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STUDY OF THE IMMUNOGENICITY OF THE CELL DEATH INDUCED BY THE ACTIVATION OF CD47 IN TUMOR CELLS

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER'S IN SCIENCE WITH ORIENTATION IN INMMUNOBIOLOGY

JULY, 2019

SPECIAL ACKNOWLEDGEMENT

The present thesis work not have been possible without the economic support and the infrastructure provided by the Laboratory of the Immunology and Virology at the School of Biological Sciences of the UANL.

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1. ABSTRACT

CD47 activation by peptides derived from the C-terminal domain of thrombospondin-1, such as PKHB1, induces regulated cell death (RCD) in different types of cancer cells. The RCD induced by the activation of CD47 is characterized by being independent of caspases, calcium dependent, generating morphological changes in the endoplasmic reticulum (ER) and the exposure of calreticulin (CRT) in chronic lymphocytic leukemia (CLL) cells. On the other hand, ER stress and CRT exposure are characteristics of immunogenic cell death (ICD). Based on the above, the objective of this thesis was to determine the immunogenicity of cell death induced by the activation of CD47 in lymphoma and breast cancer cells. To this end, ICD's characteristics were evaluated, such as damage associated molecular patterns (DAMPs) exposure or release and their effect on the activation of an antitumor immune response. In addition, the extent of the immunogenicity of death was evaluated by prophylactic and therapeutic vaccinations, with lysates of cancer cells treated with PKHB1 (PKHB1-TCL) on the tumor establishment of lymphoma and breast cancer (*in vivo*), as well as their capacity to generate a long-term anti-tumor immunological memory.

The results obtained allow us to demonstrate that PKHB1 induces CRT exposure and release of CRT and HSP90 in lymphoma and breast cancer cells. It was also demonstrated that the PKHB1-TCL promotes the maturation of dendritic cells (DCs) increasing the expression of maturation markers CD80, CD86 and the release of TNFα. Furthermore, the PKHB1-TCL activated the antitumor immune response (*ex vivo*), promoting the specific cytotoxicity of tumor cells by T lymphocytes and induced the inflammatory cytokines IL-2 and IFNγ release. Finally, it was demonstrated that the PKHB1-TCL is able to prevent the tumor establishment in a lymphoma and murine breast cancer model, and to induce a long-term immunological anti-tumor memory.

Finally, when observing that the immunogenic characteristics of death are shared among the different tumor models, we can propose that the CD47 activation by PKHB1 induces a conserved ICD mechanism, enhancing its application and making it a promising antitumor therapy.

2. RESUMEN

La activación de CD47 por péptidos derivados del dominio C-terminal de la trombospondina-1, como el PKHB1, induce muerte celular regulada (MCR) en diferentes tipos de células cancerosas. La MCR inducida por la activación de CD47 se caracteriza por ser independiente de caspasas, dependiente de calcio, generar cambios morfológicos en el retículo endoplásmico (RE) y exposición de Calreticulina (CRT) en células de leucemia linfocítica crónica (LLC). El estrés del RE y la exposición de CRT son característias de la muerte celular inmunogénica (MCI). Con base en lo anterior, el objetivo de esta tesis fue determinar la inmunogenicidad de la muerte celular inducida por la activación de CD47 en células de linfoma y cáncer de mama. Para ello se evaluaron características de MCI, como la liberación de patrones moleculares asociados a daño (DAMPs) y su efecto en la activación de la respuesta inmune antitumoral. Además, se evaluó el alcance de la inmunogenicidad de la muerte mediante vacunaciones profilácticas y terapéuticas, con lisados de células cancerosas tratadas con PKHB1 (PKHB1-TCL) sobre el establecimiento tumoral de linfoma y cáncer de mama (*in vivo*), así como su capacidad de generar una memoria inmunológica antitumoral a largo plazo.

Los resultados obtenidos nos permiten demostrar que el PKHB1 induce la exposición de CRT y liberación de CRT, HSP90, ATP y HMGB1 en células de linfoma y cáncer de mama. También se demostró que el PKHB1-TCL promueve la maduración de células dendríticas (DCs) aumentando la expresión de marcadores de maduración CD80, CD86 y la liberación de TNFα. Asimismo, el PKHB1-TCL activó la respuesta inmune antitumoral (*ex vivo)*, promoviendo la citotoxicidad específica de células tumorales por linfocitos T e indujo la liberación de citocinas inflamatorias IL-2 e IFNγ. Además, se demostró que el PKHB1-TCL es capaz de prevenir y disminuir el establecimiento tumoral hasta lograr la remisión en un modelo de linfoma y cáncer de mama murino, además de inducir una memoria inmunológica antitumoral a largo plazo.

Finalmente, al observar que las características inmunogénicas de la muerte se comparten entre los diferentes modelos tumorales, podemos proponer que la activación de CD47 por el PKHB1 induce un mecanismo conservado de MCI, potenciando su aplicación y haciéndola una prometedora terapia antitumoral.

