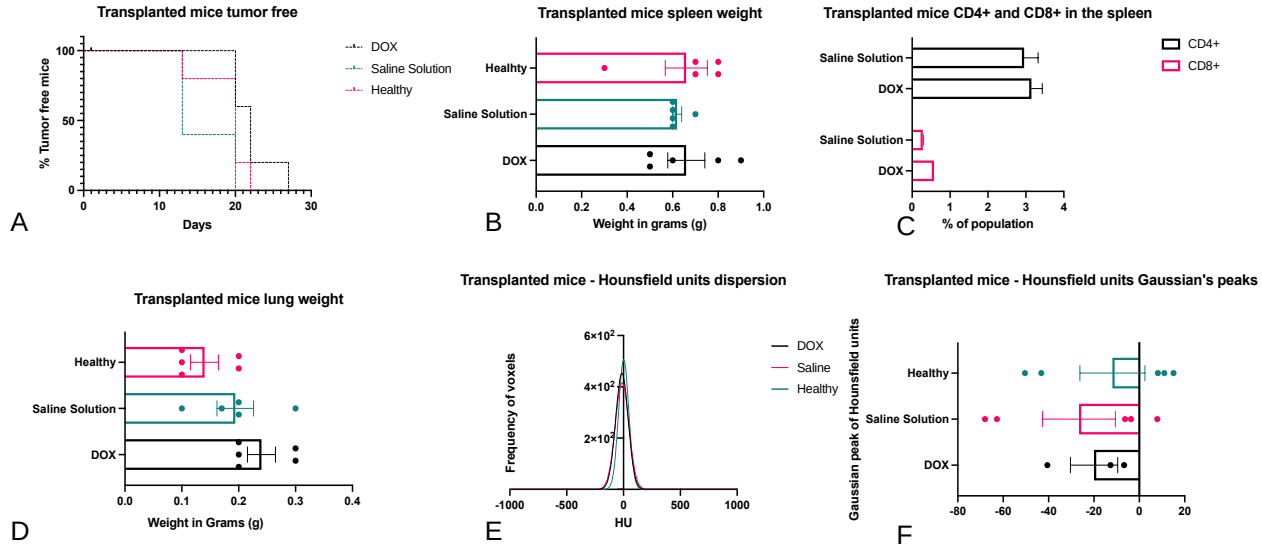

Figura 10 - **Transplanted mice evaluation**. (A) Percentage of tumor-free mice represented as a survival curve. A difference can be observed in the appearance curve, in which mice who received the lymphocytes from the vaccinated group performed significantly better, where the Long-rank test presented a p= 0.0185. No difference could be observed among the populations of CD4+ and CD8+ in vaccinated group or saline solution group (C), or in any of the other evaluated variables.

The result to be highlighted in the nude mice is the appearance of the tumors, Figure 11 (A). Even though all the mice presented primary tumors in 30 days, a difference can be observed in the appearance curve, in which mice who received the lymphocytes from the vaccinated DOX group performed significantly better. This corroborates what was previously observed in the BALB/c vaccinated mice. However, the other results were not as encouraging as before. No significant difference could be observed in the spleen or lung weights of the nude mice, as presented in Figures 4 (B) and (C). What is interesting is that at the moment of euthanasia, the population of CD4+ and CD8+ cells were still found in the nude mice (C). This result corroborates the notion that the CD4+ population effect is not only restricted to being the

orchestrator of the immune response but the immune cytotoxic response itself could be a real explanation for the immune response observed.

Moreover, we also evaluated lung metastasis development in these nude mice. Following the metastasis analysis, the same evaluation performed on BALB/c mice was also done on nude mice, but no significant difference between the groups could be observed regarding the tomographies and correlations analyzed. This is not necessarily a bad outcome, since the three-dimensional reconstruction of the nude lungs presented a healthy pattern in all the groups analyzed. However, their Hounsfield unit dispersion was more dislocated to the right than the one observed in the BALB/c, Figure 9 (E). This transplant protocol has some limitations, with the site of lymphocyte donation being the main one. A collection directly from the bone marrow could be more efficient in transplanting the CD8+ population to the nude mice, which could significantly alter the immune response observed in these mice.

