UNIVERSIDADE DE SÃO PAULO
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**Nanoengineered cell membrane-based nanoparticles for tumor and immunocompetent
cells modulation.**

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EDSON JOSÉ COMPARETTI

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"You have no obligation under the sun other than to discover your real needs, to fulfill them, and to rejoice in doing so".

François Rabelais.

ABSTRACT

COMPARETTI, E. J. **Nanoengineered cell membrane-based nanoparticles for tumor and immunocompetent cells modulation.** 2021. 141 p. Tese (Doutorado em Ciências) – Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, 2021.

In 2020, cancer caused 10 millions of deaths and 17 million new diagnoses have been registered worldwide. Human hepatocellular carcinoma, pancreatic carcinoma, and prostate carcinoma were the third, seventh, and eighth neoplasms, respectively, with the highest casualties, with the frequency of relapses and metastasis requiring the development of new therapeutic procedures. Amid scientific and technological innovation that contribute to patient survival, nanomedicine and immunotherapy are responsible for the most satisfactory clinical results, creating new therapies currently applied to chronic diseases. Although neoplastic cells have the ability to evade immune surveillance and to modulate tumor microenvironment, nanocomposites are capable to prevent expression of immunosuppressive proteins and to increase cellular immunity. Recent advances in nanoengineering use the main components from cell membrane to create nanovesicles to deliver a large amount of antigenic material to antigen-presenting cells (APC). In the blood, phagocytic cells impair particles deposition in target sites, activating inflammatory response to modulate tumor microenvironment. Instead of targeting tumor mass, nanostructures are expected to re-educate the immune system and to increase the major mechanisms in circulatory system and peripheral lymphoid organs, helping to repair leukocytes activity lost after exposure to suppressive stimulus. In this thesis we synthesized nanoparticles with lipids and proteins from plasma membrane of hepatic neoplastic cells and pancreatic cancer cells (MNPs), aiming at developing nanocarriers to transport antineoplastic and immunomodulatory agents. We combined MNPs with first-line drugs used in clinical treatment (gemcitabine (GEM), paclitaxel (PTX), cabazitaxel (CTX)) to produce a cytotoxic response in cancer cells, and with an oligonucleotide sequence (siRNA) from an oncogene, to establish a pro-inflammatory response in human peripheral blood monocytes. The nanoparticles were used to deliver antineoplastic agents to cancer cells, as well as to deliver antigenic material to antigen-presenting cells for modulating immune system and promote an inflammatory response in the tumor sites. A discuss on the nanoparticles

modulatory mechanisms and their effects on cancer immunogenicity is presented, investigating the next-generation of nanomaterials developed to decrease the neoplastic cells ability of evading surveillance system.

Keywords: Nanomedicine. Vaccine. Cancer. Immune system.

RESUMO

COMPARETTI, E. J. **Nanopartículas engenheiradas a base de membrana celular para aplicação na modulação de células tumorais e imunocompetentes**. 2021. 141 p. Tese (Doutorado em Ciências) – Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, 2021.

Em 2020, as neoplasias causaram 10 milhões de mortes e 17 milhões de novos diagnósticos foram registrados em todo o mundo. Nesse cenário, o carcinoma hepatocelular humano, carcinoma pancreático e o carcinoma prostático foram a terceira, a sétima e a oitava neoplasias com maiores números de óbitos, respectivamente, com a frequência de recidivas e metástases exigindo o desenvolvimento de novas abordagens terapêuticas. Em meio às inovações científicas e tecnológicas que contribuem para a sobrevivência dos pacientes, a nanomedicina e a imunoterapia são responsáveis pelos resultados clínicos mais satisfatórios atualmente, quando aplicadas às doenças crônicas. Embora as células neoplásicas tenham a capacidade de escapar da vigilância imunológica e modular o microambiente tumoral, os nanocompósitos são capazes de prevenir a expressão de proteínas imunossupressoras e aumentar a imunidade celular. Um recente avanço na nanoengenharia usa os principais componentes da membrana celular para criar nanovesículas e entregar uma grande quantidade de material antigênico às células apresentadoras de antígenos (APC). No sangue, as células fagocíticas reduzem a deposição de partículas em locais-alvo, mas sua ativação pode modular o microambiente tumoral. Atualmente, espera-se que novas nanoestruturas reeduquem o sistema imunológico e aumentem os principais mecanismos do sistema circulatório e dos órgãos linfoides periféricos, ajudando a reparar a atividade dos leucócitos após a exposição a estímulos supressores. Nesse trabalho de doutoramento, sintetizamos nanopartículas com lipídios e proteínas da membrana plasmática de células neoplásicas pancreática, prostática e hepáticas (MNPs), com o objetivo de desenvolver nanocarreadores para transportar agentes antineoplásicos e imunomoduladores. Combinamos as MNPs com medicamentos de primeira linha usados na clínica (gemcitabina (GEM), paclitaxel (PTX) e cabazitaxel (CTX)) para produzir uma resposta citotóxica em células cancerosas, e uma sequência de oligonucleotídeo (siRNA) de um oncogene, para estabelecer uma resposta pró-inflamatória em monócitos do sangue periférico humano. As nanopartículas foram também

utilizadas para entregar material antigênico às células apresentadoras de antígeno, modulando o sistema imunológico e promovendo uma resposta inflamatória. Uma discussão sobre os mecanismos imunomodulatórios das nanopartículas e seus efeitos na imunogenicidade do câncer é apresentada, investigando a próxima geração de nanomateriais para diminuir a capacidade das células neoplásicas de evadir o sistema imunológico.

Palavras-chave: Nanomedicina. Vacina. Câncer. Sistema imunológico.

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ABBREVIATIONS AND ACRONYMS

7AAD -	7-Aminoactinomycin D
Alexa 488 -	Alexafluor 488
ANOVA –	Análise de variância
APC –	aloficocianina
APC-H7 -	aloficocianina-cyanine tandem 7
CTX -	cabazitaxel
DCs -	Células dendríticas
DMSO -	Dimetilsulfóxido ou sulfóxido de dimetilo
EDC -	1-Etil-3-(3-dimetilaminopropil carbodiimida
FITC -	Isotiocianato de fluoresceína
FTIR -	Espectroscopia na região do Infravermelho com transformada de Fourier
GEM -	Gemcitabina
iDC -	Células dendríticas imaturas
mDC -	Células dendríticas maduras
MEV -	Microscopia Eletrônica de Varredura
MLR -	Reação mista de leucócitos
MNPs –	Membrane nanoparticles
MTT -	3-(4,5-dimetiltiazol-2-yl)-2,5-difenil brometo de tetrazolina
NHS -	N-Hidroxisuccinimida
PBMCs -	Célula mononuclear do sangue periférico
PE -	ficoeritrina
PeCy7 -	ficoeritrina-cianine tandem 7
PEI –	Polietilenimina
PercP -	clorofila peridinina
PTX -	Paclitaxel
TEM -	Microscopia Eletrônica de Transmissão

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INTRODUCTION

Cancer caused 10 million of deaths and 17 million new cases were registered in 2020.¹ Lung cancer is the most incident and responsible for the highest casualties in men (14%), followed by prostate cancer (13%), whereas the most prevalent in women are breast and colorectal cancers, representing 24% and 9% of the cases, respectively. In children, leukemia and brain tumors are responsible for 50% of the diagnoses, with neuroblastoma, kidney, and Hodgkin lymphoma completing the ranking.²

Poor nutrition, infections, obesity, smoking, and genetic factors increase the risk to develop carcinomas.¹ In developed countries, early detection reduces the number of deaths by cancer, but neoplasms with highest rate of mortalities were registered in underdeveloped or developing territories as a result from the lack of medicines and treatment interruption.¹ Under these circumstances, innovative techniques that present a short period of therapeutic intervention are needed in underprivileged areas.

Nanotechnology is one of the areas that contribute to increase the specificity of treatments and the development of new materials applicable to human health.³ Nanomedicine aid diagnosis techniques and disease treatments, like magnetic resonance imaging, hyperthermia, and drug delivery, using concepts from chemistry, physics, and biotechnology.⁴ For example, organic and inorganic nanoparticles can be modified to “address” cytotoxic activity in diseased tissues and organs.³ For example, the deposition of cellular biomarkers (specific for each cell class) on nanoparticle’s surface allows the delivery of drugs or contrast agents to the cells of interest, in order to increase the specificity of treatments and improve diagnostic accuracy, respectively.³

In the medical and biomedical areas, nanoparticles (NPs) carry antineoplastic agents, such as proteins, nucleic acids, and drugs, delivering high concentrations of the drugs within target cells in order to minimize unwanted toxicity in healthy tissues.³⁻⁴ In the clinic, paclitaxel-loaded albumin particles (nab-paclitaxel) complement treatment with taxoids to prolong patient survival, showing high efficiency against pancreatic cancer and metastatic breast cancer.⁵⁻⁶ Studies have already demonstrated the efficacy of nab-paclitaxel together with nedaplatin (an alkylating agent) in 27 patients with advanced cervical cancer. Doses of 175 mg/m² nab-paclitaxel and 80 mg/m² nedaplatin were intravenously injected in two cycles of 21-days and produced efficacy in 50% of patients, extending the survival period up

to 20 months.⁷ In addition, nanotechnology also contributes to overcome the cell resistance to antineoplastics. For example, nanoparticles inhibit the main mechanisms of cellular pumps, keeping drugs inside cancer cells for longer periods than drug molecules alone, decreasing therapeutic resistance.⁸⁻⁹

The accumulation of proteins and particles in the tumor microenvironment occur by passive and active mechanisms, as the so-called Permeability and Retention Effect (EPR), and with the use of targeting molecules on nanocompound's surface, respectively.¹⁰⁻¹¹ However, NPs are also retained in the lung, liver, kidney, and spleen, ensuring that a small fraction of *ca.* 1% reaches the disease sites when administered intravenously, with most of them producing cytotoxic side effects in highly vascularized tissues, making clinical application unfeasible.¹² Despite the low concentration in the tumor microenvironment, FDA has approved liposomes loaded with doxorubicin (Doxil) to treat ovarian cancer, as well as nab-paclitaxel to treat pancreatic and lung neoplasms.¹⁰

It is necessary to highlight that before reaching target tissues, nanoparticles migrate through the bloodstream, changing the phenotype of immunocompetent cells. Such modulatory mechanism can be used to trigger an anti-tumor activity of white cells.¹³ In the body, leukocytes and proteins from the complement system are the first barriers to be overcome by nanomaterials, since their size, shape, and surface functionalization influence their phagocytosis.^{12,14} While the formation of the protein corona stabilizes NPs dispersion in electrolytes and other components (eg, albumin, immunoglobulins G, and fibrinogen), immune regulatory proteins adsorbed on nanostructure activate transcription factors in human defense cells.¹⁵⁻¹⁶ The PEGylation and conjugation with biological components help to preserve nanoparticles in the circulatory system, but this strategy is not efficient to prevent inflammatory stimuli and their elimination before reaching the tumor site.¹⁷ Consequently, it is necessary novel strategies to develop versatile nanoplatforms to ensure the application of nanocomposites in cancer therapy.

A recent advance in nanoengineering uses the main components from cellular plasma membrane to camouflage the nanostructures in organisms.¹⁸ Lipids and proteins are isolated from organelles by ultracentrifugation and incorporated on particle surface by extrusion or sonication. Its biophysical properties prolong the nanostructure circulatory period in the bloodstream and the presence of adhesion proteins facilitates specific interactions with diseased tissues.¹⁸⁻¹⁹

Here, we aimed to evaluate the efficiency of nanoparticles synthesized from the main constituents of the plasma membrane of tumor cells (MNPs) to transport antineoplastic agents to human pancreatic, liver, and prostate cancer cells, and also verify their immunomodulatory capacity in antigen-presenting cells (APCs) (graphical abstract).

THESIS ORGANIZATION

In Chapter I we present an overview of the immune response triggered by nanomaterials *in vitro* and *in vivo*, including a brief description of the interactions between nanoparticles and peripheral blood leukocytes. We also describe different biomimetic systems for cancer treatment, addressing their immunomodulatory properties and their challenges in clinical use. The chapter ends with a perspective of novel nanomedical tools;

In Chapter II we describe the main components from pancreatic tumor cell membrane (PANC-1) for the synthesis of nanoparticles (MNPs) and the encapsulation of two first-line drugs used in clinical treatment, *viz.*: gemcitabine (GEM) and paclitaxel (PTX). Our results demonstrate that MNPs-GEM-PTX is very stable and induces greater cytotoxic effects on PANC-1 cells, compared to the use of pure GEM+PTX. The most interesting finding was the interaction of MNPs with monocytes and dendritic cells, increasing the expression of costimulatory molecules CD80, CD83, CD86, and HLA-DR that indicate leukocyte activation. This study was published in Materials Advances, doi.org/10.1039/D0MA00367K;

In Chapter III is presented a study aimed to isolate the main components from the plasma membrane of prostatic carcinoma cells in order to encapsulate cabazitaxel (CTX) into MNPs. After exposure to prostate cancer cells (PC-3), the cytotoxic analysis revealed low efficacy of CTX complexed to nanoparticle, compared to pure CTX. Furthermore, the uptake was analyzed by fluorescence microscopy, confirming the capture of MNPs by cancer cells. The nanoparticles did not promote a better antineoplastic response *in vitro*. Thus, further studies are required to investigate the feasibility of MNPs in targeting antineoplastic agents to cancer cells as a new strategy to improve their effectiveness.

Chapter IV brings a description on the nanoparticles synthesized with lipids and proteins from the plasma membrane of liver cancer cells (MNPs) to deliver a large amount of antigenic material to immunosuppressive macrophages (M2). To establish a pro-inflammatory response, MNPs were incorporated with monophosphoryl lipid A and siRNA to silence the c-MYC oncogene. The nanocarriers were tested for: a) nanoparticles internalization in cancer cells and immunocompetent cells, b) MNPs immunomodulatory activity through the expression of cell surface markers, and c) cytotoxicity of the nanocarriers. The adsorption of plasma proteins on the surface of nanoparticles and their influence on cell uptake was also investigated.

Graphical Abstract – Synthesis of MNP to incorporate antineoplastic agents and target their activity at tumor cells. Cells were exposed to nanoparticles for 48h and then collected for analyses phenotypic changes and verified cell viability.

Source: By the author.

CHAPTER 1 IMMUNOMODULATORY PROPERTIES OF NANOSTRUCTURED SYSTEMS FOR CANCER THERAPY

ABSTRACT

Cancer was responsible for approximately 10 million deaths in 2020, and 17 million new cases were diagnosed worldwide. Novel therapies combining nanomedicine and immunotherapy have shown important clinical results, being capable of hindering neoplastic proliferation and to reduce adverse events in the body, thereby promoting immune destruction of diseased cells. Nanostructured systems have been proven to elicit specific immune responses that may enhance anti-tumor activity. A new generation of nanomedicines, based on biomimetic and bioinspired systems, has been proposed to target tumors by providing immunomodulatory features and by enabling recovery of human immune destruction capacity against cancer cells. This chapter provides an overview on the mechanisms by which nanomedicines can be used to enhance clinical procedures using the immune modulatory responses of nanoparticles in the host defense system. We discuss the conventional and new treatment strategies providing a description of the human host defense system and the basic principles of nanoparticle interactions with monocytes, leukocytes, and dendritic cells for the modulation of antitumor immune responses. A report on different biomimetic and bioinspired systems is also presented and their particularities in cancer treatments are addressed, highlighting their immunomodulatory properties and the main challenges for future use in clinical practice.

1.1 AN OVERVIEW OF CANCER STATISTICS AND CONVENTIONAL TREATMENTS

Despite the recent advances in clinical treatments, cancer remains one of the leading causes of death globally, representing a major public health issue. Incidence and mortality data are usually available 2 to 4 years after the corresponding period, considering the time required for data acquisition, compilation, and dissemination.²⁰ In an attempt to provide an estimate of the contemporary cancer burden, GLOBOCAN reported 9.9 million deaths and 19 million new cases in 2020. These data represent underestimated numbers due to the absence of high-quality cancer registries, especially in low- and middle-income countries.¹

In adults, lung cancer is the most commonly reported malignancy. It is responsible for the highest number of casualties in men (14.3%), followed by prostate cancer (14.1%). In contrast, breast and colorectal cancers are the most prevalent types reported in women, representing 24.5% and 9.4% of the cases, respectively.¹ In children, leukemia and brain tumors are responsible for 50% of the diagnoses, with neuroblastoma, kidney, and Hodgkin lymphoma responsible for the remaining cases.² In developed countries, early detection helps in the reduction of the number of deaths. The highest rate of mortality was registered in underdeveloped or developing territories due to a lack of availability of medicines and treatment interruption.¹

Conventional treatments for cancer include different options. Surgical removal is the first recommended procedure for treating considerable and localized malignancies.^{21, 22} Since its approval over 60 years ago, standard treatment recommendations continue to be based on chemotherapy, which targets rapidly growing and dividing cells, and radiation therapy, which targets all cells within the localized tumor microenvironment (TME), both using well-established protocols.²³

As an initial or complementary treatment to surgery, conformational radiotherapy approaches, including intensity-modulated radiation therapy (IMRT) and image-guided radiation therapy (IGRT), aid the optimization of the dose delivered to the target tissues and fractionation of the total dose of radiation in therapeutic sessions to minimize DNA damage in normal cells.²⁴ However, infliction of injuries to healthy tissues and discomfort in the irradiated regions may occur as side effects of subjection to such treatments.²⁵

If surgery and radiotherapy are not recommended, patients should be administered with cytotoxic molecules (chemotherapeutics) through local and systemic administration, oral administration, or even alternative routes.²⁶⁻²⁷ Conventional drugs used in chemotherapy regimens exhibit different mechanisms of action, and most can be categorized as i) alkylating agents for the inhibition of DNA transcription;²⁸ ii) antimetabolites used as an analog of cell compounds for inducing blockade of metabolic pathways in the S phase;²⁹ iii) microtubule-targeting agents used for the destruction or stabilization of the cytoskeleton in the G0/G1 and/or G2/M phases;³⁰ iv) topoisomerase inhibitors;³¹ and v) anthracyclines.³² Owing to a lack of specificity, chemotherapy includes the occurrence of common side effects observed during drug administration, which may change according to the period of exposure, the type of drug administered, and the concentration of the therapeutic agent in the body. The

destruction of healthy cells results in the occurrence of several side effects, including dermatological, cardiac, pulmonary, neurological, hematological (leukopenia, thrombocytopenia, and anemia), gastrointestinal (nausea, vomiting, mucositis, and diarrhea), metabolic changes, allergic reactions, and anaphylaxis (Figure 1).³³

Figure 1.1 – Most common side effects of chemotherapeutics reported in patients.

Source: Adapted from JENKINS *et al.*³³

As the incidence rate and mortality of cancer have increased markedly, substantial efforts to prolong survival, to reduce local recurrence, and to minimize the side effects of conventional therapies have become increasingly dependent on modern robotic surgery, tumor adjuvant therapy, and other new technologies, among which the application of nanomedicines should be highlighted.²³

The next generation of cancer treatments is mainly based on the characterization of molecular features and the identification of a plethora of effective targets for therapy. In the present era, the hallmarks of cancer, first reported by Weinberg and Hanahan, which are used to describe and identify remarkable characteristics of malignant tumor cells, provides evidence that the acquisition of knowledge based on such biological aspects has important implications for the realization of successful cancer therapies.³⁴⁻³⁵ Among such new

strategies, the recognition of tumor cell capacity in the immune system enables the design and development of novel strategies that can effectively guarantee the success of different immunotherapeutic strategies.