3. INTRODUCTION

CD47 is an ubiquitously expressed transmembrane receptor, member of the immunoglobulin superfamily, with diverse functions in the immune system. It is a counter-receptor for the signaling protein alpha (SIRPα) and also acts as a receptor for thrombospondin-1 (TSP1). CD47 acts as a "don't eat me" signal when interacting with SIRPα, a receptor that is highly expressed in professional phagocytes, such as macrophages and dendritic cells, which regulates programed cell removal (Matozaki et al. 2009). The interaction of CD47 with the C-terminal domain of TSP1 or peptides derived from this domain (such as 4N1K and PKHB1) regulates distinct cellular activities, such as cell-cell interactions, migration, proliferation and death, depending on the cellular context. It has been observed that the TSP1-CD47 interaction induces regulated cell death (RCD) in different types of cancer cells (Denèfle et al. 2016a). In chronic lymphocytic leukemia (CLL) cells PKHB1 induces calreticulin (CRT) exposure on the cell surface (A. C. Martinez-Torres et al. 2015). CRT is an ER chaperone protein that can be exposed to the cell membrane under conditions of ER stress. When it is exposed, it functions as an "eat me" signal with a crucial role in the immunogenicity of cell death, favoring the activation of an anti-tumor immune responses (Obeid et al. 2007).

Immunogenic cell death (ICD) has been recently recognized as a critical determinant for the efficiency of cancer therapy, since it is a cell death modality that is capable of stimulating an immune response against dead cell antigens, particularly when they are derived from cancer cells. The ICD is characterized by the exposure and release of damage associated molecular patterns (DAMPs) such as; CRT, heat shock proteins (HSP70 and 90), ATP and HMGB1 (Garg et al. 2015).

In recent years, distinct immunogenic cell death inductors have been discovered, including conventional chemotherapeutics, oncolytic viruses, targeted antitumor agents and other biological and physicochemical therapies. However, it has been observed that although different treatments can induce the DAMPs exposure and release in vitro, its application in cancer patients is not always efficient (Kepp et al. 2014). Thus, to conclude that a certain drug is an ICD inducer the analysis of DAMPs should be complemented by *ex vivo* and *in vivo* analyses of the antitumor immune responses (Garg et al. 2015). Therefore, the

vaccination assays are the “gold standard” that prove the ability to reject tumors in immunized hosts (Garg et al. 2015).

Due to the importance of ICD in the efficacy of antitumor therapies, the activation of CD47 by agonist peptides such as PKHB1 is an attractive strategy for the induction of ICD, since the activation of CD47 by PKHB1 induces ER morphological changes and CRT exposure in CLL, two main characteristics of the ICD.

For all the above, this thesis work was focused in the evaluation of the immunogenicity of cell death induced by the activation of CD47 in different types of tumor cells.

4. BACKGROUND

4.1 Cell death

Every day our cells carry out endless processes that are necessary for the maintenance and the proper functioning of the organism. Within these processes is the death *per se* of the cells, especially of the cells with physical damage, infected with pathogenic agents or carrying genetic alterations, which prevent them from maintaining their functions. Cell death can be carried out in different ways, which unleashes ambiguities when describing the pathways through which the cell dies. From a conceptual point of view, cell death can be defined as the permanent cessation of vital cellular functions. However, for the precise identification of a dead cell the Cell Death Nomenclature Committee (NCCD) has proposed the following three criteria:

1. The permanent loss of the barrier function of the plasma membrane.
2. The breakdown of the cell into discrete fragments (apoptotic bodies).
3. Immersion of the cell by professional phagocytes or other cells with phagocytic activity (L Galluzzi et al. 2012).

Currently the NCCD classifies cell death into two broad categories, described below and schematized in Figure 1:

Accidental cell death (ACD)

A type of almost immediate death, caused by physical (high temperatures or pressures), chemical (powerful detergents or variations in pH) or mechanical damage, insensitive to pharmacological or genetic interventions of any kind.

Regulated cell death (RCD)

It involves a genetically encoded molecular machinery; therefore, it can be altered pharmacologically or genetically, using as a target the key components of this machinery. RCD often occurs due to the failed attempts of the cell to restore homeostasis.

It should be noted that RCD does not occur exclusively as a consequence of microenvironmental disturbances, but also in the context of embryonic development, tissue

homeostasis, and immune response. Such completely physiological cases are generally referred to as programmed cell death (L Galluzzi et al. 2012).

4.1.1 Programed cell death (PCD)

It is an evolutionarily conserved pathway for embryonic development and tissue homeostasis. PCD acts as a normal physiological response to different stimuli that leads to the elimination of abnormal cells, infected or unable to perform their functions correctly (Sjostrom 2001).