6. CONCLUSION

In the near future, the use of vaccines for cancer therapy will be a reality for oncology approaches. As presented in our results, it was possible to develop an effective therapeutic vaccine to impair both tumor and distant metastasis growth in aggressive stage IV pre-clinical 4T1 tumor-bearing mice. By inducing immunogenic cell death in these tumor cells (4T1 Breast Cancer Cells), after exposure to doxorubicin, it was possible to prepare a laboratory vaccine. This ICD vaccine was able to promote an immunological response in the mice after three applications, and this protocol impaired the progression of this aggressive tumor model. Moreover, we also showed that the systemic lymphocyte was impacted by the vaccination, and the transfer of these cells to nude mice improved the immune response against the same tumor cells in these immunodeficient mice. Bringing these results together, they indicate good prospects for real clinical preventive cancer vaccines.

BIBLIOGRAPHY

Ahmed, A., & Tait, S. W. G. (2020). Targeting immunogenic cell death in cancer. *Molecular Oncology*, *14*(12), 2994–3006. https://doi.org/10.1002/1878-0261.12851

Bezu, L., Gomes-de-Silva, L. C., Dewitte, H., Breckpot, K., Fucikova, J., Spisek, R., Galluzzi, L., Kepp, O., & Kroemer, G. (2015). Combinatorial Strategies for the Induction of Immunogenic Cell Death. *Frontiers in Immunology*, *6*. https://doi.org/10.3389/fimmu.2015.00187

Borst, J., Ahrends, T., Bąbała, N., Melief, C. J. M., & Kastenmüller, W. (2018). CD4+ T cell help in cancer immunology and immunotherapy. *Nature Reviews Immunology*, *18*(10), 635–647. https://doi.org/10.1038/s41577-018-0044-0

Brignone, C., Gutierrez, M., Mefti, F., Brain, E., Jarcau, R., Cvitkovic, F., Bousetta, N., Medioni, J., Gligorov, J., Grygar, C., Marcu, M., & Triebel, F. (2010). First-line chemoimmunotherapy in metastatic breast carcinoma: Combination of paclitaxel and IMP321 (LAG-3Ig) enhances immune responses and antitumor activity. *Journal of Translational Medicine*, *8*(1), 71. https://doi.org/10.1186/1479-5876-8-71

Cao, X. (2016). Self-regulation and cross-regulation of pattern-recognition receptor signalling in health and disease. *Nature Reviews Immunology*, *16*(1), 35–50. https://doi.org/10.1038/nri.2015.8

Casares, N., Pequignot, M. O., Tesniere, A., Ghiringhelli, F., Roux, S., Chaput, N., Schmitt, E., Hamai, A., Hervas-Stubbs, S., Obeid, M., Coutant, F., Métivier, D., Pichard, E., Aucouturier, P., Pierron, G., Garrido, C., Zitvogel, L., & Kroemer, G. (2005). Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *Journal of Experimental Medicine*, *202*(12), 1691–1701. https://doi.org/10.1084/jem.20050915

Cooper, M. D., & Alder, M. N. (2006). The Evolution of Adaptive Immune Systems. *Cell*, *124*(4), 815–822. https://doi.org/10.1016/j.cell.2006.02.001

de Lima, L. I., Faria, R. S., Franco, M. S., Roque, M. C., Arruda Pacheco, T. J., Rodrigues, M. C., Muehlmann, L. A., Moya, S. E., Azevedo, R. B., de Oliveira, M. C., & Figueiró Longo, J. P. (2020). Combined paclitaxel-doxorubicin liposomal results in positive prognosis with infiltrating lymphocytes in lung metastasis. *Nanomedicine*, *15*(28), 2753–2770. https://doi.org/10.2217/nnm-2020-0201

dos Santos Câmara, A. L., Nagel, G., Tschiche, H. R., Cardador, C. M., Muehlmann, L. A., de Oliveira, D. M., Alvim, P. Q., Azevedo, R. B., Calderón, M., & Figueiró Longo, J. P. (2017). Acid-sensitive lipidated doxorubicin prodrug entrapped in nanoemulsion impairs lung tumor metastasis in a breast cancer model. *Nanomedicine*, *12*(15), 1751–1765. https://doi.org/10.2217/nnm-2017-0091

Dou, L., Chen, Y.-F., Cowan, P. J., & Chen, X.-P. (2018). Extracellular ATP signaling and clinical relevance. *Clinical Immunology*, *188*, 67–73. https://doi.org/10.1016/j.clim.2017.12.006