1.2 HOST DEFENSE SYSTEM AND IMMUNOTHERAPY

The importance of the human immune system in conferring protection against different pathogens is well established. However, whether cancer prevention is a primary function of the host immune system remains debatable.³⁶ It is well established that the immune system establishes intricate communication with tumor cells over the entire process of disease development and progression to metastasis. This complex crosstalk established between immunity and cancer development can both result in the inhibition and enhancement of tumor growth, a phenomenon that has been outlined as an important hallmark of cancer.³⁷

Immunocompetent cells establish interaction with tumor cells in a three-phase multistage process referred to as the “3 Es”, namely elimination (also referred to as immunosurveillance), equilibrium, and escape.³⁸⁻³⁹ The concept of cancer immunosurveillance originally stated that transformed cells frequently develop, but are recognized and eliminated by the immune system before leading to the development of clinically observable diseases.⁴⁰ However, frequent cases of cancer are reported even in individuals with a highly functional and robust immune system. Hence, researchers believe that immunosurveillance is only one facet of the interaction established between immunocompetent cells with tumor cells. Indeed, tumors derived from immunocompetent hosts are less immunogenic than those isolated from immunodeficient hosts,⁴¹⁻⁴² indicating that few phenotypical features of tumor cells are acquired via the interaction established with the immune system.³⁸

The immune system not only performs the recognition of tumor cells, but also helps modulate tumor cell immunogenicity, establishing an equilibrium phase with selected “silent” variants. These selected variants are resistant to immune effectors and retain genes associated with survival, immune evasion, and the ability to escape to pre-metastatic niches.⁴³⁻⁴⁴ Importantly, such niches comprise limited or absent tissue-resident memory cells responsible for immunosurveillance.⁴⁵ Therefore, cancer cells, with the ability to invade the

basement membrane and to migrate to distant sites, detect a convenient environment for metastasis. Despite the ability of cancer cells to escape immunosurveillance and specific immune responses, several immunocompetent cells, including dendritic cells (DCs), helper and cytotoxic T lymphocytes, tumor-infiltrating macrophages, and natural killer cells, are recruited to mount immune responses against tumor cells.

DCs are the chief antigen-presenting cells (APCs) that perform continuous surveillance and recognition of the microenvironment of tissues and organs where they remain as immature cells (iDCs). iDCs can capture soluble and particulate antigens^{46, 47} through several surface receptors, such as FcγR,⁴⁷ mannose receptor (MR),⁴⁸ DC-SIGN,⁴⁹ type C lectin receptors (DEC-205),⁵⁰ as well as *Toll*-like receptors.⁵¹ These antigens are then processed and disintegrated into peptides that are subsequently presented to T lymphocytes in the context of the major histocompatibility complex (MHC),^{47,51} inducing their differentiation into Th1, Th2, Th17, or Th9 lymphocytes, which exhibit different roles in the antitumor response.⁵²

Conventional DCs are identified by the expression of CD11c, CD1a, or CD83,^{47, 51} and are subdivided into CD11c⁺ (BDCA1⁺ cells) and CD141⁺ (BDCA3⁺) subsets.⁵³⁻⁵⁴ Mature DCs express CD80, CD86, CD40, and CCR7.⁵⁵ Importantly, during maturation, the co-receptors ICOSL, TNFSF4, and TNFSF8 as well as receptors for IL-2, IL-1, IL12, and IL-18 are also found.⁵⁶ It is important to clarify that during maturation, DCs lose the ability to capture and process antigens, but demonstrate increased efficiency at presenting processed peptides to T lymphocytes, thereby triggering a specific immune response against cancer cells.^{47,51,57}

In addition to the development of target responses, while CD4⁺ lymphocytes perform the recognition of tumor antigens processed and presented by professional APCs to initiate a specific immune response, CD8⁺ lymphocytes demonstrate the ability of direct recognition of tumor antigens expressed on the tumor cell surface, targeting them for cell-mediated cytotoxicity.⁵² Therefore, CD8⁺ cytotoxic T lymphocytes (CTL) are classically considered as the main antitumor effector cells, as they recognize tumor antigens in a restricted manner and exhibit clonal expansion, with their activation and evolution into cytolytic antitumor cells, thereby improving the antitumor status.⁵⁸⁻⁵⁹

The presence of cytotoxic lymphocytes at the tumor site has been associated with good clinical outcomes during therapy.⁶⁰ Theoretically, an efficient antitumor response driven by the immune system is related to the release of pro-inflammatory cytokines, both by the

APCs and the helper T cells generated by the stimulation of CD4⁺ lymphocytes. However, the ability of the human host immune system to resist or eradicate the formation and progression of incipient neoplasias, late-stage tumors, and metastasis remains an unresolved issue. Scientific evidence suggests that the immune system may perform functions as a significant barrier to tumor formation and progression, at least in certain types of cancer.³⁴ In fact, cancer cells that may evade immune destruction by disabling components of the immune system should be recognized, with an aim to eliminate them.^{34,36,61} Understanding these immune inhibitory processes is crucial for predicting the mechanisms by which cancers escape the normal immune system.⁶¹

Considering the antitumor immunity as a significant barrier to tumor formation and progression, the immunosuppressive cancer cell phenotype is recognized as one of the hallmarks of cancer, which should be addressed to improve novel therapeutic strategies.³⁷

As the above-mentioned approaches necessary for an active immune response do not occur in an effective manner for cancer cells, novel therapies are designed with an aim to “re-educate” antitumor responses using cytokine inhibitors. Blockade of the expression of classical biomarkers in regulatory cells has been shown to exhibit excellent results in different cancers, including stomach, neck, kidney, bladder, esophagus, lung, breast, and aggressive malignant melanoma.⁶² Immunotherapeutic drugs, such as ipilimumab, pembrolizumab, talimogene laherparepvec (T-Vec), and trastuzumab, which are used to block suppressor receptors on lymphocytes (CTLA-4 and PD-1),⁶² are not indicated for all classes of tumors and do not present the same efficacy in all patients, thereby highlighting the necessity of the standardization of individual protocols, which may substantially increase treatment costs.⁶³ Despite the use of immunotherapeutic drugs, another promising strategy is to potentiate the naturally occurring immune response of the patient, which can be considered together with nanomedicine strategies in the present era.³⁶

1.3 NANOMEDICINE IN CLINICAL PROCEDURES

The extensive investigation of effective therapeutic regimens in cancer treatment has been the focus of renowned research groups worldwide. Nanomedicine, which is the application of nanotechnology in diagnosis, prevention, and treatment, represents one of the most advanced technologies for the treatment of different neoplasia types.

Nanotechnology has been applied in medical and biomedical practices to improve conventional procedures and to develop novel therapeutic regimens.^{10,64} The possibility of modulating the physical and chemical properties of nanosystems according to the desired application, and the ability of nanosystems to cross biological barriers and to accumulate in tumor tissues, contribute to the evident benefits of their utilization as advanced platforms for cancer therapies.^{10,64} Therefore, several classes of nanosystems have been extensively studied for anticancer drug delivery, including polymeric nanoparticles, polymeric micelles, liposomes, nanocapsules, dendrimers, inorganic nanoparticles (NPs), nanoemulsions, nanogels, and others, and few have already reached clinical trial stages (Table 1.1).^{10,64} Nanostructured systems loaded with different anticancer agents have shown promising results.^{10,64} The possibility of encapsulating different molecules within nanoparticles with controlled surface chemistry, size, shape, and superficial charge allows the targeting of cancer cells to reduce undesired activity in healthy tissues. Herein, we highlight the improvements provided by these nanostructures against cancer cells, emphasizing their immunomodulatory capacity in the TME, blood, and lymphoid organs.

Nanostructured systems overcome the lack of specificity and efficacy associated with the usual clinical techniques, improving therapeutic procedures in primary and metastatic sites.¹⁰ For example, chemotherapeutics adsorbed or covalently bound to nanomaterials can reduce drug withdrawal from the cytoplasm by ATP-binding cassette (ABC) transporters.⁶⁵ Nanoparticles inhibit the main mechanisms of cellular pumps, retaining antineoplastic agents inside cancer cells for extensive periods compared to free drug molecules. Such peculiarity minimizes the low bioavailability of hydrophobic drugs and improves their delivery with low therapeutic resistance.⁶⁵⁻⁶⁶ Recently, active molecules released from nanostructures were demonstrated to maintain the minimal effective dose for longer periods in the TME.⁶⁷⁻⁷⁰ In fact, from an immunological perspective, the use of nanocarriers may help stimulate antitumor immunity by promoting the activation of DCs, CTLs, NKs, and the depletion of Treg suppression.⁷¹ For example, commercially available nanostructured systems, such as Abraxane® and Doxil®, when administered, are internalized by immune-suppressed macrophages that drive their immune response to an inflammatory profile.⁷²⁻⁷³ Daily administration is not necessary for these nanoformulations, as they occur with the use of their free drug molecules, subsequently reducing cytotoxicity in white blood cells (WBCs).⁷²⁻⁷³

Drug delivery nanosystems exhibit fewer side effects, including leucopenia, thrombocytopenia, anemia, fatigue, peripheral neuropathy, and neutropenia, as described for paclitaxel and doxorubicin (DOX). Based on the improvement provided in terms of therapeutic efficacy and its immunological aspects, paclitaxel-loaded nanoparticles (Abraxane[®]) exhibit not only a slight cytotoxicity in peripheral monocytes in the bloodstream, but also does not induce phenotypic changes.⁷⁴⁻⁷⁵ Exposure of peripheral blood monocytes with NPs-PTX during their *in vitro* differentiation to DCs did not decrease the expression of CD11c, CD209, and MHC-II.⁷⁵ Additionally, NPs-PTX do not interfere with the stimulatory ability of DCs or suppress the capacity to stimulate naive T cells, with no phenotypic and functional changes observed in mature DCs.⁷⁵

1.4 NANOMEDICINES FOR THE MODULATION OF HUMAN IMMUNE RESPONSE

The immunomodulatory properties of intravenously administered nanoparticles have been extensively studied for a wide range of clinical applications. Their successful investigation in clinical trials should be based on their immunostimulatory potential, while considering their uptake, presentation of cancer antigens by APCs, and the elicitation of an immune response (Figure 2).⁷⁶

Table 1.1 - Nanoparticles (NPs) in clinical use.

NP types	Purposes	Approved by FDA and/or EMA	Indication
Lipid	Treatment	Abelcet®	Fungal infections
		Caelyx®	Kaposi's sarcoma, ovary, breast, myeloma
		Doxil®	Ovarian and Breast cancer; myeloma
		Marqibo®	Lymphoblastic leukemia
		MEPACT®	Osteosarcoma
		Myocet®	Breast cancer
		Oncaspar®	Lymphoblastic leukemia
		Onivyde MM-398®	Pancreatic cancer
		Visudyne®	Pathologic myopia, ocular histoplasmosis
		VYXEOS CPX-35®	Myeloid leukemia
Protein-based	Diagnosis	Definity®	Cardiovascular ultrasound enhancement
		SonoVue®	Contrast agent for liver, spleen, and kidney trauma
	Treatment	Abraxane®	Lung, breast, and pancreatic cancer
		Ontak®	T-cell lymphoma
	Polymeric	Adynovate®	Hemophilia
		Cimzia®	Crohn's disease and rheumatoid arthritis
		Copaxone®	Multiple sclerosis
		Eligard®	Prostate cancer
		Krystexxa®	Chronic gout
		Mircera®	Anemia
		Neulasta®	Neutropenia
		Oncaspar®	Leukemia
Metallic	Treatment	Pegasys®	Hepatitis B, hepatitis C
		Renagel®	Chronic kidney disease
		Welchol®	Type II diabetes
		Feraheme®	Iron deficiency
		Ferinject®	Anemia
		Hensify®	Squamous cell carcinoma
		Injectafer®	Anemia
		Venofer®	Anemia
	Diagnosis	Feridex®	Liver lesions (drug withdrawn from the market)
		Resovist®	Magnetic resonance imaging

Source: By the author.

Figure 1.2 – New therapeutic strategies using NPs for the activation of phagocytic cells and inhibition of the suppressive profile. The nanoparticles do not need to reach the tumor tissues to produce cytotoxic activity in diseased cells, they can contribute to modulate an individual's immune response in blood and TME. Nanocarriers loaded with antigenic material and adjuvants induce maturation of B lymphocytes, macrophages, and DCs in tissues.

Source: By the author.

The capture of NPs by WBCs may occur via phagocytosis, endocytosis, or adsorption. Among the APCs, DCs play an important role in tumor control via the induction of tumor-specific T-cell responses; therefore, they are an ideal target for the conduction of immunotherapy mediated by nanomedicine usage.⁷⁷ As an adjunct therapy, poly(lactic-co-glycolic acid) (PLGA) NPs with different surface modifications can be produced to collect and

deliver tumor-derived protein antigens to DCs and macrophages after radiotherapy or chemotherapy.⁷⁶ It has also been verified that PLGA NPs may capture damage-associated molecular patterns (DAMPs), which are defined as biomolecules that can help initiate and perpetuate a noninfectious inflammatory response and potentiate immune activity. Particularly, nanostructured systems perform the adsorption of tumor antigenic materials to stimulate DC activation and to enhance antineoplastic immune responses. After capturing nanocarriers loaded with stimulatory drugs or tumor antigens, DCs increase the expression of activation markers to proceed with antigen presentation to naïve lymphocytes. Antigenic materials are degraded by proteases in endosomes and associate with intracellular vesicles carrying MHC class II for further exposure on the cell surface and for establishing interaction with CD3⁺ T lymphocytes. Cytosolic antigens are processed by proteasomes for association with MHC class I in the endoplasmic reticulum (ER). When the peptide-MHC complex is established, MHC-I leaves the ER for localization at the cell surface to recognize CD8⁺ T cells. These processes ensure that fusogenic nanostructures release extracellular proteins into the cytoplasm, inhibiting vesicular degradation and enhancing peptide presentation via MHC class I (Figure 3).⁷⁸

Figure 1.3 – The three main routes through which nanoparticles are captured by macrophages and dendritic cells: phagocytosis, endocytosis, and fusion in the plasma membrane. Solid nanoparticles can undergo endocytosis and liposomes can undergo fusion in APC plasma membranes, delivering tumor antigens into the cytoplasm for future processing in the endoplasmic reticulum and fusion with the major histocompatibility complex in the Golgi apparatus.

Source: By the author.

With the exhibition of a series of DC subsets, human skin can be an attractive and accessible site for antigen-based immunotherapy. Based on this principle, Boks and co-workers developed a novel therapy using liposomes for the simultaneous delivery of tumor antigens and adjuvants to human skin-resident DCs, exploring their potential for intradermally delivered vaccines. The incorporation of TLR4 monophosphoryl lipid A ligand (MPLA) into liposomes stimulates the skin APCs to instruct tumor-specific CD8⁺ T cell responses. Interestingly, this was not observed when free MPLA was applied.⁷⁷ Once the antigen is captured, peripheral DCs may migrate to lymphoid tissues, triggering the activation of T cells. It is important to highlight that the high levels of the mannose receptor and scavenger receptors on DCs may be more appropriate to target nanostructure capture and to stimulate immune responses.⁷⁹

DC-targeted biomaterials, including hydrogels or other antigen delivery systems, have attracted considerable attention for cancer immunotherapy.⁸⁰ Such carriers can help modulate host DC populations by spatiotemporally controlling biochemical molecules, adjuvants, or cytokines. For example, a PEGylated polypeptide hydrogel was designed to encapsulate antigens and a toll-like receptor 3 agonist. The system could stimulate the DC phenotype during activation and *in vitro* and *in vivo* maturation, increasing antigen presentation to T lymphocytes and eliciting a response to kill cancer cells.⁸⁰

In addition to the immune system, macrophages and phagocytic cells found in the lung (dust cells), liver (Kupffer cells), kidney (mesangial phagocytes), brain (microglia), bone (osteoclasts), spleen, among others, can undergo activation and differentiation as pro-inflammatory and anti-inflammatory, referring to classic M1 macrophages and alternative M2-type, both derived from immature M0 macrophages.⁸¹ Tumor-associated macrophages (TAMs) usually exhibit the M2 phenotype, providing a favorable environment for cancer progression and producing suppressive signals for the amplification of the Th2 cytokine response.⁸²⁻⁸³

Therefore, new therapeutic strategies that are aimed at re-education of human TAMs and inhibition of their tumor-promoting functions have been proposed.⁸⁴ Nanostructures have been proven to disturb biological interactions within tumors by modulating TAM phenotypes, a process known as polarization.⁸⁵ Commercially available clodronate liposomes can be used to efficiently inhibit TAMs and to restore the immune response against liver

cancer, colon cancer, and lymphoma.⁸⁶⁻⁸⁹ Indeed, studies suggest that such nanoparticles increase the M1/M2 cell ratio by reducing the activity of the transcription factor, STAT3.⁸⁶⁻⁹⁰

A series of scientific reports regarding macrophage polarization suggest that while polymeric nanoparticles and liposomes aid the provision of M2-like polarization, inorganic nanostructures must provide M1-like polarization.⁸⁵ Particularly, gold nanoparticle (AuNP) uptake by human monocyte-derived macrophages was preferentially driven by the M2-type and alternatively activated cells according to the clear hierarchy M2c>M2>M2b>M2a>M1.⁹¹ Additionally, AuNPs changed the conformational arrangement (denaturation) of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), inhibiting the production of proangiogenic proteins by cancer-associated fibroblasts (CAFs) in the stroma.⁹²⁻⁹³ As VEGF expression is upregulated by cancer and infiltrating cells, cationic AuNPs have been proposed to inhibit their signaling cascade *in vitro* in a structure-dependent manner.⁹²⁻⁹³ Regarding the angiogenic blood vessels, cationic liposomes strongly bound to endothelial cells were internalized, while anionic, neutral, or sterically stabilized neutral liposomes did not demonstrate cellular uptake. This highlights the cationic nanostructures that selectively target angiogenic blood vessels in cancer therapy.⁹²⁻⁹³

1.5 MAIN CHALLENGES IN THE USE OF NANOPARTICLES IN IMMUNOTHERAPY

Before reaching the targeted cells, NPs can produce side effects in healthy tissues and phenotypic changes in immunocompetent cells, which can impair their clinical applications.¹² Therapeutic strategies in nanomedicines consider that both normal and transformed cells internalize nanoparticles through passive and active mechanisms, and promising results may be achieved with the combination of both procedures (Figure 4).

Figure 1.4 – Schematic illustration depicting the combination of target therapies and immunomodulatory techniques for retardation of cancer cell growth.

Source: By the author.

Owing to the process of angiogenesis, the main mechanism responsible for the passive accumulation of macromolecules and nanostructures in the TME is the well-known and so-called permeability and retention effect (EPR), which leads to the accumulation of structures with molecular weights ranging between 10 and 40 kDa.¹¹ NPs are also retained in hypervascularized tissues, such as the lungs, liver, kidneys, and spleen, ensuring that only a tiny fraction of the administered nanosystems reach the cancer sites.¹² In addition to the aforementioned aspects, several intrinsic factors may influence their efficiency, including pre-existing disease conditions and an individual's genotype.