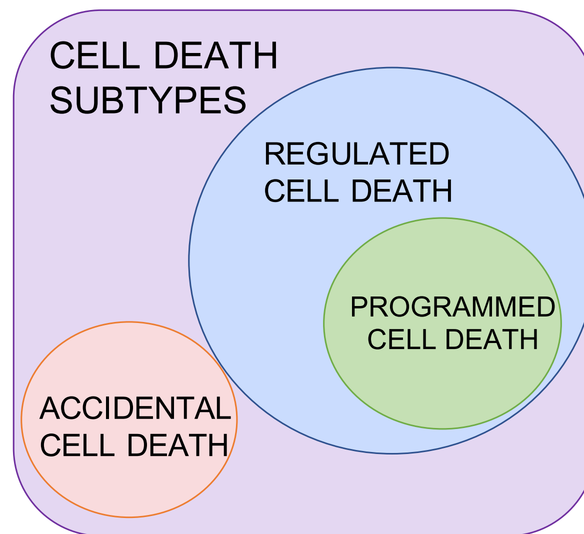

Figure. 1 Types of cell death. Cells exposed to physical, chemical and mechanical extreme stimuli succumb in a completely uncontrollable way of cell death, with the immediate loss of structural integrity. This type of death is known as: accidental cell death (ACD). On the other hand, cell death can be initiated by a genetically encoded machinery, this death is called regulated cell death (RCD). The term programmed cell death (PCD) is used to indicate cases of RCD that occur as part of a developmental program or to preserve adult tissue homeostasis.

Table 1 shows different RCD modalities classified biochemically, according to the dependence on caspases. Caspases are cysteine proteases that hydrolyze peptide bonds and can also generate signaling molecules that participate in different processes, such as regulated cell death.

Table 1. Modalities of regulated cell death

Modalities of Regulated Cell Death			
Types of Cell Death		**Subtypes/Triggers**	**Main Features**
Caspase-Dependent	**Extrinsic Apoptosis**	Cell death receptors (FAS, TNFR) Dependent Receptors (DCC or UNC5B when its ligan netrin-1 is missing)	Initiated by perturbations of the extracellular microenvironment. Need caspase 8 and Caspase 3.
	Intrinsic Apoptosis	Mediated by: DNA damage Oxidative stress ER stress	RCD initiated by perturbations of the extracellular or intracellular microenvironment, demarcated by MOMP, and need executioner caspases
	Atypic cases	Anoikis: lack of external membrane signal-dependent	Specific variant of intrinsic apoptosis initiated by the loss of integrin-dependent anchorage and some grown factor like EGFR
		Cornification	Ocurrs in the epidermis and involves the activation of transglutaminases
		Pyroptosis: formation of plasma membrane pores by members of the gasdermin protein family,often as a consequence of inflammatory caspase activation.	RCD Caspase 1-dependent. Display morphologics characteristics of both apoptosis and necrosis
		Lysosome-dependent cell death	Demarcated by lysosomal membrane permeabilization and precipitated by cathepsins, with optional involvement of MOMP and caspases.
Caspase-Independent	**Autophagy**	A massive vacuolization of the citoplasm; trigger by innanition or stress	Induce lipidation MAP1LC3 and degradation of SQSTM1
	Regulated Necrosis	Necroptosis: triggered by perturbations of extracellular or intracellular homeostasis	Depends on MLKL,RIPK3, and (at least in some settings) on the kinase activity of RIPK1.
		Parthanatos	Initiated by PARP1 hyperactivation and precipitated by the consequent bioenergetic catastrophe coupled toAIF-dependent and MIF-dependent DNA degradation.
		Mitochondrial permeability transition (MPT)-driven necrosis: RCD triggered by perturbations of the intracellular microenvironment	Generally manifests with a necrotic morphotype and relying on CYPD.
	Mitotic catastrophe and Mitotic death	Oncosuppressive mechanism for the control of mitosis-incompetent cells by RCD	Per se, mitotic catastrophe does not constitute a form or RCD.
	Atypic cases	NETotic cell death	A ROS-dependent modality of RCD restricted to cells of hematopoietic derivation and associated with NET extrusion.
		Entotic cell death is a form of cell cannibalism that occurs in healthyand malignant mammalian tissues	A type of RCD that originates from actomyosin-dependent cell-in-cell internalization (entosis) and is executed by lysosomes.

Adapted from (Martínez-Torres 2013; and Galluzzi et al. 2018).

5. Deregulation of cell death and associated pathologies

Cell division and death are predominant physiological processes that regulate tissue homeostasis in the body. Deregulation of these processes are involved in the pathogenesis of diseases, such as neurodegenerative disorders, myocardial infarction, cerebrovascular accidents, atherosclerosis and cancer (the most important group of diseases for this thesis work). Therefore, attempts to find cell cycle and cell death modulators are being made with

the hope of creating new therapeutic approaches to treat these diseases. It is clear that a better understanding of how cells balance the life and death processes is crucial for this development.

Furthermore, the deregulation in the cell death machinery can result in death resistance, which is associated with carcinogenesis. In fact, the resistance to cell death is one of the 10 hallmarks of cancer cells, as well as one of the principal targets of anti-cancer therapies (Hanahan and Weinberg 2011).

6. Resistance to cell death in cancer

Tumor cells develop different strategies to limit or elude apoptosis. The most common is the loss of function of the tumor suppressor protein (p53), alternatively, tumors can achieve similar purposes by increasing the expression of antiapoptotic regulators (Bcl-2, Bcl-x) or survival signals (Igf1 / 2), by negatively regulating proapoptotic factors (Bax, Bim, Puma) or by blocking the extrinsic pathway of ligand-induced death (Hanahan and Weinberg 2011). The structure of the RCD machinery and the strategies used by cancer cells to evade their functions, reflects the diversity of cancer cell populations during their evolution to the malignant state (Zhivotovsky and Orrenius 2010).