Dumitriu, I. E., Baruah, P., Manfredi, A. A., Bianchi, M. E., & Rovere-Querini, P. (2005). HMGB1: Guiding immunity from within. *Trends in Immunology*, *26*(7), 381–387. https://doi.org/10.1016/j.it.2005.04.009

Fuchs, Y., & Steller, H. (2015). Live to die another way: Modes of programmed cell death and the signals emanating from dying cells. *Nature Reviews Molecular Cell Biology*, *16*(6), 329–344. https://doi.org/10.1038/nrm3999

Fucikova, J., Kralikova, P., Fialova, A., Brtnicky, T., Rob, L., Bartunkova, J., & Špíšek, R. (2011). Human Tumor Cells Killed by Anthracyclines Induce a Tumor-Specific Immune Response. *Cancer Research*, *71*(14), 4821–4833. https://doi.org/10.1158/0008-5472.CAN-11-0950

Galluzzi, L., Bravo-San Pedro, J. M., Vitale, I., Aaronson, S. A., Abrams, J. M., Adam, D., Alnemri, E. S., Altucci, L., Andrews, D., Annicchiarico-Petruzzelli, M., Baehrecke, E. H., Bazan, N. G.,

Bertrand, M. J., Bianchi, K., Blagosklonny, M. V., Blomgren, K., Borner, C., Bredesen, D. E., Brenner, C., … Kroemer, G. (2015). Essential versus accessory aspects of cell death: Recommendations of the NCCD 2015. *Cell Death & Differentiation*, *22*(1), 58–73. https://doi.org/10.1038/cdd.2014.137

Galluzzi, L., Buqué, A., Kepp, O., Zitvogel, L., & Kroemer, G. (2017). Immunogenic cell death in cancer and infectious disease. *Nature Reviews Immunology*, *17*(2), 97–111. https://doi.org/10.1038/nri.2016.107

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of Cancer: The Next Generation. *Cell*, *144*(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Kepp, O., Senovilla, L., Vitale, I., Vacchelli, E., Adjemian, S., Agostinis, P., Apetoh, L., Aranda, F., Barnaba, V., Bloy, N., Bracci, L., Breckpot, K., Brough, D., Buqué, A., Castro, M. G., Cirone, M., Colombo, M. I., Cremer, I., Demaria, S., … Galluzzi, L. (2014). Consensus guidelines for the detection of immunogenic cell death. *OncoImmunology*, *3*(9), e955691. https://doi.org/10.4161/21624011.2014.955691

Khakh, B. S., & Burnstock, G. (2009). The Double Life of ATP. *Scientific American*, *301*(6), 84–92. https://doi.org/10.1038/scientificamerican1209-84

Kim, H.-J., & Cantor, H. (2014). CD4 T-cell Subsets and Tumor Immunity: The Helpful and the Not-so-Helpful. *Cancer Immunology Research*, *2*(2), 91–98. https://doi.org/10.1158/2326-6066.CIR-13-0216

Klune, J. R., Dhupar, R., Cardinal, J., Billiar, T. R., & Tsung, A. (2008). HMGB1: Endogenous Danger Signaling. *Molecular Medicine*, *14*(7–8), 476–484. https://doi.org/10.2119/2008-00034.Klune

Kroemer, G., Galluzzi, L., Kepp, O., & Zitvogel, L. (2013). Immunogenic Cell Death in Cancer Therapy. *Annual Review of Immunology*, *31*(1), 51–72. https://doi.org/10.1146/annurev-immunol-032712-100008

Lees, J. G., White, D., Keating, B. A., Barkl-Luke, M. E., Makker, P. G. S., Goldstein, D., & Moalem-Taylor, G. (2020). Oxaliplatin-induced haematological toxicity and splenomegaly in mice. *PLOS ONE*, *15*(9), e0238164. https://doi.org/10.1371/journal.pone.0238164

Liu, P., Zhao, L., Kepp, O., & Kroemer, G. (2020). Quantitation of calreticulin exposure associated with immunogenic cell death. In *Methods in Enzymology* (Vol. 632, pp. 1–13). Elsevier. https://doi.org/10.1016/bs.mie.2019.05.011

Loibl, S., Poortmans, P., Morrow, M., Denkert, C., & Curigliano, G. (2021). Breast cancer. *The Lancet*, *397*(10286), 1750–1769. https://doi.org/10.1016/S0140-6736(20)32381-3