After administration, WBCs and proteins generated by the humoral response represent the first barriers that must be overcome (or managed) by nanomaterials in the body.^{12,14} Under exposition with foreign organisms, APCs perform the activation of innate and adaptive immunological responses that may affect the stability of myelopoiesis and lymphopoiesis. PEGylation or conjugation with biological targeting components is widely known to aid the preservation of nanoparticles in the circulatory system.¹⁷ However, PEGylation may not be efficient at preventing inflammatory stimulus and partial clearance of the NPs before reaching the tumor site.¹⁷ Bio-polymers or biocompatible materials applied at the nanoparticle surface result in the reduction of immune cell recognition. Furthermore, the prolonged half-life in the circulatory system increases the deposition of plasma components on the protein corona layer, inducing complement system activation, contact kallikrein reaction, and blood coagulation.⁹⁴

The complement system is activated via three pathways. Notably, all models require the cleavage of plasma C3 protein into C3a and C3b. In the classical model, antibodies (IgG and IgM) are deposited on the surface of foreign invaders.⁹⁵⁻⁹⁶ C1q proteins bind to the Fc

region and trigger the cleavage of C4 and C2 proteins into C4a+C4b and C2a+C2b for the formation of C3 convertase (C4bC2a).⁹⁵⁻⁹⁶

Alternative activation occurs without the presence of antibodies at the beginning of the process. In blood, the pre-established C3b binds to the carboxylic and amino groups present on the particle surface and results in the generation of the C3 convertase after binding with complement factor B protein.⁹⁵⁻⁹⁶ Another possibility of activation is dependent on the plasmatic lectin that recognizes mannose fragments on pathogens and triggers C4 and C2 cleavage to generate C3 convertase.⁹⁵⁻⁹⁶

After intravenous administration, albumin, immunoglobulin G, and fibrinogen are the first proteins to establish interaction with NPs in blood.⁹⁷ Immunoglobulins represent 40% of the proteins in human plasma and are considered the main constituent on the biomaterial surface, stabilizing their dispersion in electrolytes and activating human defense cells after adsorption of immune regulatory proteins.^{15-16,98} Therefore, it is essential to elucidate the activity of blood cascade systems to avoid the occurrence of nonspecific reactions.⁹⁹

Nanomaterials may also induce complement activation-related pseudoallergy (CARPA), a process related to tumor growth in few patients undergoing nano-therapies.¹⁰⁰ In clinical settings, even after validation through toxicological studies, few patients presented with hypersensitivity and severe allergic reactions (anaphylaxis) following the administration of Doxil (Figure 5).^{98,101-102} Continuous activation of the complement system can aggravate the patient's condition.⁹⁷ Therefore, monitoring of the hemodynamic and hematological conditions is essential to avoid the occurrence of allergic reactions induced by nanostructures.¹⁰³ Importantly, complement activation can aid the recruitment of suppressive immune cells to control the inflammatory response produced by nanoparticles in the cancer microenvironment, inducing tumor cell progression in certain neoplasms.¹⁰⁴⁻¹⁰⁵

Figure 1.5 – A study on the hypersensitivity caused by Doxil. (A) Doxil dispersion was conducted in the plasma of 20 healthy donors (DO) for 30 min, and levels of the complement protein (iC3b) were determined by ELISA ($n = 3$). The results were compared with those obtained from samples without submission to treatments (negative control -NC), with cobra venom factor considered as a positive control (PC). (B) The individual variability of complement response was classified with stimulation index (SI) as follows: low ($SI \leq 2$), medium ($SI 2-6$), and high ($SI \geq 6$).

Source: Adapted from NEUN *et al.*¹¹⁰

Considering the toxic aspects, several efforts have been engaged to understand NP opsonization by plasma proteins to modulate allergic reactions and to avoid the occurrence of severe side effects during treatment.⁹⁹ Nanoparticle size, shape, surface charge, surface functionalization, and concentration in blood are known to dictate the complement activity; however, more studies are warranted.¹⁰⁶ Ensuring the control of the complement responses triggered by the amino and hydroxyl groups present on the nanocomposite surface is a major challenge.¹⁰⁷

When nanoparticles are subjected to conjugation with polymers, different structural conformations may produce distinct complement responses, even for the same surface composition.^{104,108} For example, the use of NPs with a diameter of 250 nm conjugated with dextran helped activate the complement cascade, while similar results were not observed

for the same NPs with a diameter of 600 nm, indicating the influence of NP size on the recruited response. Polymeric chains may demonstrate thermosensitive and pH-responsive properties and in such cases, when escape from the blood circulatory system is exhibited by NPs to infiltrate in tumors, the reduction in pH results in a conformational change in the polymeric matrix, subsequently exposing or hiding functional groups for protein adsorption.¹⁰⁹

Few studies have confirmed that complement depletion does not result in the stimulation of the delivery of antigenic materials from NPs to phagocytic cells and consequently inhibits the adaptive immune response against pathogens.¹¹⁰ Moreover, studies conducted using animal models demonstrated that adsorption of complement proteins on polyhydroxylated-PPS-NPs (25 nm) increased the expression of activation markers, CD80, CD86, and CD40 in immature DCs.¹¹⁰ Particularly, in the case of nanovesicles, their lipid composition was demonstrated to change the humoral response. The presence of cholesterol molecules modulates the pseudoallergic reaction of liposomes and PEGylated lipid structures, followed by the occurrence of intense leukopenia and thrombocytopenia in mice.^{103, 111}

Furthermore, the mechanisms by which adsorbed complement proteins on NP contribute to modulating humoral immunity and activation of WBCs warrant further studies to aid the design of versatile nanoplatforms and to improve their application in cancer therapy.

1.6 Novel strategies to boost nanotherapeutic use via application of bioinspired and biomimetic systems

As previously described, despite several benefits regarding the ability of nanostructures to modulate immunological response, these systems require improvement before their clinical applications to overcome eventual problems that can undermine their safety.

Recent advances in nanoengineering have helped propose the design of bioinspired and biomimetic systems that can ensure nanoparticle camouflage, targeting, and accumulation in the tumor microregion via specific functionalization of NPs with biological elements or even with whole natural cell membranes, thus providing higher specificity.^{74,112-}

¹¹⁶ In this process, cellular vesicles are separated from organelles by ultracentrifugation and adsorbed on the NP surface to incorporate biological properties into the nanostructures, thereby increasing biocompatibility, decreasing allergic reaction *in vivo*, and enhancing their effectiveness against cancer.¹¹⁷ The resultant nanostructure carries the full array of cancer cell antigens, thereby offering a robust and innovative platform that is applicable to multiple modalities of anticancer therapy. These engineered biomimetic features are currently under exploration as advanced drug delivery systems in which natural components are used for theragnostic purposes, bypassing macrophage uptake and systemic clearance and improving therapeutic outcomes.¹¹⁸⁻¹¹⁹

In this section, we describe different biomimetic and bioinspired systems engineered through the consideration of different cell structures, covering their immunomodulatory properties with regard to cancer therapy.

Cellular plasma membrane-derived NPs

Cell membranes may represent the most basic, structural, and functional units of organisms carrying different biomarkers that can assist cell recognition and signal transduction, among other functions.¹²⁰ The isolation of extracellular vesicles (exosomes and microvesicles) and their conjugation with therapeutic agents have been addressed in this context.¹²¹ Their reconstruction as nanocarriers can help deliver antineoplastic and immunomodulatory drugs to diseased and healthy cells, replacing synthetic liposomes or polymeric platforms. Plasma membranes isolated from cancer cells, erythrocytes, and leukocytes increase nanoparticle functionality in the body, with their immune-stimulatory capacity complementing traditional therapies.¹²²

Cancer cell membrane

The deposition of proteins and lipids from the plasma membrane onto the particle surface aids the development of new nanotherapeutic approaches for the treatment of chronic diseases, such as cancer, subsequently ensuring increased applicability of a wide range of solid nanoparticle systems.¹²⁰ Based on the excellent results observed with the application of biological vesicles, studies conducted by our group reported the use of cellular membrane-derived nanoparticles (MNPs) to enhance the specificity of external source

therapies. PEG-coated gold nanorods incorporated in MNPs derived from lung cancer cell membranes were loaded with the anticancer drug, β -lapachone, and were used to provide a specific multifunctional system combining chemotherapeutic and photothermal cell destruction in a synergic manner.¹¹² In another study, we reported the use of MNPs for conducting encapsulation of two first-line drugs used in pancreatic cancer treatment.⁷⁴ The MNPs were isolated from the pancreatic membrane and incorporated with gemcitabine and paclitaxel to induce apoptosis in PANC-1 cell lines *in vitro*. Furthermore, the antigenic material carried in the nanovesicles activated human monocytes and DCs in the presence of chemotherapeutic molecules. Such properties can avoid the evasion of cancer cells from the immune system in a safer and more effective manner during treatment.¹²⁰

In fact, biomimetic NPs covered with cancer cell membrane components can be used as vaccines to modulate the immune system.¹²³⁻¹²⁵ Yang and co-workers developed tumor vaccines for cancer prevention and treatment by performing coating of R837-containing poly-(D,L-lactide-co-glycolide) PLGA NPs with mannose-modified tumor cell membranes. The nanovesicles showed enhanced uptake by APCs, such as DCs, which were stimulated to maturation, triggering antitumor immune responses, thereby representing an important potential for clinical translation.¹²⁶

Lipid nanoparticles synthesized with tumor plasma membranes were used to deliver a substantial amount of antigenic material to the APCs. In such cases, macrophages processed antigenic proteins and exposed them to T lymphocytes via MHC, triggering anti-tumor responses in peripheral lymphoid organs.^{123,125} The delivery of antigenic material by lipid NPs stimulates a higher primary and secondary antibody response than that generated after pure antigen administration.¹²⁷

Undoubtedly, the use of cell membrane-coated NP technology provides an excellent nanostructure with immunomodulatory potential. Murine B16-F10 cell-derived NPs exhibited uptake by immature leukocytes, improving WBC activation in draining lymph nodes.¹²⁸ After 6 h of subjection to subcutaneous administration, phagocytes demonstrated the expression of maturation markers, CD80/CD86, while Th1 cells secreted a considerable amount of IFN- γ and IL-2. The presence of membrane antigens on the NP surface promoted pro-inflammatory activity in TME and adhesion protein expression (such as selectin ligands, integrin, and chemokine receptors) in addition to the targeting of nanocarriers to tumor cells.¹²⁹⁻¹³⁰

Red blood cell membranes (RBCm)

Erythrocytes, or red blood cells, were the first cell type used to establish the lipid and protein isolation protocol to fabricate biomimetic and bioinspired systems. Their abundant “self-markers” such as proteins, glycan, and sialic acid moieties play a critical role in suppressing an immune attack.¹³¹ After hemolysis, erythrocyte lysates are subjected to density gradient centrifugation to obtain membrane components¹³²⁻¹³⁴ and used to reduce immune clearance of the nanoparticles (Figure 6).¹¹³⁻¹¹⁵ Although proteins derived from the RBCm inhibit the capture of nanomaterials by phagocytic cells,¹¹³ they do not prevent their retention in the liver, lung, and spleen.¹¹⁴ To avoid such interactions, antibodies and aptamers can be bound to RBCm to enhance specificity and cell internalization. For example, RBC nanovesicles have been used in fusion with tumor-penetrating peptides for the treatment of metastatic breast cancer, subsequently promoting an increased interaction with tumor tissues.¹¹³ In another study, DOX-containing RBCm-PLGA nanoparticles could cross the microvascular endothelium for glioblastoma treatment. Tumor angiogenesis showed reduction for a few days after its administration to mice.¹³⁵ DOX carried by RBCm-NPs has been shown to prevent toxicity in neutrophils, lymphocytes, and monocytes, and to avoid the reduction of plasma compounds, such as albumin, bicarbonate, and creatinine, indicating the absence of organ dysfunction.¹³⁶

Figure 1.6 – (A) Schematic illustration of nanoparticles loaded with erythrocyte plasma membrane. (B) Size and (C) surface zeta potential of upconversion nanoparticles (UCNPs), RBC membrane vesicles and UCNPs fused with the RBC membrane. (D) TEM images of UCNPs and (E-F) RBC-UCNPs (scale bar = 25 nm) revealed incorporation of the erythrocyte membrane on the nanostructure surface. (G) Polyacrylamide gel electrophoresis conducted for the detection of plasma proteins in the samples of RBCs and RBC-UCNPs.

Source: Adapted from RAO *et al.*¹¹⁴

Currently, PLGA-based NPs are the most commonly used polymeric structures for designing cell membrane-derived systems. Even when used at high concentrations, PLGA does not interfere with cell phenotype and cell viability.¹³⁷⁻¹³⁹ More importantly, RBC-covered PLGA NPs have been shown to exhibit increased blood circulation time, showing the importance of both long circulation and tumor penetration for improved therapeutic outcomes.¹¹³

In another study that used the RBCm coating technology, a novel antigenic peptide delivery system involving PLGA-NPs was constructed to target carbohydrate receptors present on macrophages and DCs. The addition of mannose was performed to actively target APCs in lymphatic organs. Using *in vivo* models, the developed nanostructures inhibited tumor growth and suppressed tumor metastasis, in addition to effectively enhancing IFN- γ secretion and CD8⁺ T cell response. The high accumulation of the nanostructures in the draining lymph nodes increased the expression of CD86 proteins and the production of TNF- α and IL-12 by phagocytic cells and IFN- γ by Th1 cells.

White blood cell membranes

Under damaged vasculatures, WBCs migrate from the capillaries to inflamed regions. Neutrophils are professional phagocytic cells that can cross the endothelial cell barrier to reach the acute inflammation site.¹⁴⁰ Therefore, neutrophil plasma membranes have also been isolated and used to design biomimetic nanovesicles providing NP camouflage for mononuclear phagocyte system clearance, overcoming the vascular barrier and localization at the target tissue.¹⁴¹ Gão *et al.*¹⁴² developed new methodologies based on nitrogen cavitation for the formation of neutrophil plasma membrane nanovesicles rich in integrin- β 2. Neutrophil nanovesicles replicate the membrane features of the source cells, which are captured by inflamed microvasculature, a method that can be applied to a wide range of diseases without inflammatory conditions.¹⁴²

Circulating tumor cells (CTCs) comprise organelles that can trigger the early stages of metastasis, rendering them a valuable target for preventing the spread of cancer. Knowledge regarding the occurrence of metastasis also highlights neutrophils as a fundamental cell in the early stages of their formation. Therefore, Kang *et al.* designed NM-NPs by coating the surface of a PLGA polymer with an inflammatory neutrophil-derived membrane. The biomimetic nanostructure was loaded with carfilzomib (CFZ) to prevent metastasis and to inhibit the growth of the already formed cancer sites. Granulocytes were previously co-cultured with LPS to increase inflammatory membrane proteins, preserving L-selectin, CXCR4, LFA-1, and β 1 integrins to establish interaction with ICAM-1, VCAM-1, and CD44 molecules on CTC surfaces and inflamed endothelial cells.¹²⁹ After binding, nanostructures are selectively endocytosed by clathrin and the caveolae pathway, eliminating cancer cells through the loss of plasma membrane integrity.¹²⁹ Furthermore, treatment was found to reduce the expression of inflammatory cytokines and the number of immune-suppressive neutrophils, which promoted cytotoxic effects against inoculated 4T1 cells.¹²⁹ As a result, the neutrophil plasma membrane reduced the size and number of metastatic nodules in the lungs, impairing the capability of CTC migration through the circulatory system.¹²⁹

Similar to the procedure adopted for neutrophil membrane nanovesicles, natural membranes derived from macrophages have been applied to coat mesoporous silica nanocapsules loaded with DOX for the treatment of breast cancer. This biomimetic nanostructure effectively integrates tumor-assisted targeting therapy with immunological

aspects. *In vivo* assays have demonstrated effective accumulation in tumors and the inhibition of tumor growth. Additionally, the membrane-coating strategy provided active targeting abilities for recognition of the tumor endothelium.¹⁴¹

Proteins derived from the leukocyte plasma membrane were employed as coatings for synthetic phospholipid bilayer nanoparticles, referred to as leukosomes, which could preferentially target inflamed endothelia both *in vitro* and *in vivo*.¹⁴³ Macrophage membrane-coated liposomes were developed by Cao *et al.*⁷⁰ to improve specific metastasis-targeting capability and to suppress secondary lung tumors resulting from breast cancer metastasis. The emtansine drug was encapsulated into pH-sensitive liposomes and coated with macrophage membranes isolated from a murine monocyte/macrophage cell line (RAW 264.7) with high expression of $\alpha 4$ and $\beta 1$ integrins. Biomimetic systems were used to effectively enhance cellular uptake and the inhibitory effects on cell viability.⁷⁰ The use of macrophage cell membrane coating also inhibited untargeted drug delivery in normal cells and decreased the retention of nanoparticles in the liver, spleen, and lungs.^{70,141}

Considering the increasing number of individuals affected by cancer and their particularities, there is an urgent need for the discovery of successful therapeutic alternatives. Furthermore, based on the molecular differences noted between patients, the next generation of cancer treatments is likely to be based on molecular features and the identification of effective targets that may constitute personalized medicine in cancer therapy. In this scenario, nanomedicine merits special attention. To improve conventional therapies, new treatments are being developed with aim to abolish the neoplastic ability to evade host defense mechanisms through nanotechnological tools. For instance, nanoparticles may improve cancer immunotherapy results, producing faster innate and adaptive responses compared to conventional treatments. Therefore, to optimize the design of safe formulations, surface-coating biomimetic technologies allow nanoparticles to travel longer distances through vascular networks and enable the establishment of interactions with the immune system without chronic inflammation, providing interesting features. Thus far, these advanced drug delivery systems have been recognized as multifunctional platforms that can trigger immunotherapy responses, thereby emerging as an extremely promising strategy that remains at initial stages of development, which also evidently holds remarkable potential.

As a novel bioinspired and biomimetic platform, advanced drug delivery systems may be effective for delivering multiple tumor antigens to conduct re-education of pro-inflammatory profiles, by exploiting intact and natural organelle functions rather than by replicating these features using synthetic techniques. Although the technique is now widely discussed, few studies have harnessed applied natural organelles as nanovesicles for coordinated delivery to the tumor sites without evaluating the immunomodulatory aspects provided by these platforms. Owing to these properties, these bioinspired nanostructures are considered excellent candidates as next-generation carriers for suppressing cancer growth and for potentiating the recovery of immunity.

We believe that in the future, the combination of cell biology and nanotechnology may be used to fabricate chimeric nanostructures that can help exploit the intrinsic properties of their origin to exert advanced drug-delivery functions. Nanostructured systems are expected to increase tumor immunogenicity and to modulate immune mechanisms, preventing suppressive effects in the cancer environment. Importantly, the advantages and disadvantages of nanomedicine and immunotherapy are complementary. Furthermore, their combination opens possibilities for the development of new alternative therapeutic strategies that require a multidisciplinary understanding of their properties in the body.

CHAPTER 2 CANCER CELL MEMBRANE-DERIVED NANOPARTICLE IMPROVE THE ACTIVITY OF GEMCITABINE AND PACLITAXEL ON PANCREATIC CANCER CELLS AND COORDINATE IMMUNOREGULATORY PROPERTIES ON PROFESSIONAL ANTIGEN-PRESENTING CELLS.

ABSTRACT

Human pancreatic carcinoma is amid the neoplasias with the highest number of deaths and the frequency of relapses has demanded novel therapeutic intervention. Currently, the simultaneous application of different chemotherapeutics improves treatment efficiency in patients. However, the low specificity from these molecules may increase cytotoxic side effects in healthy cells. Nanocarriers conjugated with aptamers and monoclonal antibodies target distinct proteins expressed on cancer cells and can be used to increase drug activity in disease sites. A recent advance in nanoengineering isolate cell membrane lipids and proteins from cytoplasmatic organelles by ultracentrifugation and use the double-layer surface to coat organic and inorganic nanoparticles to prolong their permanence in the bloodstream. Additionally, plasmatic cell components form lipid nanovesicles able to deliver antineoplastic molecules. The present study aims to isolate the major components from pancreatic tumor cell membrane (PANC-1) to fabricate nanoparticles (MNPs) encapsulating two first-line drugs used in clinical treatment, viz., gemcitabine (GEM) and paclitaxel (PTX). Our results demonstrated that the MNPs-GEM-PTX were very stable and induced higher cytotoxic effects on PANC-1 cells, in comparison to the use of pure GEM+PTX. The most interesting finding was that MNPs also interact with monocytes and dendritic cells, increasing the expression of costimulatory molecules CD80, CD83, CD86, and HLA-DR, indicating that drug-carrier property of MNPs upregulates activation of white blood cells. Therefore, a novel class of nanoparticle was used to deliver antineoplastic agents to cancer cells and antigenic material to antigen-presenting cells. We also investigated their ability to modulate immune cells aiming at promoting pro-inflammatory response in tumor sites.