7. Emerging therapies: the immunogenicity of cancer cells

Every day, several millions of cells succumb to mechanisms of regulated cell death, and these cells are eliminated efficiently without causing local or systemic inflammation. This homeostatic cell death, that mainly occurs by apoptosis, is considered to be tolerogenic (promoting tolerance towards one's own) or as a null effect without impact on the immune system (Kroemer et al. 2013).

However, for some years now we have witnessed the appearance of a new concept; Immunogenic cell death (Kroemer et al. 2013), that is, a cell death modality that stimulates an immune response against antigens of dead cells, particularly when they are derived from cancer cells. This model was proposed for the first time in the context of anticancer chemotherapy, based on clinical evidence that indicates that tumor-specific immune

responses can determine the effectiveness of anti-cancer therapies with conventional cytotoxic drugs (Kroemer et al. 2013; Showalter et al. 2017).
For this reason, the immunogenicity of dying cancer cells to improve the efficacy of cancer therapy is a paradigm that has gained significant impulse in the last 5 years (Garg et al. 2015).

8. Immunogenic cell death: a new paradigm

The most notable advances in anti-cancer therapies involve different forms of cell death that broaden the scope of the classical cell death modalities, such as apoptosis, through the use of new cytotoxic agents (Hanahan and Weinberg 2011).
Classical cancer regimens induce cancer cell death mainly through apoptosis, a cell death process that has generally been considered immunosuppressive or even tolerogenic. However, recently it has emerged a selected class of cytotoxic agents (for example, anthracyclines) that can induce an immunogenic form of apoptosis in tumor cells, through the emission of distinct damage associated molecular patterns (DAMPs) and thus, these dying tumor cells can induce an effective antitumor immune response (Krysko et al. 2012).
Extensive studies have revealed two different types of immunogenic cell death (ICD) inductors, classified according to their form of inducing stress in the endoplasmic reticulum (ER). Importantly, ICD inductors are able to restart the immune responses that are usually suppressed by the tumor microenvironment (Inoue and Tani 2014).
For this reason, the anti-cancer emerging therapies focus on the induction of immunogenic cell death (which will be described below) in order to favor the tumor elimination and prevent their possible recurrence, through the anti-tumor immunological memory establishment.

8.1 Immunogenic cell death and cancer

The immunogenicity of cancer cells has been recognized as a critical determinant in the anti-cancer therapies. Therefore, in addition to developing direct immunostimulatory regimens, which include dendritic cell-based vaccines, cancer checkpoint blocking therapies and adoptive T cell transfer, researchers have begun to focus on the general immunobiology of neoplastic cells (Kroemer et al. 2013). Now it is clear that cancer cells may succumb to some

anti-cancer therapies in a peculiar form of cell death that is characterized by an increased immunogenic potential, due to the emission of DAMPs. The emission of DAMPs by cells that succumb to ICD favors the establishment of a productive connection with the immune system (Kroemer et al. 2013).

It has also been shown that the induction of cancer cell death pathways associated with the pre-mortem emission of DAMPs, considerably increase the immunogenicity of these dying cells (Kepp et al. 2014), and can result in the activation of anti-tumor immune responses (Garg et al. 2015).

8.2 Damage associated molecular patterns (DAMPs)

DAMPs are intracellular molecules that are not recognized by the immune system under normal physiological conditions. However, under conditions of cellular stress or tissue injury, these molecules are passively released into the extracellular environment by dying cells, thus allowing their binding to receptors in immune cells. It is noteworthy that the DAMPs can have pro and anti-inflammatory effects, as well as chemotactic, mitogenic and tissue remodeling effects depending on the context in which they are recognized by the host (Land 2015). Table 3 summarizes the most outstanding DAMPs characterized to date and their mode of emission, the cell death path to which they are associated and their related receptors (Garg et al. 2015).

On the other hand, it is important to consider that not all DAMPs can act as immunogenic warning signs. As shown in Table 2, there are several DAMPs that are crucial for the maintenance of tissue homeostasis and the repression of autoimmune responses, since they exert immunosuppressive effects, including Annexin A1 (ANXA1), death domain 1α (DD1α) and some molecules derived from the extracellular matrix (Garg et al. 2015).

Table 2. Prominent damage-associated molecular patterns (DAMPs) associated in immunogenic cell death