Longo, J. P. F., Muehlmann, L. A., Miranda-Vilela, A. L., Portilho, F. A., de Souza, L. R., Silva, J. R., Lacava, Z. G. M., Bocca, A. L., Chaves, S. B., & Azevedo, R. B. (2016). Prevention of Distant Lung Metastasis After Photodynamic Therapy Application in a Breast Cancer Tumor Model. *Journal of Biomedical Nanotechnology*, *12*(4), 689–699. https://doi.org/10.1166/jbn.2016.2208

Lu, Y. (2020). The Role of Cyclin-Dependent Kinases on the Metastasis of Breast Cancer. *Novel Approaches in Cancer Study*, *4*(4). https://doi.org/10.31031/NACS.2020.04.000594

Michalak, M., Corbett, E. F., Mesaeli, N., Nakamura, K., & Opas, M. (1999). *Calreticulin: One protein, one gene, many functions*. 12.

Netea, M. G., Schlitzer, A., Placek, K., Joosten, L. A. B., & Schultze, J. L. (2019). Innate and Adaptive Immune Memory: An Evolutionary Continuum in the Host's Response to Pathogens. *Cell Host & Microbe*, *25*(1), 13–26. https://doi.org/10.1016/j.chom.2018.12.006

Obeid, M., Panaretakis, T., Tesniere, A., Joza, N., Tufi, R., Apetoh, L., Ghiringhelli, F., Zitvogel, L., & Kroemer, G. (2007). Leveraging the Immune System during Chemotherapy: Moving Calreticulin to the Cell Surface Converts Apoptotic Death from "Silent" to Immunogenic. *Cancer Research*, *67*(17), 7941–7944. https://doi.org/10.1158/0008-5472.CAN-07-1622

Obeid, M., Tesniere, A., Ghiringhelli, F., Fimia, G. M., Apetoh, L., Perfettini, J.-L., Castedo, M., Mignot, G., Panaretakis, T., Casares, N., Métivier, D., Larochette, N., van Endert, P., Ciccosanti, F., Piacentini, M., Zitvogel, L., & Kroemer, G. (2007). Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nature Medicine*, *13*(1), 54–61. https://doi.org/10.1038/nm1523

Oh, D. Y., & Fong, L. (2021). Cytotoxic CD4+ T cells in cancer: Expanding the immune effector toolbox. *Immunity*, *54*(12), 2701–2711. https://doi.org/10.1016/j.immuni.2021.11.015

Parretta, E., Cassese, G., Barba, P., Santoni, A., Guardiola, J., & Di Rosa, F. (2005). CD8 Cell Division Maintaining Cytotoxic Memory Occurs Predominantly in the Bone Marrow. *The Journal of Immunology*, *174*(12), 7654–7664. https://doi.org/10.4049/jimmunol.174.12.7654

Pasparakis, M., & Vandenabeele, P. (2015). Necroptosis and its role in inflammation. *Nature*, *517*(7534), 311–320. https://doi.org/10.1038/nature14191

Pellegatti, P., Falzoni, S., Pinton, P., Rizzuto, R., & Di Virgilio, F. (2005). A Novel Recombinant Plasma Membrane-targeted Luciferase Reveals a New Pathway for ATP Secretion. *Molecular Biology of the Cell*, *16*(8), 3659–3665. https://doi.org/10.1091/mbc.e05-03-0222

Pol, J., Vacchelli, E., Aranda, F., Castoldi, F., Eggermont, A., Cremer, I., Sautès-Fridman, C., Fucikova, J., Galon, J., Spisek, R., Tartour, E., Zitvogel, L., Kroemer, G., & Galluzzi, L. (2015). Trial Watch: Immunogenic cell death inducers for anticancer chemotherapy. *OncoImmunology*, *4*(4), e1008866. https://doi.org/10.1080/2162402X.2015.1008866

Reina-Campos, M., Scharping, N. E., & Goldrath, A. W. (2021). CD8+ T cell metabolism in infection and cancer. *Nature Reviews Immunology*, *21*(11), 718–738. https://doi.org/10.1038/s41577-021-00537-8