2.1 INTRODUCTION

Chronic diseases responded for 15% of world deaths,¹⁴⁴ with 9.5 million of casualties registered in 2018 and 17 million new cases expected for 2020.¹⁴⁵ Pancreatic cancer represents the 14th most frequent and the 7th largest mortality amid neoplasms, with diagnosis usually confirmed in late stages when disease already progressed in the organism.¹⁴⁴⁻¹⁴⁵ Surgery and chemotherapy are the main therapeutic strategies against abnormal cells in the pancreas, but patients survive just a couple of months after initiating the treatments.¹⁴⁶⁻¹⁴⁷

In chemotherapy, the most common classes of drugs include alkylates to inhibit DNA transcription, antimetabolites - cellular compounds analog – that block metabolic pathways in S-phase, microtubule-stabilizing agents in G₀/G₁ and/or G₂/M phases, and topoisomerase inhibitors.³² Gemcitabine (GEM) is an antimetabolite drug used to treat pancreatic cancer because they interfere in DNA replication, incorporating in polynucleotides and inhibiting deoxyribonucleotides synthesis in S phase, which leads to anti-cancer effects during cell division. In late stages, pancreatic carcinoma is highly resistant to chemotherapy,¹⁴⁸ and to improve treatment efficiency, researchers have pointed to the simultaneous application of different drugs, such as the combination of GEM with cisplatin, oxaliplatin, taxol, and capecitabine. However, only therapies using GEM+Taxol demonstrated effects better than monotherapy with GEM in clinical trials.¹⁴⁹ Simultaneous administration of GEM and paclitaxel (PTX) increases the survival rate at least 2 months in patients with metastases,¹⁵⁰ being considered a novel therapeutic procedure for pancreatic cancer.

Recent studies revealed the feasibility of nanoparticles (NPs) to deliver a high concentration of drugs within target cells and to reduce undesirable toxicity in other tissues. Nanoparticles can carry antineoplastic agents to improve drug activity in diseased cells, however, their applicability depends on their ability to move through organs and tissues in the human body.¹⁵¹ The "addressing" for primary and metastatic sites is done upon coupling antibodies and aptamers on the nanostructure surface, establishing greater specificity for target tissues.¹⁵² Such strategy increases hydrophobic drugs release in disease sites and minimizes the bioavailability to healthy cells. Nanomaterials also inhibit the expulsion of chemotherapeutic molecules from the cytoplasm by ATP carrier proteins (ABC transporters) before producing any cytotoxic effects.⁶⁵ Liposomes used in drug delivery may reduce

hematological and vascular toxicity observed in clinical treatments with chemotherapeutics, restoring the normal level of platelets, monocytes and T cells.¹⁵³⁻¹⁵⁴

A recent breakthrough in nanoengineering uses the major components from the cell membrane to camouflage nanostructures in the organism and to conduct their activity to cells where membrane residues came from.¹⁸ In the latter strategy, Lipids and proteins are isolated by ultracentrifugation and used to synthesize nanovesicles with immunomodulatory properties.¹²⁸ Currently, studies already used lipids and proteins from the plasma membrane (MNP) from red cells, immune cells (macrophages, dendritic cells, and T cells), and cancer cells in the preparation of nanostructures. The presence of cellular adhesion proteins aids interaction with the desire target cell¹⁸⁻¹⁹ and membrane lipids (phospholipids, glycolipids, and cholesterol) increasing the stability in biological medium, keeping drugs biodisponibility for weeks in circulatory system.¹⁵⁵⁻¹⁵⁷

Neoplastic cell growth and expansion begin with their ability to modulate tumor microenvironment and evade immune surveillance. To use nanoparticles in clinical treatments, it is necessary to investigate their toxicity and phenotypic changes in leukocytes from human peripheral blood. Nanocomposites prevent the production of immunosuppressive cytokines and increase the activity of the main mechanisms of cellular immunity. In this study, we synthesized lipid nanoparticles with the main components from pancreatic cancer cell membrane (PANC-1) to deliver a large amount of antigenic material to antigen presenting cells (APCs). The nanoparticles also carried low doses of GEM and PTX to reduce minimal effective doses in malignant cells and side effects in healthy cells. According to our knowledge, this is the first study using this class of nanovesicle to deliver two chemotherapeutic drugs simultaneously. Novel therapeutic approaches administrate low and ultra-low doses of chemotherapeutics to inhibit negative side effects in the organism, like severe immunosuppression and cancer resistance.¹⁵⁸ Metronomic therapy becomes more attractive than periodic application of maximum tolerated doses because it reduces immune suppression of macrophages and lymphocytes in tumor microenvironment, activating white blood cells against neoplasias.⁶⁷⁻⁶⁹ The MNPs prepared here were tested *in vitro* in tumor and healthy cell lines to verify cytotoxic activity of GEM/PTX nanocarriers (MNPs-GEM-PTX). The ability of the nanoparticles to target specific cells were performed by exposing them to PANC-1 cells, HEPA-RG cells and peripheral blood monocytes from healthy donors. We observe an excellent stability of MNPs-GEM-PTX, which exhibited higher

cytotoxic effects than GEM or PTX alone. Therefore, we proposed to improve drug delivery to pancreatic cells and improve antigenic material processing into monocytes and dendritic cells using nanovesicles created from cancer cell membrane components.

2.2 METHODOLOGY

2.2.1 Nanoparticles synthesis

Plasma membranes from pancreatic carcinoma cells were isolated by ultracentrifugation, as describe by Lung *et al.*¹¹⁷ and extruded 20 times through 100 nm membrane for nanovesicles fabrication. Briefly, cells were detached from culture bottles, washed three times with PBS, lysed in a hypotonic buffer for 20 min at 4 °C and homogenized. The lysed cells were centrifuged at 15,000 g for 15 min at 4 °C to remove cell debris. The supernatant was collected and centrifuged at 100,000 g for 2h, using an Optima MAX-XP ultracentrifuge (Beckman Coulter, USA). The pellet containing the cell membranes was dispersed in 1 ml of PBS containing Complete Protease Inhibitor (Roche Diagnostics, Mannheim, Germany) and stored at -80°C. Membrane proteins present in the vesicles were quantified by the Bradford colorimetric assays (Sigma-Aldrich) to determine protein concentration in the samples.

2.2.2 Ultraviolet–visible spectroscopy (UV-VIS) and Fourier-transform infrared spectroscopy (FTIR)

A Hitachi UV-VIS spectrophotometer model U2008 was used to monitor the amount of chemotherapeutic encapsulated in MNPs and also its release over 48h. FTIR analyses were performed in a Nicolet iS-50 spectrophotometer.

2.2.3 Microscopy analyses

Nanoparticles morphology was analyzed using Field Emission Scanning Electron Microscopy (MEV-FEG, ZEISS, model SIGMA), Transmission Cryo-Electron Microscopy (TEM, JEOL 1400) and Atomic Force Microscope (AFM, Nanosurf model EasyScan 2 FlexAFM) for

measurement of external density and dimensions, as well as to verify the presence of adsorbed or incorporated materials. Confocal microscopy was also used to evaluate nanoparticles interaction with the cells. A Leica TCS SP8 was employed.

2.2.4 Nanoparticle stability in aqueous solutions

Dynamic light scattering (DLS) and zeta potential (spectrometer Zetasizer Nano ZS90) were used to evaluate nanoparticle polydispersity, size distribution, and surface potential. Nanoparticle tracking analysis (NTA) was carried out using a Malvern NanoSight NS300 instrument to also study nanoparticle size. DLS analyses were employed to evaluate the stability of the nanoparticles incubated in DMEM culture medium with 10% Fetal Bovine Serum (FBS) under agitation for 4h, 8h, 12h, 24h, 36h, and 48h.¹⁵

2.2.5 Incorporation of chemotherapeutic drugs into MNPs

Gemcitabine (GEM) and paclitaxel (PTX) were co-extruded with the hepatocellular carcinoma membranes through a 100 nm polycarbonate membrane using a mini-extruder (Avanti Polar Lipids). MNPs-GEM and MNPs-PTX were prepared by mixing 1 ml of MNPs suspension at 10^9 particle/ml with 5.2 μ l of GEM at 100 μ M and 7.12 μ l of PTX at 5 nM dissolved in PBS and extruded through a 100 nm membrane in a Malvern nano-extruder at room temperature. The solution was centrifuged at 100,000 g for 2h and washed with PBS. After removal of unbound GEM and PTX, the complexes (MNPs-GEM, MNPs-PTX, and MNPs-GEM-PTX) were resuspended in 1 ml of PBS under gentle sonication for 30 min. at 40 kHz. UV/visible spectroscopy of pure MNPs and samples with GEM/PTX was carried out to estimate the amount of encapsulated chemotherapeutic molecules.

2.2.6 MNPs staining with fluorescein isothiocyanate and analysis of their interaction with cells

Nanovesicles label was prepared with 10^{10} particles dispersed in 1 ml of PBS and 0.1 mg of fluorescein isothiocyanate (FITC) stirring in the dark at 4 °C for 12h. MNPs-FITC was separated from unconjugated fluorescein by 12h dialysis on a 12K Dalton membrane.

Following, the suspension was ultracentrifuged and resuspended in PBS. FITC⁺ nanoparticles were used to prove preferential uptake of MNPs by mononuclear cells (monocytes) and pancreatic cancer cells (PANC-1), incubated with 10^8 particles/ml for 48h. Flow cytometer (model FACS Calibur) analyzed the samples and the results were processed in FlowJo software, version vX. 10.6.

2.2.7 Tumor cells culture

Pancreatic cancer cells (PANC-1) and hepatic cell lines (HEPA-RG) were cultured in complete medium [DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% non-essential amino acids, 1% antibiotic and antimycotic (Life Technologies), and HEPES (Sigma)] at 37 °C under 5% of CO₂. We used 0.05% trypsin-EDTA (Gibco) to release cells from bottle surface before washing in complete culture medium to use in the assays.

2.2.8 Cytotoxicity assay (MTT)

We analyzed the nanoparticle interaction with the cell lines using MTT (3-[4,5-dimethyl-thiazol-2-yl] -2,5-diphenyltetrazolium bromide) colorimetric assay. This salt reduced to formazan crystals through mitochondrial metabolism, reflecting the activity of living cells as an indicator of viability. The treatment was performed by adding the particles and chemotherapeutics in cell culture (1×10^4 cells per well) in a flat bottom plate of 96 wells for 24 and 48h. As contrasts we tested a) pure MNPs; b) MNPs encapsulated with GEM (MNPs-GEM); c) MNPs with PTX (MNPs-PTX); d) MNPs carrying GEM and PTX (MNPs-GEM-PTX) and d) pure GEM and PTX. Cells exposed to DMSO were used as positive control in the assay (maximal lysis). The living cells were incubated with solution of MTT for 3h at 37 °C under 5% of CO₂. Dimethyl sulfoxide (DMSO) solubilized formazan crystals in mitochondria for reading in spectrophotometer at 580 nm.

2.2.9 Necrosis and apoptosis assays

Flow cytometry confirmed cytotoxic activity of MNPs at 10^8 particles per ml and 10 μ M GEM and 10 nM PTX. After incubation for 48h in 12-well plates, the particles were removed from supernatant, as well as the cell debris. About 1×10^5 cells were collected and centrifuged at 10,000 rpm for 30 seconds and resuspended in isotone (PBS, containing 0.5% BSA) for 20 min at room temperature with Annexin V and 7AAD and acquisition in flow cytometer.

2.2.10 Human monocytes culture.

Human monocytes were obtained from the peripheral blood of 3 healthy donors with ages ranging from 20 to 40 years. The investigation was carried out according to the Helsinki Declaration and approved by the Institutional Research Ethics Committee. All the cell donors were informed about the procedures, objectives and risks involved in the study, and signed an agreement form. Human peripheral blood monocytes were separated from red blood cells and plasma by centrifugation at 1600 rpm for 35 min in Ficoll-Isopaque gradient. Cell suspension was centrifuged in 51% of Percoll gradient to separate monocytes from lymphocytes and dispense mononuclear cells into sterile plates (2×10^5 per well) for performing the interaction assays.

2.2.11 Dendritic cells differentiation

Monocytes from healthy donors were differentiated in dendritic cells (DCs) after supplemented culture medium with 80 ng/ml of IL-4 and GM-CSF. Positive control group (Control⁺) was incubated with activation cocktail composed by IL-1, IL6, TNF, and PGE₂ to obtain complete mature DCs differentiation. After 6 days, adherent cells were harvested and washed with complete medium, being concentrated to 2×10^5 per well. DCs were treated with nanoparticles carrying antineoplastic agents to evaluate cell viability, phenotype, and functional assays.

2.2.12 Evaluation of monocytes and DCs phenotype after exposing to MNPs

We used flow cytometry to analyze surface markers from monocytes and dendritic cells after 48h of treatment with monoclonal antibodies (BD Bioscience). In monocyte (CD14⁺) and DCs populations (CD11c⁺), we observed co-stimulation molecules (CD80, CD83, and CD86); major histocompatibility complex class II (HLA-DR); suppressor molecule (PD-L1) and cell viability (7AAD⁺), with analysis based on gates quadrant of working groups.

2.2.13 Mixed lymphocyte reaction (MLR)

Mixed Lymphocyte Reaction (MLR) investigates the DCs allogeneic response in lymphocyte proliferation. In 7th day of differentiation, DCs treated with previously described nanoparticles were co-cultured with lymphocytes. In experiments of allogeneic response, it was used the ratio of 1:10 between DCs and lymphocytes. The culture was maintained for 5 days and total allogeneic lymphocytes incubated with MTT salt at the end of the period for proliferative analysis.

2.2.14 Statistical analysis.

All assays were repeated at least 3 times and data analysis was submitted to ANOVA followed by Tukey Mean Difference tests, considering differences with error probability less than or equal to 5% ($\alpha \leq 0.05$).

2.3 RESULTS

2.3.1 Nanoparticles characterization

The plasma membrane derived nanoparticles were produced by extrusion after membrane extraction from pancreatic cancer cell line (PANC-1). Dynamic light scattering and nanoparticle tracking analysis revealed particles with an average size between 100-200 nm. All nanoparticles exhibited negative Zeta potential when dispersed in PBS at pH 7.4. However, in buffer solution at pH 4.4, only the experimental groups encapsulated with PTX

presented a negatively superficial charge (Table 2.1). A negative charge was expected since the MNP contain antigenic material from PANC-1 in phospholipid bilayers. For example, MNP zeta potential is -11 mV in saline buffer at pH 7.4 and, in the presence of chemotherapeutics, the MNPs-GEM, MNPs-PTX and MNPs-GEM-PTX had their zeta potential increased to -4, -5 and -4 mV, respectively. Drug encapsulation did not change MNPs size drastically, but the presence of GEM and PTX shifted the nanoparticle zeta potential close to zero, which could interfere in the nanoparticle stability. We analyzed MNPs superficial charge in lysosomes pH (4.4) and observed a negative zeta potential in the vesicles loaded with PTX. We also investigated nanoparticles stability in DMEM culture medium (Dulbecco's Modified Eagle Medium) supplemented with fetal bovine serum (FBS). All groups of nanoparticles were incubated for 4, 8, 12, 24, 36 and 48h at 37°C in a shaker before analyses of size and zeta potential (Figure 2.1).

Nanoparticle stability and aggregation phenomena are important parameters to be optimized for biological applications. As shown in Figure 2.1, nanoparticles kept in cell culture medium maintained their hydrodynamic diameter despite adsorption of proteins that created a protein corona on MNPs surface. The interaction with FBS constituents reduced MNPs-GEM-PTX zeta potential to -13mV, in contrast with his superficial charge in saline solution at pH 7.4 (-4 mV). After 48h of interaction with DMEM, we did not observe drastic variations in the size and superficial charge, demonstrating good stability in all experimental groups. Therefore, in physiologic pH values, the nanoparticles with and without chemotherapeutics are stable *in vitro* during the period analyzed.

Table 2.1 - Particles size and zeta potential based on NTA and DLS data.

Nanoparticles	pH 4.4			pH 7.4		
	Size (nm)		Zeta Potential ζ (mV)	Size (nm)		Zeta Potential ζ (mV)
	NTA	DLS		NTA	DLS	
MNPs	238 ± 4	450 ± 50	4.33 ± 1	187 ± 5	242 ± 2	-11.23 ± 1
MNPs-GEM	155 ± 5	410 ± 80	1.54 ± 1	130 ± 1	142 ± 2	-4.26 ± 2
MNPs-PTX	226 ± 7	360 ± 60	-6.92 ± 1	122 ± 2	139 ± 3	-5.02 ± 2
MNPs-GEM-PTX	270 ± 20	250 ± 60	-8.61 ± 1	154 ± 5	152 ± 4	-4.15 ± 1

Source: By the author.

Figure 2.1 – Size (a) and zeta potential (b) of MNPs-GEM, MNPs-PTX and MNPs-GEM-PTX at 4, 8, 16, 24, 36, and 48h with DMEM 10% FBS.

Source: By the author.

Atomic force microscopy (AFM) and field emission scanning electron microscopy (FEG-MEV) were used to investigate the morphology of plasma membrane derived nanoparticles from pancreatic cancer cell line (Figure A1). Transmission electron microscopy (TEM) images confirmed the results from DLS and NTA, with nanoparticles size around 100-400 nm. We also analyzed MNPs morphology and size distribution by cryo-microscopy (Cryo-TEM). Figure 2.2 shows the nanoparticles containing PTX and GEM, which presented a vesicular shape. Additionally, we visualize a fuzzy shade in the lipid bilayer from MNPs-GEM-PTX (Figure 2.2d-f), indicating an interaction between chemotherapeutics and membrane proteins on nanoparticles surface as verified by the change in zeta potentials. The distinct dimensions of MNPs-PTX and MNPs-PTX-GEM are related to drug retention and adsorption of culture medium proteins on nanoparticle surface. As demonstrated by cryo-microscopy, the arrows indicate a shade created by protein corona constituents and chemotherapeutic molecules on the lipid bilayer. Protein-stabilizing molecules (PTX) or drugs to interfere in DNA replication (GEM) may increase and decrease MNPs dimension, folding the polypeptides by chemotherapeutic agents activities. In Figure 2.1, we observed a difference among MNPs just 4h after dispersion in DMEM, turning similar along 48h. In PBS pH 7.4, the size of MNPs-PTX and MNPs-PTX-GEM are very close after the extrusion (~139nm and ~152nm, respectively) (table1), thus indicating the interfere from the culture medium proteins in nanoparticle stability during in vitro experiments.

Figure 2.2 – Cryo-TEM images of plasma membrane nanoparticles (MNPs) extracted from the cancer cell (a and b), after extrusion (c), and with MNPs-GEM-PTX (d-f). The arrows indicate a shade created by the interaction of protein corona constituents and chemotherapeutic molecules on the lipid bilayer.

Source: By the author.

Incorporation of chemotherapeutic agents in MNPs was investigated by UV/Visible spectroscopy using the Beer–Lambert law for pure MNPs, MNPs-GEM, and MNPs-PTX (Figure 2.3a). It was estimated approximately 50 μM of drug loaded in 1×10^9 particles, measuring the absorbance value of MNPs and GEM at 262 nm and 270 nm, respectively. Drug release was carried out in PBS (pH 4.8 and pH 7.4) and the concentration determined using GEM standard curves (Figure 2.3b-c).

Figure 2.3 – Absorbance spectrum at different concentrations of MNPs (a), and GEM (b). From the absorbance values, their respective standard curves were used to estimate the concentration of GEM in MNPs. Drug release of GEM from MNPs at pH 4.8 and pH 7.8 (c). FTIR spectra of MNPs encapsulated with gemcitabine and paclitaxel (MNPs-GEM, MNPs-PTX, and MNPs-GEM-PTX) (d).

Source: By the author.

We estimate the protein concentration in the MNPs using Bradford's assay and gel electrophoresis. Approximately 150 $\mu\text{g}/\text{ml}$ of 50-65 kDa-protein was extracted from 2×10^7 cells. We decided to confirm MNPs composition by infrared spectroscopy, analyzing the chemical bonds present in polypeptide chains. Because of the high concentration of proteins, bands related to carbon, oxygen, and hydrogen are highlighted in the spectrum, verifying the effectiveness of chemical treatments in functionalized walls. The molecular vibration of O-H stretches, N-H bonds and Secondary Amines are assigned in the graph between 3200 cm^{-1} to 3700 cm^{-1} in all experimental groups (region 1, Figure 2.3d). C-H, secondary amines, angular deformation of N-H and $\text{CH}_2\text{-NH}_2$ bonds are observed in the region from 1500 cm^{-1} to 1750 cm^{-1} (region 2). The third region corresponds to carboxylic acid and C-N stretching from primary amines (1100 cm^{-1} and 1200 cm^{-1} , respectively).