DAMPs	Receptors	Localization and mode of emission	Relevant cell death pathway	Functions
Annexin A1	FPR-1	Surface exposed or actively	Apoptosis	Guides the final approach of APCs to dying cells
Adenosine Triphosphate	P_2Y_2 and P_2Y_7	Actively or passively released	ICD, Apoptosis / Necrosis	Favours the recruitment of APCs and their activation
Calreticulin	LPR1 (CD91)	Mostly surface exposed sometimes passively released	ICD	Promotes the uptake of dead cell-associated antigens
Cellular RNA, dsRNA, ssRNA, dsDNA	TLR3, TLR7, CDSs	Passive release	ICD/Necrosis	Promotes the synthesis of pro-inflammatory factorsincluding type I IFNs
Type I IFNs	IFNAR	-	ICD	Promote CXCL10secretion by cancercells and exert immunostimulatory effects
Thrombospondin 1 and its heparin-binding domain	αvβ3 integrin	Passively released or surfaceassociated	Apoptosis	Mediates the protein's interaction with calreticulin, and integrins during cellular adhesion. Low-density lipoprotein receptor-related protein during uptake and clearance
Heat shock proteins (HSP70/90,60,72)	LRP1,TLR2, TLR4 SREC-1 and FEEL-1	Surface exposure, active secretion or passive release	ICD, Apoptosis / Necrosis	Stimulates the uptake of dead cell-associatedantigens
High-mobility group box	TLR2, TR4, RAGE and TIM3	Mostly passively relesed; sometimes actively released	ICD/Necrosis	Promotes the synthesis of pro-inflammatory factorsincluding type I IFNs

Adapted from (Garg et al. 2015; Lorenzo Galluzzi et al. 2017)

The principal DAMP associated with ICD is calreticulin (CRT), which is normally located in the light of the ER, but when it is exposed to the plasma membrane it has been shown to function as a potent "eat me" signal, allowing the phagocytosis of stressed or dying tumor cells by dendritic cells (DCs) (Garg et al. 2015). In addition, CRT is able to stimulate the production of IL-6, tumor necrosis factor (TNFα) and improves the antigen presentation to T lymphocytes by DCs, facilitating the polarization of Th1 lymphocytes (lymphocytes that activate macrophages, dendritic cells and NK cells). For this reason, CRT is the main protein of immunogenic cell death (Garg et al. 2015).

The high mobility group 1 protein (HMGB1) is a non-histone chromatin binding protein that influences transcription and other nuclear functions (Tesniere et al. 2008), which, once present in the extracellular space, can act as a signal of tissue damage and initiate an inflammatory response through binding to a range of receptors, including the receptor for

advanced glycosylation end products (RAGE), and toll like receptors (TLR2 or TLR4) on DCs (Tesniere et al. 2008). When bound to these receptors it activates the production of proinflammatory cytokines and it participates in the correct antigen presentation. In addition, it has been found that extracellular HMGB1 suppresses the activity of immunosuppressive cells such as regulatory T lymphocytes (Garg et al. 2015).

Extracellular ATP is a potent short-range "find me" signal (Elliott et al. 2009), it promotes the immune cells recruitment at ICD sites and their differentiation, this effect depends on the P2Y purinergic receptor (coupled to a G protein). Extracellular ATP also promotes the activation of DCs (Ghiringhelli et al. 2009), causing the activation of the NLRP3 inflammasome and proinflammatory cytokines release such as IL-1β and IL-18 (Garg et al. 2015).

On the other hand, heat shock proteins (HSP) 70 and 90 have also been found on the cell surface during ICD (Showalter et al. 2017). During the tumor cell death, HSP70 and HSP90 can be transferred to the plasma membrane to facilitate the uptake and processing of dead tumor cells' antigens by DCs and to present "cross-linked" peptides derived from tumors to CD8 + T cells, which leads to a cytotoxic response. The HSPs exposure takes place relatively late compared to CRT and could therefore play a secondary role (with CRT being the primary "eat me" signal) in the decision for antigen taking and processing by CD (Kepp et al. 2011).

Overall, the exposure of the proteins described above (as well as ATP) is crucial for the immunogenicity of dead cancer cells, Figure 2 shows a diagram of how the main DAMPs are implicated in ICD (Garg et al. 2015).