Rodrigues, M. C., de Sousa Júnior, W. T., Mundim, T., Vale, C. L. C., de Oliveira, J. V., Ganassin, R., Pacheco, T. J. A., Vasconcelos Morais, J. A., Longo, J. P. F., Azevedo, R. B., & Muehlmann, L. A. (2022). Induction of Immunogenic Cell Death by Photodynamic Therapy Mediated by Aluminum-Phthalocyanine in Nanoemulsion. *Pharmaceutics*, *14*(1), 196. https://doi.org/10.3390/pharmaceutics14010196

Rodrigues, M. C., Vieira, L. G., Horst, F. H., de Araújo, E. C., Ganassin, R., Merker, C., Meyer, T., Böttner, J., Venus, T., Longo, J. P. F., Chaves, S. B., Garcia, M. P., Estrela-Lopis, I., Azevedo, R. B., & Muehlmann, L. A. (2020). Photodynamic therapy mediated by aluminium-phthalocyanine nanoemulsion eliminates primary tumors and pulmonary metastases in a murine 4T1 breast adenocarcinoma model. *Journal of Photochemistry and Photobiology B: Biology*, *204*, 111808. https://doi.org/10.1016/j.jphotobiol.2020.111808

Schreiber, J. J., Anderson, P. A., Rosas, H. G., Buchholz, A. L., & Au, A. G. (2011). Hounsfield Units for Assessing Bone Mineral Density and Strength: A Tool for Osteoporosis Management. *Journal of Bone and Joint Surgery*, *93*(11), 1057–1063. https://doi.org/10.2106/JBJS.J.00160

Steeg, P. S. (2016). Targeting metastasis. *Nature Reviews Cancer*, *16*(4), 201–218. https://doi.org/10.1038/nrc.2016.25

Tseng, J.-C., & Kung, A. L. (2012). In Vivo Imaging of Inflammatory Phagocytes. *Chemistry & Biology*, *19*(9), 1199–1209. https://doi.org/10.1016/j.chembiol.2012.08.007

Tsimberidou, A. M., Fountzilas, E., Nikanjam, M., & Kurzrock, R. (2020). Review of precision cancer medicine: Evolution of the treatment paradigm. *Cancer Treatment Reviews*, *86*, 102019. https://doi.org/10.1016/j.ctrv.2020.102019

Turner, S. J., Bennett, T. J., & La Gruta, N. L. (2021). CD8 $^{+}$ T-Cell Memory: The Why, the When, and the How. *Cold Spring Harbor Perspectives in Biology*, *13*(5), a038661. https://doi.org/10.1101/cshperspect.a038661

Vacchelli, E., Aranda, F., Eggermont, A., Galon, J., Sautès-Fridman, C., Cremer, I., Zitvogel, L., Kroemer, G., & Galluzzi, L. (2014). Trial Watch: Chemotherapy with immunogenic cell death inducers. *OncoImmunology*, *3*(3), e27878. https://doi.org/10.4161/onci.27878

van Kempen, T. S., Wenink, M. H., Leijten, E. F. A., Radstake, T. R. D. J., & Boes, M. (2015). Perception of self: Distinguishing autoimmunity from autoinflammation. *Nature Reviews Rheumatology*, *11*(8), 483–492. https://doi.org/10.1038/nrrheum.2015.60

Yang, H. (2005). The cytokine activity of HMGB1. *Journal of Leukocyte Biology*, *78*(1), 1–8. https://doi.org/10.1189/jlb.1104648

Zhu, J., Yamane, H., & Paul, W. E. (2010). Differentiation of Effector CD4 T Cell Populations. *Annual Review of Immunology*, *28*(1), 445–489. https://doi.org/10.1146/annurev-immunol-030409-101212

Universidade de Brasília
Comissão de Ética no Uso Animal

Brasília, 19 de outubro de 2020.

DECLARAÇÃO

Declaramos que o projeto intitulado **"AVALIAÇÃO DA EFICÁCIA DE UMA QUIMIOIMUNOTERAPIA EM CONTROLAR A PROGRESSÃO TUMORAL EM UM MODELO DE CÂNCER MAMÁRIO MURINO ",** Protocolo n.º 026/2020, sob responsabilidade do Professor Dr. João Paulo Figueiró Longo, 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 (105 fêmeas). A presente declaração é válida pelo período de: 19/10/2020 a 19/10/2021.

Dr. José Luiz Jivago de Paula Rôlo

Coordenador da CEUA – UnB

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