2.3.2 Interaction of nanoparticles with tumor cells and monocytes from healthy donors.

We used nanoparticles previously stained with fluorescein isothiocyanate (FITC) to investigate their interaction with pancreatic cancer cells, healthy liver cells, and immunocompetent cells. FITC fluorochrome emits green light when excited with light wavelength of 488 nm and can be detected by flow cytometry. This assay was performed only with FITC-labeled MNPs to prove interaction with tumor cells. As a result, we observed a high percentage of tumor cells FITC⁺, in contrast with the hepatic cell lines (Figure 2.4a-d). The frequency of nanoparticles on cell surface was estimated by examining the median fluorescence intensity (MFI). After treatment with MNPs, pancreatic cancer cells FITC⁺ double the value of MFI over their respective control group (cells not exposed to MNPs). This result is not observed in HEPA-RG cell lines, indicating less interaction on the surface of hepatic cells and great specificity to PANC-1.

Monocytes, macrophages, neutrophils, and dendritic cells capture nanoparticle very quickly, limiting the delivery of chemotherapeutic agents in disease sites^{159, 160}. Consequently, we decided to investigate MNPs interaction with monocytes from healthy donors to confirm if peripheral blood cells phagocyte a large number of fluorescein-labeled particles (Figure 2.4e-f). Our results indicate that 97% of monocytes are FITC⁺ with MFI values 10x greater than white blood cells treated with FITC stock solution on the same molar concentration adsorbed at MNPs (control group=37.5; FITC⁺-MNPs =372).

Figure 2.4 – Incorporation of MNPs-FITC into pancreatic cancer cells (a) and healthy liver cells (green) (c) based on size and granularity parameters. Histograms presenting the percentage of nanoparticles incorporation into pancreatic cancer cells (b) and normal liver cells (d), in which orange and green histograms represent FITC⁺ cells (cells incubated at 10⁸ and 10⁹ particles/ml, respectively) and the red histogram represents the unlabeled cell population. Incorporation of MNPs-FITC into monocytes (green) was also observed based through size and granularity analysis (e) and histogram (f), demonstrating the percentage of nanoparticles incorporation into white blood cells.

Source: By the author.

Confocal microscopy confirms the specific internalization of MNPs in pancreatic cancer cells (Figure A2). In PANC-1, red fluorescence from cell membrane surrounded green fluorescence that did not interfered in blue fluorescence from the cell nucleus, suggesting internalization of MNPs-FITC⁺. The images revealed the nanoparticles distribution in

cytoplasm without membrane residue. Meanwhile, the presence of antigenic material improved particle association on pancreatic cell surface, turning the intensity of MNPs more regular in PANC-1 cytoplasm than in the control group with HEPA-RG cells (Figure A2), as previously described by MFI values in Figure 2.4b and Figure 2.4d.

2.3.3 Nanoparticles cytotoxicity

We conducted methyl tetrazolium (MTT) colorimetric assay to evaluate the toxicity of MNPs and chemotherapeutics in PANC-1 and HEPA-RG cell lines, determining their concentration to be used in the *in vitro* experiments. Our data indicated the exposure of pancreatic cancer to pure antineoplastic agents (Figure 2.5a-c) did not promote the reduction in cell viability in all concentrations tested. These results were confirmed by apoptosis (annexin⁺) and necrosis (7AAD⁺) assays performed in flow cytometer (Figure 2.5d-g). In contrast, pure GEM-PTX increased treatment effectiveness in HEPA-RG (Figure 2.5h-j), demonstrating the importance to use nanoparticle to inhibit side effects in untargeted cells.

Pure MNPs did not exhibit toxicity after 24h and 48h (Figure A3a). Meanwhile at 1×10^9 part./ml, MNPs-GEM reduced tumor cell viability in 30% after 24h of incubation with an increase in toxicity after 48h (Figure A3b).

For nanoparticles encapsulated with PTX, cell viability was between 60% and 80% after 24h, and the low concentration of drugs allowed tumor cells to retake growth after 48h (Figure A3c). The simultaneous delivery of two first-line chemotherapeutics (MNPs-GEM-PTX) increased the nanoparticle toxicity, exhibiting greater efficacy at low concentrations in the period of 48h (Figure A3d). Consequently, we chose to work with 1×10^8 parts./ml in the further tests with cancer cells and healthy peripheral blood cells.

Because nanoparticles contained parts of pancreatic tumor cell membranes, we determined their specificity upon their incubation with healthy liver cell lines (HEPA-RG). The toxicity of MNPs-GEM and MNPs-PTX (Figure A4b-c) was similar to PANC-1 after 48h, but MNPs-GEM-PTX did not inhibit cell proliferation at low concentrations (Figure A4d). Additionally, in Figure 2.6, we sum up the statistical relevance between PANC-1 and HEPA-RG after treatment with GEM+PTX and nanovesicles loaded with both molecules (MNPs-GEM-PTX), demonstrating a higher activity of chemotherapeutics in neoplastic pancreatic cells than hepatic cell lines when delivered by MNPs at the same molar concentration.

Figure 2.5 – Cytotoxicity analysis of GEM and PTX in pancreatic cancer cells (PANC-1) by methyl tetrazolium reduction (MTT). PANC-1 (1×10^4 cells per well) was exposed to five concentrations of pure GEM (100; 75; 50; 25; 5 μ M) (a), PTX alone (5; 3.75; 2.5; 1.25 and 0.25 nM) (b) and simultaneous exposure with both drugs (GEM-PTX) (c). We also analyzed cancer cell death by flow cytometry after treatment with GEM/PTX, labeling with annexin-V (FL2 channel), and 7AAD (FL3 channel). PANC-1 (1×10^5 cells per well) were exposed to 100 μ M of GEM (d), 5nM of PTX (e), and both GEM-PTX (f) to observe the cytotoxic response at the maximum drug concentration used in MTT assay. Mean and SD of 3 independent assays (g). HEPA-RG (1×10^4 cells per well) were exposed to GEM (100; 75; 50; 25; 5 μ M) (h), PTX (5; 3.75; 2.5; 1.25 and 0.25 nM) (i), and GEM-PTX (j) to observe chemotherapeutic activity in healthy cell line.

Source: By the author.

We observed the specificity and the superior cytotoxic effects produced by plasma-membrane derived nanoparticles in contrast to synthetic lipid nanostructures (Figure A5). Antineoplastic agents were also delivered by liposomes synthesized with phosphatidylcholine, phosphatidylserine, and cholesterol (products obtained from Avanti® Polar Lipids) into pancreatic cancer cells. Synthetic liposomes loaded with Gemcitabine and/or Paclitaxel (Lipid-GEM, Lipid-PTX, and Lipid-GEM-PTX) demonstrated to be less effective than MNPs groups (Figure A5a-d). Interestingly, MNPs-GEM-PTX exhibited a

superior activity in comparison to Lipid-GEM-PTX, indicating that cell membrane components aid particle interaction in tumor cells.

2.3.4 Cell death induced via apoptosis and/or necrosis

Although MTT experiments indicated metabolic activity in the cells, we decided to verify cancer cell death using Annexin-V/7AAD assay by flow cytometry. Aiming to investigate whether the MNPs-GEM-PTX enhances the effectiveness against pancreatic cancer cells, the induction of apoptosis and/or necrosis cell death was analyzed by labeling annexin-V and 7-AAD. Annexin-V bind to phosphatidylserine on the cellular membrane. Cells undergoing apoptosis expose this phospholipid on the external surface, enabling the binding of annexin. Moreover, osmotic lysis creates pores on the plasma membrane, allowing 7-AAD to infiltrate and labeling necrotic cells. After exposure to MNPs-GEM-PTX, PANC-1 cells undergone a higher cell death ($7\text{AAD}^+ = 26$; Annexin $\text{V}^+ = 27\%$) (Figure 2.7a) than HEPA-RG ($7\text{AAD}^+ = 6\%$; Annexin $\text{V}^+ = 8\%$) (Figure 2.7b). The combination of PTX and GEM in a single nanoparticle (MNPs-GEM-PTX) increased the cytotoxic effects in neoplastic cells in comparison to MNPs-GEM and GEM alone, indicating that microtubule-stabilizing agent favors gemcitabine activity as revealed by previous studies in clinical trials with pure anti-neoplastic drugs.¹⁶¹

Figure 2.6 – Cytotoxicity analysis in pancreatic cancer cells (PANC-1) (a) and healthy liver cells (HEPA-RG) (b) by methyl tetrazolium reduction (MTT). PANC-1 and HEPA-RG (1×10^4 cells per well) were exposed to chemotherapeutics molecules (GEM+PTX) and MNPs encapsulated with both drugs (MNPs-GEM-PTX) in the same molar concentration to verify their specificity and toxicity (* $p \leq 0.05$).

Source: By the author.

Figure 2.7 – Effects of plasma membrane derived-nanoparticles on viability of tumor (PANC-1) (a) and healthy cells (HEPA-RG) (b) (Annexin V⁺ and 7AAD⁺) after 48h exposed to MNPs, MNPs-GEM, MNPs-PTX, MNPs-GEM-PTX (*p ≤ 0.05).

Source: By the author.

2.3.5 MNPs effects on monocyte and DCs phenotype from healthy donors

Since monocytes intensely phagocyte MNPs, we examine if such events would affect cell phenotype and immune responses. Human monocytes and dendritic cells (DCs) were exposed to nanoparticles and the expression of surface markers necessary for antigen-presenting activity was evaluate. Nanoparticles fabricated with GEM and PTX (MNPs-GEM-PTX) increased HLA-DR expression while other treatments did not exhibit equal response (Figure 2.8a-b and 2.9a-b). We also observed that MNPs activate co-stimulatory markers, such as CD80, CD83, CD86, and PD-L1 (Figure 2.8c-j and 2.9c-j), whereas pure GEM-PTX treatment did not exhibit the same effect. The exposure to MNPs promoted an intense phenotype change in APCs, indicating that nanoparticles interfere in immune cells activation, as confirmed by HLA-DR⁺, CD80⁺, CD83⁺, and CD86⁺ expression.

2.3.6 Mixed Lymphocyte Reaction (MLR) assays.

The main function of DCs is to present antigenic material to T lymphocytes. DCs capture foreign antigens, process and express the fragments (epitopes) associated with major histocompatibility complex (MHC) to be recognized by T cells. Since lymphocytes are

stimulated to proliferate after binding the alloantigen molecules on DCs, they are co-cultivated in Mixed Lymphocyte Reaction (MLR) assays to reproduce immune cell's interaction between MHC and T-cell receptors *in vitro*. Here, DCs were previously treated with MNPs for 48h and co-cultured with allogeneic lymphocytes (from different individuals) at the end of this period. After 5 days, we analyzed the total T cell proliferation ($CD3^+$, $CD4^+$, and $CD8^+$), as show in Figure 2.10b, indicating a reduction in DCs ability to induce an allogenic response after exposure to MNPs-GEM-PTX, as well as GEM-PTX alone. Although we observed an inefficient allogeneic response from DCs after incubated with nanoparticles carrying chemotherapeutics, MNPs alone increased lymphocyte interaction with pancreatic cancer cells surface (Figure 2.10c and 2.10e). The proteins present in nanovesicles may be processed in DCs and show the antigenic material to total T cells, allowing lymphocytes to recognize pancreatic cell lines. These results were not reproducible in immature DCs (iDCs), immune cells not exposed to antigenic material present in nanovesicles and to any other experimental treatments (Figure 2.10d and 2.10f).

Figure 2.8 – Molecules expression on monocytes after different treatments. We observed the percentage of cells expressing HLA-DR⁺ (a and b), CD80⁺ (c and d), CD83⁺ (e and f), CD86⁺ (g and h) and PD-L1⁺ (i and j). The treatment groups were composed by MNPs, MNPs-GEM-PTX and GEM-PTX (*p ≤ 0.05).

Source: By the author.

Figure 2.9 – Expression of molecules on dendritic cells after different treatments with MNPs. We observed the percentage of cells expressing HLA-DR⁺ (a and b), CD80⁺ (c and d), CD83⁺ (e and f), CD86⁺ (g and h) and PD-L1⁺ (i and j). The treatment groups were composed by MNPs, MNPs-GEM-PTX and GEM-PTX. Results were normalized by Control⁺ (representing DCs that were treated with activation cocktail to differentiate into mature DCs -mDCs) and the Control⁻ (representing DCs that no receive treatments) (*p ≤ 0.05).

Source: By the author.

Figure 2.10 – Cytotoxicity of nanoparticles in human monocytes and DCs are illustrated by 7AAD⁺ (a) labeling after exposure to MNPs, MNPs-GEM-PTX, and GEM-PTX treatments. Total lymphocytes were co-cultured for 5 days with DCs to verify lymphoid cell proliferation. Analysis was performed after incubating total T cells with MTT salt, revealing the rate of proliferation after exposure to DCs previously treated with MNPs, MNPs-GEM, MNPs-PTX, MNPs-GEM-PTX, and GEM-PTX only (b). Optical microscopy images (10x and 60x magnification) exhibit the contact between total T cells on pancreatic cell surface. Blue square shows lymphocytes adsorbed on tumor cells membrane and in the yellow square just lymphocytes at the bottom of the culture well plate. The total T cells most frequently interact on neoplastic cells after exposed to MHC molecules from DCs treated with MNPs (c and e). The same rate of interaction is not observed using lymphocytes co-cultured with the negative control group (DCs that not received any treatment), increasing the number of lymphocytes alone (yellow square) (d and f).

Source: By the author.

2.4 DISCUSSION

We investigated plasma membrane-derived lipid nanoparticles to transport gemcitabine and paclitaxel, testing their effectiveness on pancreatic cancer cells and also their effects on immunocompetent cells. The toxicity of experimental groups was evaluated in tumor cells and their immunoregulatory properties in human lymphocytes, monocytes and dendritic cells (DCs) generated *in vitro* from healthy donors. We synthesize nanovesicles from the main components of neoplastic cells membrane to deliver two first-line chemotherapeutics since high expression of adhesion molecules favor their association in cancer receptors. Macromolecules residues, ex., from proteins, contribute to increase

nanoparticle dispersivity in aqueous suspensions, aiding to avoid particle agglomeration and facilitating the encapsulation of additional substances.¹⁶²

To incorporate larger amounts of antineoplastic agents in the nanoparticles, we extruded the real membranes with chemotherapeutic molecules through a polycarbonate membrane of 100 nm.¹⁶² After extrusion, physicochemical characterizations were performed to analyze the differences in nanocarriers structure before and after incorporation of different agents. Initially, we verified the presence of protein radicals on MNPs by FTIR. Specifically, we observed the bands at 1150, 1625, and 3400 cm^{-1} corresponding to C-N, $\text{CH}_2\text{-NH}_2$, and $\text{CH}_2\text{-NH-CH}_2$ groups presented in the primary and secondary amines from amino acids. The same pattern was also observed in experimental groups encapsulated with GEM and PTX. AFM, SEM and TEM microscopies revealed that none structural changes occurred in the nanoparticles spherical format after encapsulation of the chemotherapeutics, which exhibited sizes between 100 nm and 250 nm. Additionally, cryo-TEM analysis (Figure 2.2) showed a fuzzy smudge in the lipid bilayer of the MNPS-GEM-PTX, which indicated an interaction of the antineoplastic agents with the outer surface of lipid nanoparticles¹⁶³. The overshadows in the images of MNPs is intensified due to interaction with proteins, fragments of DNA, and RNA left by this method of isolation.¹⁶⁴ The presence of drug molecules on MNPs surface was confirmed by the zeta potential values close to zero in MNPs-GEM, MNPs-PTX, and MNPs-GEM-PTX groups at pH 7.4.

After labeling the MNPs with FITC, we analyzed the interactions between nanoparticles and tumor cell cultures and human monocytes by flow cytometry and confocal microscopy (Figure 2.4 and A2, respectively). MNPs-FITC⁺ revealed the interaction between nanoparticles with PANC-1 tumor cells, HEPA-RG cells and primary monocytes from healthy donors, observing a high median fluorescence intensity (MFI) in pancreatic cells FITC⁺ (which plasma membrane was extracted to fabricate the nanocomposite) and white blood cells.

Encapsulation efficiency was 50% for GEM, with a cumulative release of 25% in 48h (10^9 part./ml) at pH 7.4. Our data indicated that PANC-1 exposure to pure antineoplastic agents (GEM=100 μM and PTX=5 nM) does not reduce cell viability at the same molar concentration used in nanoparticles synthesis (5A to 5F). Previous results indicated a half-maximal inhibitory concentration (IC_{50}) of GEM and PTX above 100 μM and 10 nM, respectively.¹⁶⁵⁻¹⁶⁶ It was chosen low concentration of nanoparticle (1×10^8 parts./ml) to check the improvement produced by MNPs in the treatments. As observed in Figure 2.6,

pure chemotherapeutics were toxic to HEPA-RG and nanoparticles reduce drug activity in this healthy cell line. Additionally, the liposomes nanocarriers showed the same behavior as the free agents when interacting with PANC-1, proving the importance of the plasma membrane proteins for delivery.

The apoptosis/necrosis assay confirmed the low toxicity of MNPs in PANC-1 and HEPA-RG cells (Figure 2.7). However, we observed similar toxicity between nanoparticles encapsulated with only one chemotherapeutic, indicating low therapeutic efficiency of the drugs when carried individually in pancreatic cancer cells. PTX incorporation (MNPs-GEM-PTX) increased the nanoparticles toxicity, increasing cell death, which leads us to conclude the presence of PTX improved the cytotoxic capacity of MNPs-GEM. Therefore, our results suggested that MNPs-GEM-PTX could be more effective than pure GEM and pure PTX in a short period of treatment. This finding is similar with previous studies with nanoparticles conjugated with GEM plus administration of pure PTX, exhibiting greater toxicity than simple treatment with GEM on lung,¹⁶⁷ mammary, and ovary cancer cells.¹⁶⁸⁻¹⁶⁹

The most interesting result was observed in MNPs-GEM-PTX group, indicating that paclitaxel favors MNPs-GEM activity in tumor cells (Figure 2.7a and Figure A3). Our purpose to deliver antineoplastic agents inside neoplastic cells using plasma membrane-derived nanoparticle was successfully achieved since MNPs-GEM-PTX induced more apoptosis/necrosis in PANC-1 than in HEPA-RG. However, it was not sufficient to prevent nanoparticle interaction with monocytes, with MNPs changing their phenotype (Figure S6 and S7) and immune function.

Phagocytic cells engulfment can be used to improve nanocarriers efficiency. In monocytes, MNPs-GEM-PTX increased HLA-DR protein and costimulatory molecules CD80, CD83, and CD86 expression in monocytes, as shown in Figure 2.8a-h. This is a relevant result because the nanocomposites can help to re-educate immune-suppressed cells, activating their cytotoxic response in the tumor microenvironment and secondary lymphoid organs. In monocytes, MFI values indicated an increase in HLA-DR expression after exposure to nanoparticles, which did not occur for non-encapsulated PTX-GEM (Figure 2.8b). The high expression of HLA-DR is not unexpected since previous studies reported that low doses of GEM and PTX increases the frequency of this activation marker in dendritic cells, increasing immune-cell ability to elicit an anti-tumor response *in vitro*.¹⁷⁰⁻¹⁷¹ Furthermore, chemotherapeutics delivered by MNPs increased the expression of CD80 and CD86

molecules on the cells. Both molecules are essential in the process of antigen presentation by macrophages and dendritic cells to present antigenic peptides to CD4⁺ T cells. The expression of CD83 (maturation marker protein) was also analyzed, remaining constant in monocytes and increasing in DCs surface (Figure 2.8e-f, 2.9e-f). For taxol interferes in assembly and disassembly of microtubules during cell division,¹⁷² treatment with MNPs-GEM-PTX increases HLA-DR expression on dendritic cells due the effect on antigen processing and presentation via MHC on cell surface. Similarly to histocompatibility complex, DCs also increase the frequency of CD83 and CD86 after treatment with MNPs-GEM-PTX, suggesting that expression of costimulatory molecules may be dependent on antigen processing.