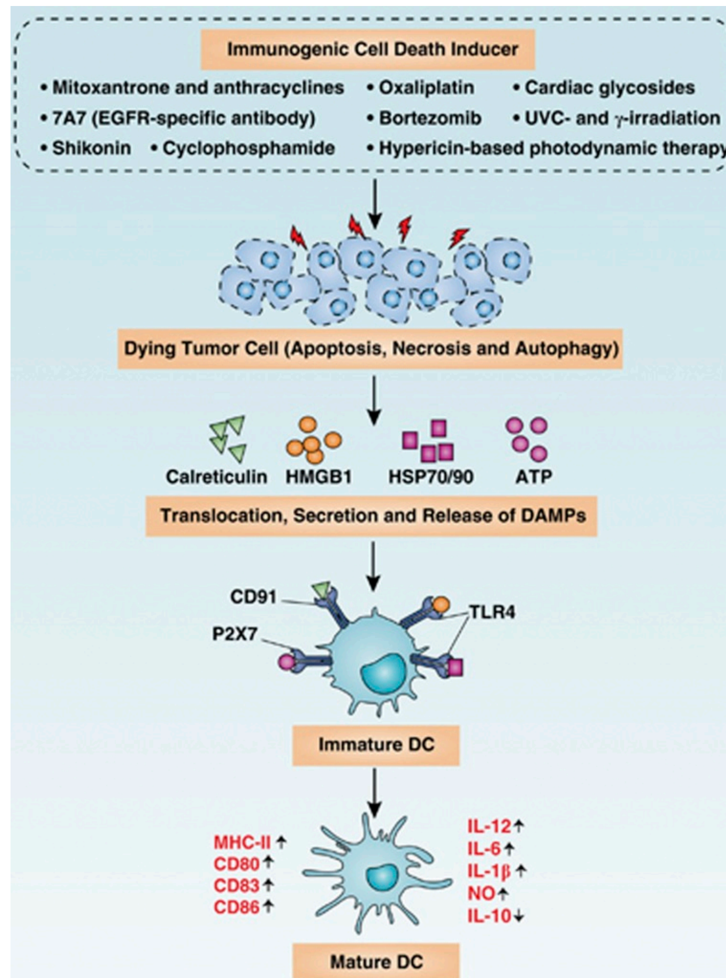

Figure 2. DAMPs implication on ICD. Cancer cells responding to ICD inducers by the exposure and release of CRT, HSP70/HSP90, ATP and HMGB1. Consequently, these DAMPs interact with receptors on immature DCs such as CRT,HSP90,HSP70-CD91 stimulating the absorption of dying tumor cells and/or their corpses (apoptotic bodies), ATP-P2X7 resulting in the assembly and activation of the NLRP3 inflammasome and thus in the secretion of IL-1β, HMGB1-TLR4 inhibits the degradation pathway of the lysosomal antigen in dendritic cells (which favors the processing and presentation of the tumor antigen) and stimulates the synthesis of the interleukin 1β precursor (Pro-IL-1β), and with unknown surface molecules. These DAMPs cause maturation of DCs, characterized by cell-surface upregulation of MHC-II, CD86, CD83, and CD80 and a distinctly pro-inflammatory cytokine pattern as indicated. Adapted from (Kepp et al. 2011; Hou et al. 2013).

8.3 Immunogenic capacity of treatments

Some anti-tumor therapies used with success for decades (chemotherapy and radiotherapy) can cause immunogenic cell death, however defects in the immune system involved with the ability to perceive a cell death as immunogenic, influence negatively among cancer patients treated with ICD inductors (Kepp et al. 2014).

Among different DAMPs, the CRT exposure is presented universally for all ICD inductors, therefore it is the most important DAMP, while ATP, HMGB1 or HSP70/90 may or not be present in certain ICD contexts. Therefore, only the analysis of molecular determinants is not reliable for the ICD characterization. For this purpose, the analysis of ICD-DAMPs should be considered secondary to the *in vivo* and/or *ex vivo* immunological analysis of the antitumor immune response, to conclude that a certain drug is an ICD inducer (Garg et al. 2015). Figure 3 shows the antitumor immune system establishment by the ICD. For this reason, the gold standard for evaluating the ability of a specific agent or modality to cause ICD is based on vaccination trials that show tumor rejection capabilities of the immunized host (Garg et al. 2015).

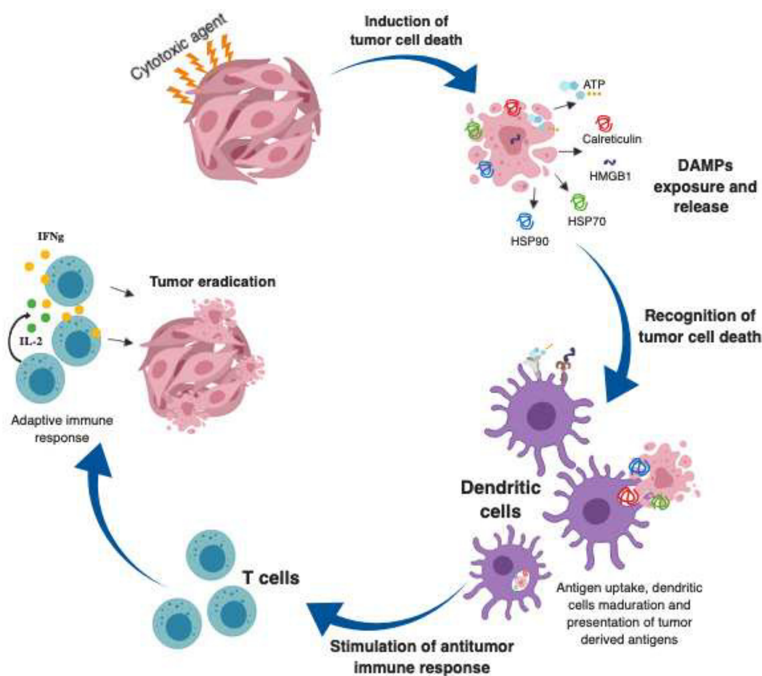

Figure 3. Establishment of the antitumor immune response by ICD. The induction of ICD leads to the recruitment of dendritic cells, then the apoptotic bodies are phagocytosed, and the antigens derived from tumors are processed and presented together with MHC and costimulatory molecules, after the maturation of DCs, to naive T lymphocytes. This process can be amplified in different steps. The selection of ICD inducers allows to improve the recognition and phagocytosis of the apoptotic residues, the maturation of DCs, the processing and the presentation of antigens derived from tumors. This leads to the establishment of the cytotoxic immune response, which involves CD4 + and CD8 + T lymphocytes, resulting in the complete eradication of the tumor cells. Adapted from (Tesniere et al. 2008).

In this context, as described in Figure 4, the cancer cells are treated *in vitro* with the drug/agent/modality/therapy to be evaluated and then the tumor cell lysate obtained after

treatment, is injected subcutaneously/intradermally in the flank of immunocompetent syngeneic mice. Seven days later, this treatment is followed by a new challenge with living cells of the same type of cancer in the opposite flank or another site of choice for the tumor. The proportion of mice that resist to the tumor establishment reveals the degree of immunogenicity of cell death for the evaluated therapy (Garg et al. 2015; Kepp et al. 2014). These rigorous standards, if maintained, would allow adequate characterization of the new ICD inducers while eliminating the possibility of false positives (Garg et al. 2015).

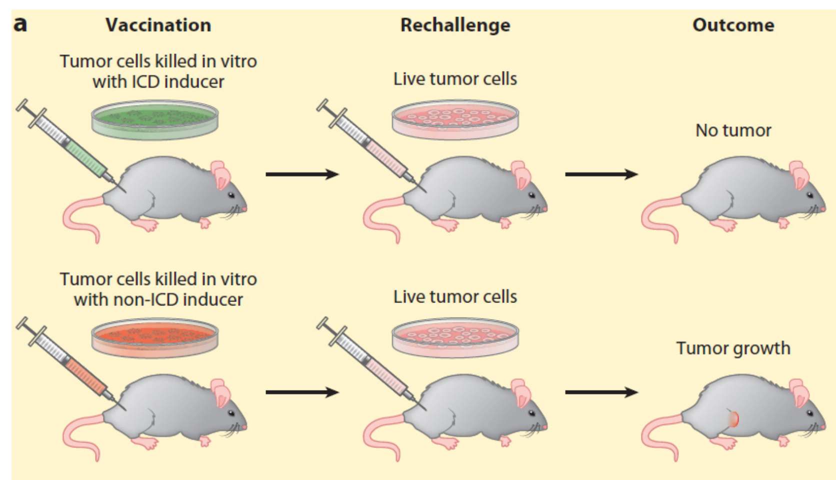

Figure 4. Operational definition of ICD. The death of cancer cells can be defined as immunogenic, based on the prevention of tumor establishment. The injection of cancer cells that succumb to ICD in immunocompetent mice should elicit a protective immune response that is specific for tumor antigens, in the absence of any adjuvant. Adapted from (Kroemer et al. 2013).

It is of great importance for ICD research to determine if other pathways of cell death could be associated in the generation of immunological memory against tumors, especially drugs, molecules, antibodies, peptides (among others) that are associated in triggering a direct or indirect damage of the endoplasmic reticulum, since this is the main source of DAMPs essential for the induction of an antitumor immunological memory.

8.4 Immunogenic cell death inductors

In recent years, several ICD inductors have been discovered, including; conventional chemotherapeutics, targeted antitumor agents and other biological and physicochemical therapies. Unfortunately, it is clear that there is no general structure-function relationship

capable of grouping all the existing ICD inductors and predicting new ones, which creates a great problem for the discovery of new ICD-inducing therapies. Nevertheless, the most peculiar characteristic of ICD inductors observed, is their capacity to induce ER stress (Garg et al. 2015).

These observations allowed the classification of ICD inductors, based on the form of ER stress induction for cell death and danger signaling. Based on this classification (Figure 5), ICD type I inductors are defined as anticancer agents that doesn't act on proteins from the ER in the induction of cell death but promote collateral stress of ER for signaling of danger, operating in multiple targets. While the ICD inductors Type II are anticancer agents that target the ER both for the induction of cell death and for danger signaling. This clearly shows that the ICD inductor type I / II concept can be used for the discovery of new ICD inductors based on their preferred orientation to the ER (Garg et al. 2015; Kepp et al. 2011).

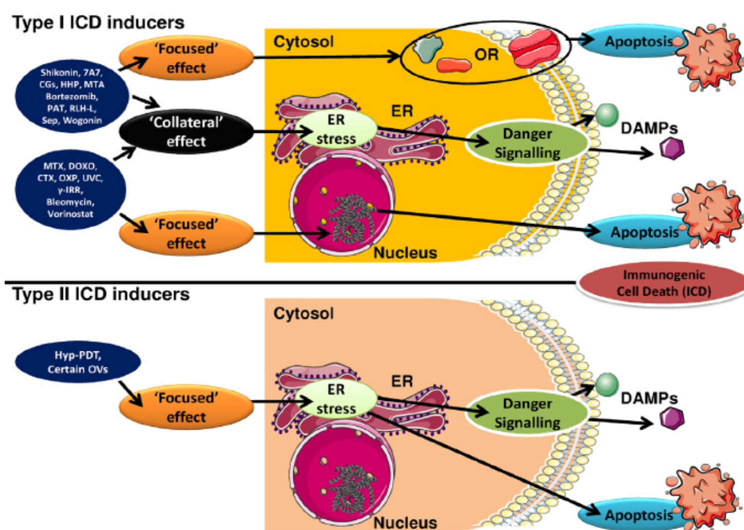

Figure 5. Activity of immunogenic cell death inductors type I and II. Type I ICD inductors induce cell death through targets unrelated to ER and danger signaling through ER stress. On the other hand, type II ICD inductors selectively target ER to induce both cell death and danger signaling. Adapted from (Garg et al. 2015).