Since nanoparticles induced low cytotoxicity in monocytes (Figure 2.10a), we evaluated DCs antigen presentation capability induced by MNPs-GEM-PTX, observing their influence on DCs in mixed lymphocyte reactions (MLR) (Figure 2.10b). Our results demonstrated a high expression of maturation markers HLA-DR, CD80, CD86, and CD83 after 48h, which did not inhibit the suppressive effects of chemotherapeutics molecules carried by cancer cell membrane-derived nanoparticles in DCs. MNPs combined with chemotherapeutics could not prevent the lymphocytopenia in future patient treatments, which could be explained by the fact that the agents are interacting in the outer membrane, decreasing the zeta potential values and releasing free working drugs. It is possible that depression in allogeneic stimulation is a consequence of the GEM-PTX cytotoxicity. Since the concentrations examined in this study interfered in lymphocyte proliferation, we reinforce the importance to observe nanoparticles activity in host immune surveillance cells to achieve a safe antitumor response and to avoid the risk of inducing tolerance.

Nevertheless, the antigenic material carried in MNPs seems to promote low allogeneic stimulation. When we examine the activity of lymphocytes co-cultured with DCs treated with MNPs (without chemotherapeutic agents), a recognition of total T cells with pancreatic cancer cells was observed (Figure 2.10c and 2.10e), as a result of the delivery cell membrane proteins. The same was not observed for DCs without any treatment (iDCs) (Figure 2.10d and 2.10f).

In summary, we developed a novel nanostructure for enhanced cancer immunogenicity and to modulate the activity of antigen-presenting cell. We synthesized nanoparticles with the main components of pancreatic cancer cell membranes to deliver

chemotherapeutics in diseased cells and to transport immunomodulatory agents to carcinoma and immunocompetent cells. Our studies revealed that MNPs are efficient carriers of chemotherapeutics for pancreatic cancer cells, producing superior cytotoxic effects than pure chemotherapy. Although highly phagocytosed by monocytes, MNPs did not reveal immune toxicity, inducing expression of HLA-DR, CD80, CD83, and CD86. Our results contribute to understanding the application of MNPs as antineoplastic agents nanocarriers and their effects on pancreatic cancer and immunocompetent cells. Therefore, this study aid nanomedicine and immunotherapy to complement each other, encompassing their knowledge to create new therapies to reduce adverse effects of treatments and to increase clinical results in the future.

CHAPTER 3 NEOPLASTIC CELL MEMBRANE-DERIVED NANOVESICLES FOR PROSTATE CANCER THERAPY

ABSTRACT

Prostate carcinoma is the second most frequent chronic disease in men, with 360,000 deaths in 2020. Currently, late diagnoses and disease relapse demand novel therapeutic interventions to improve treatment efficiency in patients. Advances in nanoengineering allow the synthesis of cellular plasma membrane-derived nanovesicles (MNPs) to prolong the permanence of antineoplastic molecules in the bloodstream and to target drug activity on cancer cells by homotypic binding. Current studies are investigating the feasibility of MNPs to address other chemotherapeutic molecules to cancer cells as a novel strategy to improve the effectiveness of antineoplastic agents. In this study, we isolate the major components from the prostate cancer membrane to form lipid nanoparticles encapsulating a first-line chemotherapeutic agent used in clinical treatments, *viz*, cabazitaxel (CTX). The MNPs were incubated in prostate cancer cells (PC-3) *in vitro*. The MNPs-CTX exhibited a diameter of 150 nm and a surface charge of -5 mV. The interaction/adherence of MNPs/CTX to target cells was analyzed by fluorescence microscopy, confirming MNPs internalization. The MNPs/CTX complexes did not promote a better antineoplastic response like CTX, but the nanoparticles were easily captured by the diseased cells.

3.1. INTRODUCTION

Prostate cancer responds for 7.3 % of chronic diseases in men, with 1.4 million new cases registered in 2020.¹ The lack of exams leads to diagnosis in the late stages, favoring future disease relapses and metastasis progression in the organism.¹⁷³ Hormone blockade and surgery are the main treatments during prostate illness.¹⁷⁴ If disease disseminates in the body, the intravenous drug prolongs patient survival for 5 years in 30% of diagnosed people.¹⁷⁵

The most common class of drugs used to treat prostate cancer include microtubule-stabilizing agents, like taxols (docetaxel, paclitaxel, and cabazitaxel).¹⁷⁶ The therapeutic use of cabazitaxel (CTX) leads to cell cycle arrest at G2/M phase, inducing chromosome

segregation, which leads to anti-cancer effects.¹⁷⁶ Although this treatment inhibits cellular division, the maximum tolerated dose application produces side effects, including neurotoxicity and neutropenia.¹⁷⁷ Whereas the antineoplastic cytotoxicity compromises the patient's ability to fight cancer, novel procedures use nanoparticles (NPs) to address drug molecules to target cells.¹⁷⁸

Currently, liposomes and polymeric nanocomposites are the most used NPs in the clinic, with their physicochemical properties improving therapeutic strategy in primary and metastatic sites.^{10,64} These nanostructures carry drugs for therapeutic uses, like Doxil to treat ovarian cancer and nab-paclitaxel to treat breast, liver, lung, pancreatic, and prostate neoplasms.^{10,64} Once captured by an endocytic process, NP avoids drug ejection by cellular pumps or ATP carrier proteins (ABC transporters), preserving a high concentration of drugs in the cytoplasm with long cytotoxic activity.⁶⁵

Lipid nanoparticles are the most investigated nanocarriers to deliver chemotherapeutics, being easily adsorbed with antibodies or aptamers and allowing the "addressing" at disease sites.¹⁷⁹ Moreover, there are novel strategies to improve NPs internalization by tumor cells. For example, nanoengineering procedures isolate lipids and proteins from cellular surfaces to synthesize nanovesicles with the major components of the plasmatic membrane (MNPs).^{74,112-116} The presence of cellular adhesion proteins improves nanostructure specificity, with excellent internalization by the cells where membrane residues came from.¹²⁹⁻¹³⁰ Recently, nanostructures loaded with gemcitabine showed a higher cytotoxic effect in pancreatic cancer cells than pure chemotherapeutic *in vitro*.¹⁸⁰ In the same study, it was demonstrated that nanovesicles fabricated with membrane lipids (phospholipids, glycolipids, and cholesterol) are very stable in the culture medium.¹⁸⁰

We synthesized lipid nanoparticles with the main components from the prostate cancer cell membrane to deliver CTX (MNPs-CTX) in malignant cells aiming at reducing drug cytotoxic activity in healthy cells. Nanoparticle specificity was checked after exposing them to PC-3 cell line, demonstrating that MNPs can be used to targeted drug molecules in diseased cells in the future.

3.2 METHODOLOGY

3.2.1 Experimental design

Proteins and lipids from the cellular plasma membrane were obtained from 1×10^7 prostate cancer cells. PC-3 cell was washed three times with PBS and, as previously described by Lung *et al.*,¹¹⁷ lysed in a hypotonic buffer for 20 min at 4 °C followed by homogenization. It was necessary to remove unlysed cells and cell debris by centrifugation at 15,000 g for 5 min at 4 °C. Finally, we collected the supernatant by centrifugation at 100,000 g for 2 h. The pellet containing cell membranes (obtained using an Optima MAX-XP ultracentrifuge (Beckman Coulter, USA)) was dispersed in 1 ml of PBS containing Complete Protease Inhibitor (Roche Diagnostics, Mannheim, Germany) and stored at -80°C. Nanoparticles (MNPs) were extruded through a 100 nm membrane and quantified the protein concentration in the samples by Bradford colorimetric assays (Sigma-Aldrich).

In order to increase CTX dispersity in aqueous solutions, drug molecules were adsorbed in polyethyleneimine (PEI - Mw ~25,000 kDa) to incorporate chemotherapeutics into MNPs. The nanocarriers were incorporate with PEI-CTX to evaluate their ability to induce apoptosis in prostate cancer cells. MNPs were labeled with fluorescein isothiocyanate to test treatment specificity and to check adherence/internalization by PC-3 cells.

3.2.2 Physicochemical characterization of MNPs

MNPs and MNPs-CTX samples were analyzed by AFM (Nanosurf model EasyScan 2 FlexAFM) to observe their external density and to verify the presence of adsorbed or incorporated materials. UV-VIS spectroscopy (Hitachi model U2008) was used to quantify chemotherapeutic molecules encapsulated in MNPs and to monitor their release over 48 h. We evaluate nanoparticle polydispersity and surface potential (ξ) in a Malvern Zetasizer Nano-ZS and confirm MNPs size distribution using nanoparticle tracking analysis (NTA - Malvern NanoSight NS300). Dynamic light scattering (DLS) and zeta potential were employed to evaluate nanoparticles stability in PBS solutions.

3.2.3 Incorporation of chemotherapeutic drugs into MNPs

First, 1 mg of EDC and 1 mg of NHS were added in PEI solution (1 mg/ml) and agitated for 30 min. Next, CTX (1 μ M) were stirred in PEI for 4h. MNPs-CTX were prepared by mixing 1 ml of MNPs suspension (10^9 particle/ml) with 100 μ l of PEI-CTX dissolved in PBS. At room temperature, these systems were extruded 20 times through a 100 nm polycarbonate membrane using a mini-extruder (Avanti Polar Lipids). The solution was sonicated for 30 min. at 40 kHz.

3.2.4 MNPs staining with fluorescein isothiocyanate and analysis of their interaction with cells

MNPs dispersions (10^9 part./ml) were labeled with 0.1 mg of fluorescein isothiocyanate (FITC) for 24 h under agitation. To separate the unconjugated fluorescein, MNPs-FITC were recovered by centrifugation, washed with PBS, and dialyzed for 12 h in a 12K Dalton membrane. These nanostructures were dispersed in PBS and kept in a dark tube at 4°C, for further assays. FITC⁺ nanovesicles were used to prove the specificity of MNPs in prostate cancer cells (PC-3), with confocal microscopy evaluating nanoparticles interaction with the cells (ZEISS LSM 900). Cell nuclei were stained with DAPI, acquired from ThermoFisher. We previously fix the cells with 4% formaldehyde and washed the cells twice with Hank's balanced salt solution (HBSS) followed by dispersion in 300 μ l of DAPI at 300 nM for 3 min. Cells were imaged in ZEISS Scanning Confocal Microscope from Nanomedicine and Nanotoxicology Group, GNano, equipped with argon diode, and HeNe laser lines, with excitation sources in 350 nm, 490 nm, and 595 nm wavelengths.

3.2.5 Tumor cells culture

Prostate adenocarcinoma (PC-3) were cultured in complete Roswell Park Memorial Institute (RPMI) 1640 Medium [RPMI medium supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% non-essential amino acids, 1% antibiotic and antimycotic (Life Technologies), and HEPES (Sigma)] at 37 °C under 5% of CO₂. When the monolayer reached 80% of confluence, we used 0.05% trypsin-EDTA (Gibco) to release cells from bottle surface

and wash in complete culture medium to future use in cytotoxic and apoptosis/necrosis assays.

3.2.6 Cytotoxicity assays (MTT)

The cell lines were seeded in 96-well plates and incubated with nanoparticles for 24 h and 48 h. MNPs cytotoxic effects were tested on tumor cells by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) based colorimetric assay. In mitochondrial living cells, the salt is reduced to formazan crystals for indicating their activity and viability. 10^4 cells/well were cultured in a flat bottom plate and challenged with: a) pure MNPs; b) MNPs extruded with PEI (MNPs-PEI); c) MNPs encapsulated with PEI-CTX (MNPs-CTX); d) pure CTX; and maximal lysis with DMSO (positive control group). Supernatants were removed and 100 μ l of MTT solution at $10 \mu\text{g}.\text{ml}^{-1}$ was added to each well at the end of the culture period. After 2 h of incubation, the formazan crystals in mitochondria were solubilized with DMSO for reading in a spectrophotometer at 580 nm.

3.2.7 Statistical analysis.

All functional assays were repeated at least 3 times and data analysis was submitted to analysis of variance (ANOVA) and Tukey-Kramer mean comparison test, considering differences with error probability less than or equal to 5% ($\alpha \leq 0.05$).

3.3. RESULTS

3.3.1 Physicochemical characterization of the nanocomposites

The main components of the plasma membrane were extracted from prostate cancer cells (PC-3) and extruded through a polycarbonate membrane of 100 nm. MNPs exhibited dimensions between 50-250 nm as revealed by DLS and NTA, with a negatively superficial charge at pH 7.4. However, only the Zeta potential of MNP without PEI presented a negative value in the buffer solution (Table 3.1). The morphology of MNPs was investigated by atomic

force microscopy (AFM), confirming previous results from DLS and NTA with nanoparticle vesicular morphology around 50 nm (Figure 3.1).

Table 3.1 - Particle size and zeta potential based on NTA and DLS data.

	Size (nm)		Zeta Potential
	NTA	DLS	ζ (mV)
MNPs	199.1 $\pm$ 3.8	207.2 $\pm$ 8.2	-7.8 $\pm$ 1.0
MNPs-CTX	189.6 $\pm$ 4.3	200.3 $\pm$ 1.0	-7.8 $\pm$ 1,0
MNPs-PEI	180.5 $\pm$ 4.3	213.9 $\pm$ 7.2	10.0 $\pm$ 0.4
MNPs-PEI-CTX	196.9 $\pm$ 4.9	229.5 $\pm$ 23	9.1 $\pm$ 1.0
Liposome	126.0 $\pm$ 1.6	129.4 $\pm$ 2.0	-5.61 $\pm$ 0.07
Liposome-CTX	136.8 $\pm$ 2.1	133.2 $\pm$ 0.7	-5.2 $\pm$ 0.6

Source: By the author.

Figure 3.1 – Atomic force microscopy (AFM) of plasma membrane nanoparticles (MNPs) reconstructed in Z-axis (A) and three-dimensional (3-D) (B) imagem. Nanoparticle tracking analysis (NTA) was used to evaluate nanoparticle's size (C) (result from three measures).

Source: By the author.

3.3.2 Specificity of MNPs to PC-3 cells

The specificity of MNPs at neoplastic cells was investigated by conjugating fluorescein isothiocyanate (FITC) on the nanoparticle's surface. FITC-labeled MNPs (MNPs-FITC) can be detected by fluorescence microscopy when excited at a wavelength of 488 nm. We confirmed MNPs-FITC internalization by confocal microscopy, labeling the cell nucleus with DAPI (blue). Fluorescence microscopy analyses show nanoparticles into cell cytoplasm and fluorescent spots in the cytoplasm (see arrows in Figure 3.2b and 3.2c), suggesting MNPs internalization in PC-3 tumor cell.

Figure 3.2 – 3D confocal microscopy of prostatic cancer cells (PC-3) before (A) and after 24 h exposition to FITC labeled nanoparticles (B-C). Incorporation of MNPs-FITC is exhibited by green fluorescence in cell cytoplasm while nucleus are stained using DAPI.

Source: By the author.

3.3.3 MNPs-CTX cytotoxic in neoplastic cells

The cytotoxic effects of MNPs-CTX was evaluated by methyl tetrazolium (MTT) colorimetric assay in PC-3 cell lines. The nanocarriers were used in the experiments for 24 h and 48 h, determining their non-lethal doses and the toxicity concentrations. In neoplastic cells, pure CTX did not present alterations in cell viability in all concentrations for 24h, and neither the blank MNPs. MNPs-CTX did not reduce PC-3 viability after 24 h and 48 h at any concentration tested, not increasing the CTX treatment efficacy (Figure 4).

Figure 3.3 – Cytotoxicity analysis of MNPs in prostatic cancer cells (PC-3) by methyl tetrazolium reduction (MTT). PC-3 (1×10^4 cells per well) were exposed to synthetic liposomes (A), liposomes loaded with CTX (B), pure MNPs (C), MNPs-CTX (D), MNPs-PEI (E), MNPs-PEI-CTX (F) and pure CTX (G) to verify their response.

Source: By the author.

3.4 DISCUSSION

The ability of nanocarriers to target tumor cells is one of the main challenges to improve their therapeutic use. We decided to fabricate lipid particles with the main constituents of the cellular membrane, in an attempt to address nanocarriers to specific cell populations.¹²⁹⁻¹³⁰ We investigated the transport of cabazitaxel by MNPs, testing their effectiveness on prostate cancer cells.

The synthesis of the nanocarriers with polyethyleneimine and the antitumor agent cabazitaxel was followed by physical characterizations that demonstrated changes in the sequence of incorporation of the different materials. Indeed, PEI improved MNPs dispersivity in PBS buffers, aiding the encapsulation of additional substances to treat prostate cancer. Initially, nanoparticle morphology was confirmed by AFM microscopy, exhibiting nanoparticles with a spherical format with sizes between 100 nm and 200 nm. We confirmed the drug encapsulation by observing the alteration in MNPs zeta potential. Further incorporation of CTX was evidenced by changing the superficial negative charge (MNPs) to positive charge (MNPs-CTX) at pH 7.4.

The incorporation of FITC on the MNPs surface allowed the imaging of their interactions with PC-3 tumor cells. Confocal microscopy revealed the FITC⁺ containing cells after incubated with nanoparticles. MTT assays confirmed the low toxicity of MNPs in PC-3 cells, while the MNPs-CTX complex showed the same toxicity as the pure CTX, inducing the same percentage of cell death. MNPs were located in the cell cytoplasm without observing an increase in toxicity, which leads us to conclude that such nanostructure has the potential to increase the specificity of treatments to desired cells. Although our results suggest that MNPs-CTX have the same result as pure CTX, nanoparticles could improve CTX treatment in a period beyond 48 h.

Our attempt to target MNPs to tumor cells was successful, as observed by confocal microscopy on PC-3 cells. This is a relevant finding because it is desirable that nanocarriers could be easily captured by tumor cells without disrupting healthy cells. However, the effectiveness of the treatment proposed here was not sufficient to improve CTX toxicity, since both MNPs-CTX and CTX caused the same rate of cell death. Thus, it is necessary to investigate other methodologies to improve drug delivery by MNPs.

CHAPTER 4 CANCER CELL MEMBRANE-DERIVED NANOPARTICLES BLOCK THE EXPRESSION OF IMMUNE CHECKPOINT PROTEINS ON CANCER CELLS.

ABSTRACT

Cancer is the most recurrent chronic disease in the world, with human hepatocellular carcinoma (HCC) being the second leading cause of death among neoplasias. The high frequency of HCC relapse and metastasis warrants the development of new diagnostic and therapeutic procedures. In advanced stages, neoplastic cells can evade immune surveillance and express immunosuppressive proteins and cytokines at tumor sites. Nanocomposites conjugated with immunomodulatory agents can increase the main mechanisms of cellular immunity. In this study, we used nanocarriers to transport oligonucleotide sequences (siRNAs) into cancer cells and leukocytes to modulate the activity of tumor microenvironment cells *in vitro*. Cell membrane-derived nanoparticles (MNPs) were fabricated with lipids and proteins from the plasma membrane of hepatic tumor cells to deliver a large amount of antigenic material to professional antigen-presenting cells (APCs), following their exposure to HCC and immunosuppressive (M2) macrophages. To establish a pro-inflammatory response, lipid MNPs were incorporated with monophosphoryl lipid A and siRNA to silence the c-MYC (myelocytomatosis) oncogene. Nanocarriers were tested for the following: a) NP internalization into cancer and immunocompetent cells; b) immunomodulatory activity by observing the expression of cell surface markers; and c) *in vitro* cytotoxicity. The adsorption of plasma proteins on the MNPs surface and their effects on cellular uptake were also investigated. Our results indicate that the nanostructures are stable in biological suspensions, and can reduce CD47 and PD-L1 expression on cancer cells and simultaneously switch APC activity for an anti-tumor response.

4.1 INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer in men and the ninth in women, with 746,000 deaths and 554,000 new cases in the world.¹⁸¹⁻¹⁸² When hepatic neoplasia is diagnosed in advanced stages, the disease progresses quickly with a short survival period among the patients.¹⁸¹⁻¹⁸² Radiotherapy and chemotherapy exhibit low

therapeutic response, necessitating surgery to remove the tumor or perform liver transplantation.¹⁸³⁻¹⁸⁴

In neoplastic sites, the tumor microenvironment is composed of several types of cell lines, with monocytes and macrophages adopting an immunosuppressive phenotype for disease progression.¹⁸⁵⁻¹⁸⁷ In tissues, macrophages are classified according to their proinflammatory potential (classical activation [M1 macrophages]) or immunosuppressed responses (alternative activation [M2 macrophages]). Both cells are important for controlling the immune system during exposure to a foreign substance, cicatrization processes, and organ transplantation. M1 and M2 macrophages express different cytokines and chemokines responsible for producing distinct cellular activities.^{186,188} M1 macrophages are involved in the inflammatory response, pathogen capture, and antitumor immunity, stimulating naïve T-cells response to T helper type 1 (Th1) cells, which have a cytotoxic response in diseased cells.¹⁸⁸ In opposition, M2 macrophages are recruited to promote pro-tumorigenic properties, such as secretion of immunosuppressive cytokines, providing a favorable microenvironment for tumor progression *in vivo*.⁸²⁻⁸³ Tumor-associated macrophages (TAMs) have a similar phenotype to alternative-activated macrophages (M2), promoting the expression of immunosuppressive cytokines (IL-4, IL-10, and IL-13) and amplifying Th2 response.¹⁸⁹ M1 or M2 cells are derived from undifferentiated macrophages (M0); consequently, there is a possibility to switch the immunosuppressive role of TAMs for a phenotype similar to M1 macrophages.⁸⁴ Recently, studies have highlighted that the proto-oncogene c-MYC (myelocytomatosis) controls cell metabolism, proliferation, and apoptosis. c-MYC also regulates the secretion of inflammatory and anti-inflammatory cytokines, with an important role in immune cell differentiation and activation.¹⁹⁰⁻¹⁹¹ In macrophages, c-MYC regulates the expression of genes associated with M2 macrophages and TAMs.¹⁹²⁻¹⁹³ The MYC protein is suppressed in pro-inflammatory cells, indicating the possibility of modulating macrophage function after suppressing the expression of genes associated with alternative activation.^{191,193}

In human neoplasms, c-MYC influences the expression of immune checkpoint proteins, such as CD44, CD47, and PD-L1.¹⁹⁴⁻¹⁹⁶ These proteins prevent tumor cell phagocytosis by the innate immune system and inhibit CD8⁺ T cell activity.^{194,196-198} Studies have shown that c-MYC blockage reduces tumor proliferation and induces cancer cell apoptosis *in vitro* and *in vivo*. For example, Casey *et al.*¹⁹⁵ confirmed the inactivation of c-

MYC using short hairpin RNA (shRNA) and showed how this mechanism interferes with mRNA transcription of CD47 and PD-L1 proteins in human hepatocellular, renal, and colorectal carcinoma cell lines.

Currently, viral vectors are used to perform genetic engineering, but the high cost of such treatment requires a new, faster, and cheaper transfection tool. In contrast to viral vaccines, nanocarriers can effectively deliver interfering nucleotide sequences (iRNA) to reduce the expression of regulatory proteins and enhance the antitumor effects of usual treatments.¹⁹⁹ In the last decade, lipid nanoparticles (NPs) deliver messenger RNA (mRNA) to induce antigen expression in low immunogenic cells, enabling adaptive and innate immune cells to identify disease sites.²⁰⁰⁻²⁰² With this knowledge, Warashina *et al.*²⁰³ reported the inhibition of tumor growth using cationic liposomes (YSK12-MEND) that escape from endosomes and release siRNA into the cytoplasm. The vaccine stimulated an inflammatory response after administration, silencing gene expression in T cells, monocytes, macrophages, and NK cells more efficiently than commercial transfection reagents, such as RNAiMAX.²⁰⁴

Although nanotechnology has been used in cancer treatment, it is necessary to improve its therapeutic procedures. For example, the lipid composition of liposomes alters the humoral response after exposure to vesicular particles. Hemodynamic and hematological conditions are observed to avoid adverse effects, such as complement activation-related allergy (CARPA).^{97,100,103} The activity of plasma proteins during enzymatic cascades, such as kallikrein-kinin and clotting processes, trigger hormonal and inflammatory responses that modify body functionalities.²⁰⁵ Studies show that the adsorption of complement proteins on lipid nanostructures produces cardiac arrhythmias, hyper or hypotension, and leukopenia in mice.¹¹¹ For example, liposomes conjugated with high concentrations of cholesterol molecules induce leukopenia and thrombocytopenia, requiring the investigation of nanoparticle activity in extracellular fluids.^{103, 111}

To overcome these side effects, recent advances in nanoengineering have yielded nanostructures coated with the main components of the plasma membrane of healthy cells or neoplastic cells for drug delivery.^{18,206} In these processes, cellular membranes are isolated by ultracentrifugation to form lipid nanovesicles with novel physicochemical properties. Plasma membrane-derived nanoparticles (MNP), which have a long blood circulation time, regulates immune cell responses.¹⁸⁻¹⁹ Lipids and proteins from the cellular surface are reconstructed as nanovesicles to deliver a large amount of antigenic material to antigen-

presenting cells (APCs). These nanocomposites prevent the production of immunosuppressive cytokines and promote immune cell responses.¹⁶² For example, Fang *et al.*¹⁶² investigated the immune cell response of polymeric nanoparticles coated with B16-F10 (murine melanoma lineage) plasma membrane to carry immune adjuvant monophosphoryl lipid A (MPLA). Synthetic MPLA (derived from lipopolysaccharide) is an FDA-approved adjuvant in vaccines with non-specific immunostimulant activity. Although less potent than LPS, but with effective immunomodulatory properties, MPLA activates immune cells, such as monocytes and dendritic cells (DCs), to produce pro-inflammatory responses.²⁰⁷ Such nanostructures stimulate murine DC maturation after the delivery of tumor antigenic materials and immune adjuvants, increasing the expression of cellular activation markers (CD40, CD80, and CD86) and enhancing co-stimulation in naive lymphocytes.¹⁶²

In the present study, we developed plasma membrane-derived nanostructures (MNPs) to enhance cancer immunogenicity and modulate the activity of APCs. MNPs were synthesized from hepatocellular carcinoma cells to deliver siRNA and MPLA into cancer cells and immune-suppressed monocytes, blocking the c-MYC oncogene to activate the pro-inflammatory response in macrophages. First, we analyzed the interaction of MNPs with human serum components using atomic force microscopy (AFM) and isothermal titration calorimetry (ITC). Furthermore, we investigated NP cytotoxicity in diseased and healthy cells by flow cytometry, with immunomodulatory properties confirmed by CD44, CD47, and PD-L1 expression in tumor cells, and observed the effects of M2 macrophage phenotype.

4.2 METHODOLOGY

4.2.1 Synthesis of the membrane-derived nanoparticles (MNPs).

The plasma membrane from hepatic carcinoma was isolated by ultracentrifugation, as described by Lung *et al.*¹¹⁷ Briefly, cells were detached from culture flasks, washed three times with phosphate-buffered saline (PBS), lysed in a hypotonic buffer for 20 min at 4°C, and homogenized. The lysed cells were centrifuged at 15,000g for 15 min at 4°C to remove cell debris. The supernatant was collected and centrifuged at 100,000g for 2 h using an Optima MAX-XP ultracentrifuge (Beckman Coulter, USA). The pellet with cell membranes

was dispersed in 1 mL of PBS containing complete protease inhibitor (Roche Diagnostics, Mannheim, Germany) and stored at -80°C. Next, nanovesicles were obtained after 20 consecutively extrusions through a 100 nm membrane. Membrane proteins present in the vesicles were quantified by Bradford colorimetric assays (Sigma-Aldrich) to determine the protein concentration in the samples.

4.2.2 Morphologic Characterization.

The morphology of MNPs was analyzed by transmission cryo-electron microscopy (cryo-TEM, JEOL 1400) and atomic force microscopy (AFM, Nanosurf model EasyScan 2 FlexAFM, Tap300-G probe) to measure the external density and dimensions of the nanoparticles, as well as to verify the presence of adsorbed or incorporated materials.

MNPs were stained with fluorescein isothiocyanate to analyze their interaction with the cell surface and cellular uptake.¹⁸⁰ Briefly, a mix of 10^9 particles dispersed in 1 mL of PBS and 0.1 mg of fluorescein isothiocyanate (FITC) was prepared for fluorescent labeling. The solution was stirred in the dark at 4°C for 12 h, and MNPs-FITC was separated from unconjugated fluorescein by dialysis on a 12,000 Da membrane. Finally, the samples were resuspended in PBS. FITC⁺ MNPs were used to confirm the incorporation of MNPs by cancer cells for 24 h, followed by analysis using confocal microscopy (ZEISS LSM 900).

4.2.3 Analysis of the MNPs stability in aqueous dispersions.

Dynamic light scattering (DLS) and zeta potential (Zetasizer Nano ZS90 spectrometer) were performed to evaluate the nanoparticle size distribution and surface potential. To evaluate the stability of MNPs, they were incubated in Dulbecco's modified Eagle's medium (DMEM) culture medium with 10% fetal bovine serum (FBS) under agitation for 4, 8, 12, 24, 36, and 48 h.¹⁵ Nanoparticle tracking analysis (NTA) (Malvern NanoSight NS300 instrument) was used to confirm the stability of MNPs in saline solution (pH 4.4 and 7.4) and culture medium supplemented with FBS.

4.2.4 Isothermal titration calorimetry (ITC) Analyses.

The interaction of MNPs with human plasma was evaluated by Isothermal Titration Calorimetry, ITC (Microcal ITC 200 Malvern). Prior to the analysis, human plasma was dialyzed in 1X PBS using a 12,000 Dalton membrane. Human plasma (5 mg mL^{-1}) was titrated into 5×10^8 particles mL^{-1} of MNPs or MNPs-MPLA at 25°C in 1X PBS. The first injection ($0.4 \mu\text{L}$) was followed by a series of $2 \mu\text{L}$ injections. Dilution heat data were acquired by titrating human plasma at the same concentration in dialyzed buffer containing 1X PBS. All ITC experiments were performed with a MicroCal 200 in triplicate, and the final curves were obtained using Origin software supplied by Malvern.

4.2.5 Incorporation of MPLA and oligonucleotides into MNPs.

A positively charged polyethyleneimine (PEI) polymer was used to facilitate the incorporation of interference oligonucleotides into MNPs.²⁰⁸⁻²⁰⁹ The membranes of hepatocellular carcinoma cells were extruded through a 100 nm polycarbonate membrane with a mini-extruder (Avanti Polar Lipids) to incorporate MPLA and the siRNA double-strand to block c-MYC activity in cancer cells and macrophages. Before extrusion, $10 \mu\text{L}$ of siRNA (10 nM) was adsorbed on PEI for 10 min on ice. MNPs-MPLA-siRNA was prepared by mixing 1 mL of the MNP suspension at 10^9 particles/mL with $2.5 \mu\text{L}$ of MPLA ($500 \mu\text{M}$) and siRNA-PEI dispersed in PBS and extruded through a 100 nm membrane in a Malvern nano-extruder at room temperature. The solution was centrifuged at $100,000g$ for 2 h and washed with PBS. After removal of unconjugated MPLA and siRNA, the complexes were resuspended in 1 mL of PBS under gentle sonication for 30 min at 40 kHz.

4.2.6 Cancer cell culture.

Human hepatocellular carcinoma cells (Hep-G2) were cultured in complete medium (MEM medium supplemented with 10% FBS, 1% sodium pyruvate, 1% non-essential amino acids, 1% antibiotic and antimycotic [Life Technologies], and HEPES [Sigma]) at 37°C under 5% CO_2 . Then, cells were released from the bottle surface using 0.05% trypsin-EDTA (Gibco) and washed in complete culture medium for use in the assays.

4.2.7 Cytotoxicity assay (MTT).

First, we analyzed the NP interaction with cells using the MTT (3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyltetrazolium bromide) colorimetric assay. MTT is reduced to formazan crystals through mitochondrial metabolism, reflecting the activity of living cells as an indicator of viability. The treatment was performed by adding the particles and chemotherapeutics in cell culture (1×10^4 cells per well) in a 96-well flat-bottom plate for 24 and 48 h. Pure MNPs and MNPs conjugated with MPLA-siRNA were tested. Cells exposed to dimethyl sulfoxide (DMSO) were used as the positive control (maximal lysis). The living cells were incubated with MTT solution for 3 h at 37°C under 5% CO₂. Then, the formazan crystals were solubilized by DMSO, and the absorbance of the solubilized dye was measured at 580 nm in a spectrophotometer (Spectramax M3).

4.2.8 Human monocyte culture.

Human peripheral blood monocytes were separated from the plasma by centrifugation at 1600 rpm for 35 min in a Ficoll-Isopaque gradient. Next, the cell suspension was centrifuged with 51% of Percoll gradient to separate monocytes from lymphocytes and dispense mononuclear cells into sterile plates (2×10^5 cells per well) for performing interaction assays.

4.2.9 Macrophage differentiation.

Monocytes from healthy donors were differentiated into macrophages (M1 and M2 polarization) in a culture medium supplemented with IFN- γ , LPS, IL-10, and TNF- α . After 6 days, adherent cells were harvested and washed with complete medium at a concentration of 2×10^5 cells per well. Macrophages were treated with MNPs carrying antineoplastic agents to evaluate cell viability, phenotype, and functions.

4.2.10 Evaluation of macrophage phenotype after exposure to MNPs.

Flow cytometry was used to analyze surface markers on macrophages after 48 h of treatment with monoclonal antibodies obtained from BD Bioscience. In monocytes (CD14⁺) and macrophages (CD64⁺ and CD163⁺), co-stimulatory molecules (CD80, CD83, and CD86), major histocompatibility complex class II (HLA-DR), suppressor molecule (PD-L1), and cell viability (7AAD⁺) were observed, based on gate quadrant analysis of working groups.

4.2.11 Indirect and direct co-culture.

To observe the ability of tumor cells to modulate the maturation and activation of monocytes after treatment with MNPs, M2 macrophages were co-cultured indirectly in Transwell plates (3 μ m pore size) with hepatic cancer cells. First, human peripheral blood monocytes were separated from the plasma by centrifugation at 1600 rpm for 35 min in a Ficoll-Isopaque gradient. Next, the cell suspension was centrifuged with 51% of Percoll gradient to separate monocytes from lymphocytes and dispense mononuclear cells into sterile plates (2×10^5 cells per well). Monocytes differentiated into M2 macrophages after stimulation with IL-4, IL-10, and TGF- β (M2 polarization). After 5 days, adherent cells were harvested and washed with complete medium at a concentration of 2×10^5 cells per well. Macrophages were treated with MNPs carrying antineoplastic agents for 48 h and evaluated using functional assays. On day 7, cell culture supernatants were removed and stored at -80°C for cytokine quantification by enzyme-linked immunosorbent assay (ELISA). Macrophages and Hep-G2 were indirectly co-cultured to check whether neoplastic proteins (present in the culture medium) can change the classical or alternative APC response. On day 9, the macrophages were collected from the Transwell and co-cultured directly with neoplastic cells for 48 h to check their capability to inhibit cancer growth. At the end of the study period, the total cells were incubated with MTT for proliferative analysis.

4.2.12 Statistical analysis.

All assays were repeated at least three times, and data were analyzed using ANOVA followed by Tukey's mean difference tests, considering differences with error probability less than or equal to 5% ($\alpha \leq 0.05$).

4.3 RESULTS

4.3.1 Morphology and stability of MNPs.

We performed cryo-TEM and AFM to analyze the morphology and distribution of MNPs (Figure 1) after isolation. The MNPs exhibited a size distribution of 40-400 nm. Cryo-TEM images allowed the visualization of a phospholipid bilayer and the vesicular spherical shape of the MNPs (Figure 1A-C). The AFM images show the MNPs with a maximum height of 44 nm (Figure 1D).

Figure 4.1 – Characterization of the isolation procedure of MNPs by cryo-TEM and AFM. Their distribution (A and B) and bilayer vesicular properties (C) are preserved after ultracentrifugation (with scales of 200 and 100 nm). Reconstruction of the Z-axis and three-dimensional (3-D) image of MNPs (D).

Source: By the author.

DLS and NP tracking analyses (NTA) were used to investigate the size and surface charge of MNPs in PBS at pH 4.4 and 7.4 (Table 4.1), as well as to estimate sample concentrations. The MNPs exhibited an average size of 160 nm after extrusion through a 100 nm pore polycarbonate membrane, confirming previous results demonstrated by cryo-TEM. The MNPs did not exhibit significant changes in their diameter after alternating from an acidic pH (4.4) to the physiological pH of arterial blood (7.4) but showed a slight reduction in the surface charge by an average of 3.5 mV.

Table 4.1 - Particle sizes and zeta potentials based on nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS) data (concentration at 1×10^9 part/mL).

	Size (nm)		Zeta Potential
	NTA	DLS	ζ (mV)
pH 4.4	160 ± 4	170 ± 2	-11.6 ± 2
pH 7.4	156 ± 1	170 ± 5	-15.2 ± 2

Source: By the author.

Before clinical trials, studies have tried to understand the opsonization of nanoparticles by plasma proteins to modulate allergic reactions and avoid severe side effects during the treatment.⁹⁹ When intravenously administrated, the protein corona of nanoparticles might be composed of albumin, fibrinogen, and other proteins of the immune system.^{97,99} Immunoglobulins account for 40% of proteins in human plasma and are the main constituent of protein corona on the surface of biomaterials.⁹⁸ Such an interaction between biomaterials and serum proteins can be reduced using specific polymeric coatings and decreasing the NP size for lower opsonization,^{99,210} but preliminary results are still inconclusive.¹⁰⁶

The stability of the MNPs in in cell culture medium (MEM) supplemented with 10% FBS was evaluated for 48 h. The average diameter and charge of the MNPs were measured by NS300 NTA equipment and Zetasizer Nano ZS, respectively. Our results indicated that the MNPs exhibited an increase in size (>200 nm) (Figure 2A) and a decrease in surface charge (<-15 mV) due to FBS protein adsorption on the nanocomposite surface (Figure 2B) when compared with saline dispersion. Both size and surface charge of the MNPs did not change after the formation of the protein corona between 4 to 48 h.

Figure 4.2 – Stability of MNPs in the culture medium over time. The size distribution was assessed by NTA analysis (a), and the surface charge was assessed by measuring the zeta potential (b) of MNPs (concentration of 10^8 particles/mL) at 4, 8, 16, 24, 36, and 48 h in RPMI 1640 medium containing 10% FBS at 37°C. The results represent the average diameter $\pm$ standard deviation of independent samples ($n=3$).

Source: By the author.

The stability of MNPs in the human plasma medium was also evaluated for 48 h. Unlike MNPs dispersed in cell culture medium, our results indicate an increase in the size (>200 nm) (Figure 3A and 3C) and a reduction in the surface charge (>-15 mV) of MNPs because of plasma protein adsorption on the nanocomposite surface (Figure 3B). DLS analyses revealed particle sizes of 200 to 800 nm, and NTA analysis revealed that MNPs had an average size of 150 nm, without significant surface charge alteration in each group for 48 h. MNPs without siRNA-PEI did not show changes in the structural parameters after the formation of the protein corona between 4 to 48 h. However, the presence of the PEI polymer increases the zeta potential of MNPs, which may indicate less adsorption of plasma proteins on the surface.

Figure 4.3 – Size (A), zeta potential (B), and NTA (C) of MNPs, MNPs-MPLA, and MNPs-MPLA-siRNA at 4, 8, 24, 36, and 48 h after exposure to human plasma.

Source: By the author.

We used AFM to analyze the morphology of MNPs after exposure to human plasma (Figure 4). The sequence in Figure 4A shows pure MNPs in different projections, allowing visualization of structural changes, such as the increase in size and appearance of hollow sphere nanostructures. MNPs functionalized with MPLA-PEI showed an increase in size, in accordance with DLS and NTA measurements, with a maximum height of 575 nm (Figure 4B). In agreement, ITC measurements showed that the human plasma titrated into the buffer (Figure 4C) had a small exothermic dilution heat due to the change in the tertiary structures of proteins at the concentrations used.²¹¹ When titrating the human plasma into MNPs, exothermic peaks were also observed (Figure 4D). Human plasma interacts with MNP-MPLA (Figure 4E) by exothermic reactions but without a saturation tendency in the analyzed injections. Comparing the heat rates with the volume titrated (Figure 4F), we observed that the interaction with MNPs overcame the effects of the dilution heat, and evaluation of three independent isotherms showed that MNPs-MPLA-PEI experimental group not reached saturation.

Figure 4.4 – Interaction analysis of MNPs and MNPs-MPLA with human plasma. Reconstruction of the Z-axis and three-dimensional (3-D) image of MNPs (A) and MNPs-MPLA (B) after the interaction with human plasma. Images at different magnifications, with scale bars at 10 and 1 μm , showed the formation of hollow MNPs with a maximum size of 500 nm. ITC isotherms of human plasma titrated into the buffer (C), MNPs (D), and MNPs-MPLA (E). Comparison of the heat rate by volume of human plasma titrated (F). The results represent the average $\pm$ standard error from three independent isotherms.

Source: By the author.

4.3.2 Establishing working concentrations.

We performed a MTT assay to evaluate the NP toxicity in hepatic cancer cells and determine the concentration of MNPs loaded with c-MYC siRNA (MNPs-MPLA-siRNA) for *in vitro* experiments. As shown in Figure 5A, MNPs at a concentration of 1×10^9 particles per mL (part/mL) reduced tumor cell replication by 20%, while the same concentration of MNPs-MPLA-siRNA (Figure 5B) practically tripled their ability to inhibit cell growth. Henceforth, we chose a concentration of 1×10^8 part/mL for use in our subsequent functional experiments.

Figure 4.5 – Cytotoxicity analysis of MNPs in hepatic cancer cells (Hep-G2) by MTT assay (A-B) and cell viability by Annexin (C) and 7AAD staining (D) (* $p \leq 0.05$).

Source: By the author.

4.3.3 Interaction of MNPs with Hep-G2 cells

We labeled MNPs with FITC molecules to analyze MNPs interactions on the cancer cell surface by flow cytometry and examine cellular uptake by confocal microscopy. MNPs-MPLA-siRNA strongly interacts with hepatic cancer cells, with a high percentage of FITC⁺ cells (Figure 6C) and high median fluorescence intensity (MFI) (Figure 6D) than MNPs only (Figure 6A-B).

Figure 4.6 – Incorporation of MNP and MNP-MPLA-siRNA into hepatic cancer cells based on the size and granularity parameters (A and C). The histograms represent the percentage of NP interaction on the cells (B and D). The green histogram represents FITC⁺ cells (cells incubated at 10⁸ particles/mL) and the gray histogram represents the unlabeled cell population.

Source: By the author.

Confocal microscopy confirmed the internalization of MNPs in Hep-G2 cells (Figure 7). In hepatic cancer cells, the green fluorescence did not interfere with the blue fluorescence from the cell nucleus. MNPs presented a regular distribution into the cytoplasm.

Figure 4.7 – Three-dimensional (3-D) confocal microscopy of hepatic cancer cells (Hep-G2) after 24 h exposure to MNPs (A-C) and MNPs-MPLA-siRNA (D-F). MNPs labeled with FITC exhibited green fluorescence in the cell cytoplasm, while the cellular nucleus was stained with DAPI. An increase in the frequency of green signals was observed in Hep-G2 cells exposed to MNPs-MPLA-siRNA.

Source: By the author.

4.3.4 The Effects of MNPs on the phenotype of tumor cells and monocytes from healthy donors.

Figure 8A reveals that a significant number of hepatic tumor cells exposed to different MNPs expressed CD44 molecules, while purified siRNA did not exhibit the same effect. MNPs loaded with siRNA/PEI (MNPs-MPLA-siRNA) reduced CD47 and PD-L1 expression on cancer cells (Figure 8B-C). We also observed that MNPs reduced CD47 and PD-L1 expression compared to the siRNA treatment and negative control groups.

Figure 4.8 – Regulatory effects of MNPs-MPLA-siRNA on the phenotype of Hep-G2 cells. Percentage (A-C) and MFI (D-F) of cells expressing CD44, CD47, and PD-L1 (* $p \leq 0.05$).

Source: By the author.

Previous results from our group revealed that MNPs were strongly internalized by monocytes.¹⁸⁰ Then, we checked whether this could influence the phenotype and immune cell function. We exposed human monocytes to different preparations of MNPs and observed the expression of classical surface markers responsible for antigen presentation activity. As shown in Figures 9 and 10, the macrophages exposed to the MNPs (and previously treated with cytokines for differentiation into M2 macrophages) showed significant changes in the expression of CD64, CD80, CD83, CD86, and CD163. The expression of these markers on the cell surface indicates interference in their activity. This is a relevant finding because nanostructures targeted to tumor cells can simultaneously interfere with the response of immunocompetent cells and induce pro-tumor activity.

Figure 4.9 – Regulatory effects of MNPs on M2 macrophage phenotype. The percentage of cells expressing CD11c, CD64, CD80, CD83, CD86, CD163, HLA-DR, and PD-L1 (* $p \leq 0.05$).

Source: By the author.

Figure 4.10 – Regulatory effects of MNPs on M2 macrophages phenotype. The MFI of CD80, CD83, CD86, CD163, HLA-DR, and PD-L1 (* $p \leq 0.05$).

Source: By the author.

4.3.5 Effects of MNPs on M2 macrophage function.

Our results indicated an increased expression of CD44 and reduced expression of CD47 and PD-L1 molecules on hepatic cancer cells after treatment with MNPs loaded with siRNA (MNPs-MPLA-siRNA). We also exposed human macrophages to different preparations of MNPs and observed the expression of classical surface markers responsible for antigen presentation activity (as shown in figures 9 and 10).

We next studied the macrophages response in Hep-G2 growth by indirect and direct co-cultures. The main function of APCs is to present antigenic material to T lymphocytes, with macrophages capturing foreign antigens, processing, and expressing the fragments (epitopes) associated with MHC to be recognized by T cells.^{95,212} Since monocytes were previously treated with MNPs carrying antigenic material from hepatic cancer cells, these cell lines were co-cultivated to reproduce the interaction of immune cells *in vitro* (Figure 11A). Here, macrophages were treated with MNPs preparations and co-cultured with Hep-G2 cells for 48 h to inhibit cell growth. As shown in Figure 11B, the total cell proliferation indicated no increase in the ability of APCs to inhibit Hep-G2 proliferation after exposure to MNPs-MPLA-siRNA.

Figure 4.11 – Macrophages were indirectly co-cultured with Hep-G2 cells for 2 days to verify if cancer cells showed changes in the expression of cytokines produced by APCs (A). Subsequently, MTT analysis revealed cancer cell proliferation after direct exposure to macrophages previously treated with MNPs, MNPs-MPLA, MNPs-MPLA-siRNA, and siRNA-MPLA only (B). Hep-G2 cells co-cultured with M2 macrophages not exposed to any MNPs served as the negative control.

Source: By the author.

4.4 – DISCUSSION

The development of new nanostructures has expanded the therapeutic strategies against cancer. The physicochemical properties of materials on the nanoscale improve the effectiveness of conventional treatments, such as chemotherapy, radiotherapy, and hormone therapy. Before human administration, it is necessary to evaluate the activity of nanoparticle in healthy cells in the body, observing their stability in the bloodstream and toxicity in normal tissues.²¹³ Studies have confirmed that less than 1% of nanostructures reach the targeted sites, most of which are captured by macrophages, natural killer cells, neutrophils, and DCs.¹² However, new research studies have been using the immunomodulatory properties of nanoparticles to increase their activity in the tumor microenvironment. Although *in vitro* and *in vivo* experiments have confirmed its safety, patients have exhibited hypersensitivity and severe allergic reactions (anaphylaxis) in the clinic.^{101,214} Although conjugation with biopolymers or biocompatible materials on nanostructure surfaces reduces immune cell activity, prolongation of the half-life in the circulatory system increases the adsorption of serum components and induces complement system activation.⁹⁴

It is still imprecise how complement systems modulate humoral immunity when serum proteins are adsorbed on the NP surface.²¹⁵ Recent studies have demonstrated the cascade system mechanisms in B-cell activation after nanoparticle administration. For example, the nanocomposites size and surface charge can trigger allergic reactions. Nanocomposites with a size of 250 nm could activate the complement response, but such structures with a size of 600 nm did not produce similar results. Activation of the complement system induces the progression of neoplasms in the tumor microenvironment, recruiting immunosuppressive cells, such as M2 macrophages, to control the inflammatory response produced by the nanostructures.^{104,108,216} White blood cells activate innate and adaptive immune responses when challenged by foreign elements. Such interactions between nanoparticle and APCs may affect granulocyte differentiation (myelopoiesis and lymphopoiesis). Nanostructure conjugated with responsive polymers are also influenced by tissue temperature and pH, producing distinct humoral responses after changing the matrix conformation.^{104,108,216} For example, the reduction in pH after nanoparticle evade the

circulatory system into the tumor microenvironment will expose or hide functional groups that allow for the adsorption of blood components.¹⁰⁹

Here, we demonstrated that the interaction of MNPs with human plasma, which led to different morphologies and increased the size of MNPs, behaved differently from the interaction of MNPs with FBS-supplemented culture media (Figures 2 and 3). Interaction studies carried out by ITC showed that the MNPs and MNPs-MPLA interacted with human plasma by different binding models (Figure 4). MNPs showed an exothermic behavior with a slight increase in size and changes in morphology, while MNPs-MPLA also showed an exothermic reaction without a saturation profile and a significant increase in size. This result suggests that proteins from the circulatory system, such as albumin, IgG antibodies, transferrin, and fibrinogen bind to the NP surface depending on the charge, generating different morphologies.^{94,217} In this process, molecules from the complement system are adsorbed on the MNPs, inducing their phagocytosis by APCs in inflamed tissues.^{94,217} However, further studies are required to understand the immune response after the activation of enzyme cascades by the MNPs. For example, polymeric nanostructures (PPS-NPs) induce the expression of CD80, CD86, and CD40 markers on immature DCs.¹¹⁰ However, PPS-NPs could not deliver antigenic materials to phagocytic cells in mice with complement depletion, consequently failing to induce an adaptive immune response against pathogens.¹¹⁰

We also examined the effectiveness of nanostructures loaded with c-MYC siRNA to decrease the expression of immune checkpoint proteins on hepatic cancer cells and modulate the immunosuppressive macrophage phenotype. Our data indicated that MNPs did not reduce Hep-G2 cell viability (Figure 5). In macrophages, MNPs-MPLA-siRNA increased the expression of classical activation markers, such as CD64, CD80, CD83, and CD86 (Figure 9), and anti-inflammatory proteins (CD163). Although CD163 was expressed on immunosuppressed cells, it is also present on M0 macrophages. The treatments exhibited the same intensity of HLA-DR (major histocompatibility complex class II) in all experimental groups, while purified siRNA did not have any effect in this study. Both HLA-DR, CD80, CD83, and CD86 are involved in antigen presentation by APCs and act as co-stimulatory signals, which are essential for the presentation of antigenic materials to CD4⁺ T lymphocytes.²¹⁸ Unexpectedly, we observed low MHC-II expression in the presence of lipopolysaccharide

(MPLA), possibly because the MNPs interfere with antigen processing and MHC assembly on the cell surface.²¹⁹

In contrast to the negative control and MPLA+siRNA, MNPs reduced the frequency of PD-L1 expression on macrophages and hepatic cancer cells (Figure 9C and 10.H). The MNPs inhibit the differentiation of naive T cells into the Th2 phenotype, similar to that caused by PD-L1 siRNA in the tumor microenvironment. This process restores lymphoproliferation after re-activating the anti-tumor activity of DCs.²²⁰⁻²²¹ Although liposomes conjugated with CD45 siRNA reduced the number of CD4⁺ T lymphocytes in the spleen and lymph nodes,²²² targeting the CD47 marker on the surface of cancer cells induces cell apoptosis and up-regulates adaptive systems.¹⁹⁷ Pre-clinical trials have used monoclonal antibodies to interfere with CD47 signaling and activating classical immunity,²²³⁻²²⁴ but recent investigations with liposomes carrying CD47 iRNA induced cancer cell death more efficiently than treatments with blocking antibodies alone.²²⁵⁻²²⁶

Here, we observed the interaction of MNPs with human plasma, their capacity to inhibit CD44, CD47, and PD-L1 expression on tumor cells, as well as the control of regulatory molecules on APCs.²²⁷ These MNPs may regulate a wide range of cell subtypes present in the abnormal tissue environment, inhibiting pro-oncogene activation and uncontrolled neoplastic cell proliferation. MNPs loaded with c-MYC siRNA blocked the expression of two checkpoint proteins, CD47 and PD-L1, on neoplastic cells and increased the expression of classical activation markers on macrophages. It is important to highlight that MNPs themselves reduce the presence of M2 cellular proteins.

4.5 CONCLUSIONS

To improve the efficacy of conventional anticancer therapies, new treatments are aimed at abolishing the neoplastic ability of cancer cells to evade host defense mechanisms. To overcome the suppressive activity of immunodeficient cells, it is necessary to generate a precise and durable immune response in the lymphatic system and tumor mass. Here, we demonstrated that MNPs improve cancer immunotherapy compared to treatments with therapeutic agents alone, eliciting faster innate and adaptive responses. To optimize safe formulations, lipid bilayer surfaces allow MNPs to travel longer distances through the vascular network and to interact with the immune system without chronic inflammation. As

a novel vaccine strategy, they have potential applications in delivering multiple tumor antigens to re-educate pro-inflammatory profiles. Moreover, the synthesis of MNPs allows the delivery of adjuvants to APCs for allogeneic responses in the body. Our studies revealed that MNPs are efficient carriers of siRNA for hepatic cancer cells, producing superior effects compared to those of pure siRNA alone. The MNPs improve the treatment activity of liver cancer cells and induce the expression of CD80, CD83, and CD86 on macrophages. In the future, it is expected that nanostructures can increase tumor immunogenicity and modulate immune mechanisms simultaneously, preventing the suppressive role in the cancer environment. Importantly, the advantages and disadvantages observed in nanomedicine and immunotherapy complement each other, and their combination provides numerous possibilities in the development of new therapeutic strategies that require a multidisciplinary understanding of their interactions with the immune system. Finally, these results contribute to the understanding of the application of MNPs as nanocarriers of antineoplastic agents and their effects on hepatic cancer and immunocompetent cells.

GENERAL CONCLUSIONS AND CONSIDERATIONS

The synthesis of nanostructures containing the main components from cellular membranes has been widely developed in our research group, which could be explored by the medical and biomedical fields in the future. In this thesis, we initially demonstrated the efficiency of MNPs to transport two chemotherapeutics usually used in the clinic (chapter II). MNPs from cancer cells proved to be efficient in delivering GEM and PTX to pancreatic cancer cells PANC-1, which toxic activity was superior to the isolated drugs. Our nanoparticles (sizes from 100 nm to 400 nm) were easily internalized by PANC-1 cells, increasing the cytotoxic effects of antineoplastic agents and the immunomodulatory properties in APCs. Our results also demonstrated that MNPs are efficient carriers of cabazitaxel for prostate cancer cells, being able to produce cytotoxicity similarly to the pure chemotherapeutics (Chapter III).

Studies with monocytes from healthy donors showed that MNPs induced phenotypic changes in defense cells (chapter II). MNPs were also phagocytosed by leukocytes, inhibiting the expression of PD-L1. Contrary to our expectations, nanoparticles alone did not cause any phenotypic changes in DCs, but the conjugation with chemotherapeutics reduced their ability to stimulate lymphoproliferation. Such treatment did not favor lymphocyte proliferation after co-culture with dendritic cells previously treated with particles, resulting in adverse effects on T cells.

After establishing the synthesis of nanoparticles, we decided to test their viability to carrier siRNA in liver tumor cells and to activate macrophages to reduce the immunosuppressive profile of the main cells present in the tumor microenvironment (chapter IV). Our results demonstrated that the targeting of MNPs to hepatic cancer cells reduces the expression of immunosuppressive proteins (CD47 and PD-L1). Finally, MNPs increase the expression of co-stimulatory molecules in alternatively activated macrophages. Although highly phagocytosed by macrophages, nanoparticles were able to induce the expression of HLA-DR, CD80, CD83, and CD86, with low toxicity to defense cells.

The physicochemical properties of MNPs are explored in the literature for the treatment of chronic diseases, with extensive studies *in vivo*. With this perspective, the results presented in this thesis contribute to understanding the activity of MNPs as drug-carrying agents and their main effects on neoplastic cells and immunocompetent cells. We

hope to contribute for improving the delivery of antineoplastic molecules to cancer cells allowing the development of new treatments that inhibit the ability of neoplasms to evade the individual's immunosurveillance. We hope that MNPs can be used to improve therapeutic results in the future, inducing a faster and more accurate immune activation profile in diseased tissues, overcoming the immunosuppressive activity produced by the tumor microenvironment.

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APPENDIX

Figure A1 – Image of plasma membrane nanoparticles (MNPs) extracted from the cancer cell by AFM, with reconstruction of Z-axis (A), amplitude (B), and three-dimensional (3-D) (C) imagens. FEG-MEV (D-F) and TEM (G-J) analysis at different magnifications, with scale bar at 1 μm and 200 nm.

Source: By the author.

Figure A2 – Confocal microscopy of pancreatic cancer cells (PANC-1) (A and B) after 24h exposed to FITC labeled nanoparticles. Incorporation of MNPs-FITC are exhibited by green fluorescence in cell cytoplasm, while Texas Red and DAPI label plasma membrane and cell nucleus, respectively. 3D confocal microscopy shows MNPs conjugated with FITC in PANC-1 cytoplasm and cell membrane (C-F). In hepatic cells (G and H), we observed a reduction in the frequency of green signals in HEPA-RG cells.

Source: By the author.

Figure A3 – Cytotoxicity analysis of MNPs in pancreatic cancer cells (PANC-1) by methyl tetrazolium reduction (MTT). PANC-1 (1x10⁴ cells per well) was exposed to A) pure MNPs, B) gemcitabine encapsulated in MNPs (MNPs-GEM), C) paclitaxel in MNPs (MNPs-PTX) and D) MNPs loaded with both drugs (MNPs-GEM-PTX) to verify their response and to estimate the working concentration in pancreatic cancer cells (*p ≤ 0.05).

Source: By the author.

Figure A4 – Cytotoxicity analysis of MNPs in healthy liver cells (HEPA-RG) by methyl tetrazolium reduction (MTT). HEPA-RG (1×10^4 cells per well) was exposed to A) pure MNPs, B) gemcitabine-encapsulated MNPs (MNP-GEM), C) paclitaxel in MNPs (MNP-PTX) and D) MNPs encapsulated with both drugs (MNP-GEM-PTX) to verify their specificity and toxicity in different concentrations of nanoparticles, fabricated from the pancreatic membrane, when exposed to healthy cell line (* $p \leq 0.05$).

Source: By the author.

Figure A5 – Cytotoxicity of synthetic liposomes encapsulated with gemcitabine and paclitaxel in pancreatic cancer cells (PANC-1) evaluated by methyl tetrazolium reduction assay (MTT). PANC-1 (1x10⁴ cells per well) were exposed to different concentrations of bare liposomes (A), GEM-encapsulated in liposomes (B), PTX-encapsulated in liposomes (C) and liposomes encapsulating both chemotherapeutics (D) to compare synthetic lipid nanoparticle activity with MNPs groups.

Source: By the author.