From this point of view, the activation of the membrane receptor CD47 (which will be described below) by agonist peptides such as PKHB1, could be an interesting therapeutic strategy for the generation of ICD, since it has been observed to induce regulated cell death in chronic lymphocytic leukemia (CLL) cells inducing ER morphological changes and CRT

exposure, additionally, it promotes the elimination of tumor cells more efficiently in immunocompetent than in immunodeficient mice (Martinez -Torres et al. 2015, Uscanga-Palomeque et al. 2018, unpublished observations). These are some of the main reasons for which the activation of CD47 is a promising strategy for the induction of ICD and a potential treatment in cancer therapy.

9. CD47

CD47 is a transmembrane protein, member of the immunoglobulin superfamily, that is ubiquitously expressed. It was first identified as a tumor antigen in human ovarian cancer in the 1980s. Since then, it has been found to be expressed in different types of human tumor cells, including acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), Hodgkin's lymphoma (LH), bladder cancer, among other solid tumors (Chao, Weissman, and Majeti 2012).

CD47 participates in diverse physiological processes, including migration, adhesion and cellular proliferation, among others, by its interaction with proteins like integrins, and thrombospondin-1. In addition, the functions of CD47 as an inhibitor of phagocytosis through its interaction with the signaling regulatory protein alpha (SIRPα) expressed in phagocytes, leads to the activation of a tyrosine phosphatase and the inhibition of myosin accumulation in the place of the phagocytic synapse (Chao, Weissman, and Majeti 2012). Thus, CD47 serves as a "don't eat me" signal that helps at the recognition of the own. The main ligands of CD47, TSP-1 and SIRPα, are described in detail below.

9.1 CD47 structure and interactions

CD47 consists of an N-terminal domain of extracellular IgV type (Figure 6), followed by a presenilin domain that contains five segments that cross the membrane and that end in a variable cytoplasmic sequence (Chao, Weissman, and Majeti 2012).

It has been shown that the IgV domain of CD47 interacts with different ligands and counter-ligands present in the extracellular matrix or anchored to the plasma membrane. The main ligand of CD47 is TSP-1, a homotrimeric glycoprotein, its C-terminal domain mediates

CD47 binding (Isenberg et al. 2009). It has been observed that the interaction of TSP-1-CD47 is also carried out in different species such as murine, bovine, porcine and rats, which indicates that this interaction is not specie-specific within mammals. The family of SIRPα, known as CD47 counter-receptors, is formed by three members: SIRPα binds with greater affinity to CD47, SIRPγ with lower affinity and SIRPβ does not bind to CD47(Soto-Pantoja, Kaur, and Roberts 2015).

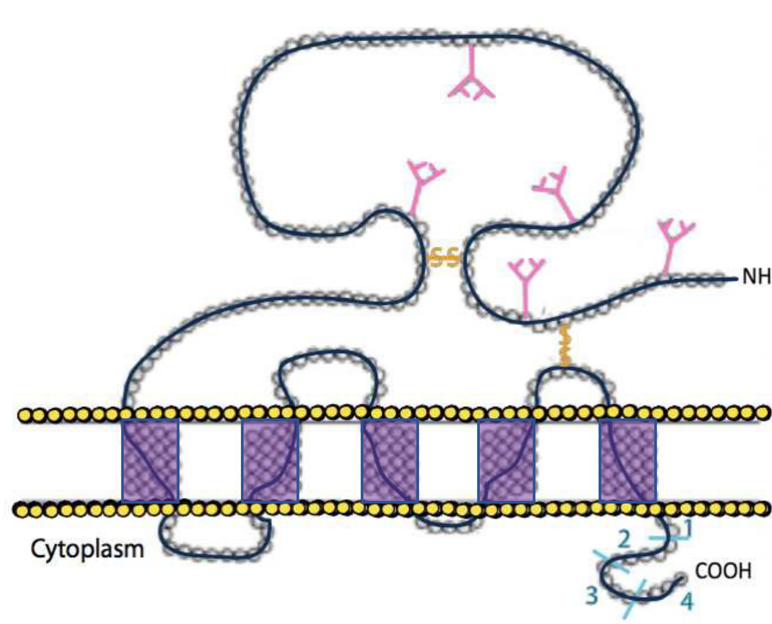

Figure 6. Structure of CD47. The figure shows the extracellular IgV domain, with its 5 glycosylation sites (in pink), followed by the 5 transmembrane segments (MMS) (in purple), disulfide bonds (s-s in orange) and finally the carboxyl terminal cytoplasmic domain with its 4 possible alternative splicings, where the isoform 4 is the complete molecule.

9.2 SIRP-α

SIRPα (also known as SHPS-1, BIT, CD172a or p84) belongs to the family of membrane proteins involved in the regulation of leukocyte function and is a counter-receptor of CD47 whose interaction has been shown to mediate cell adhesion. SIRPα is expressed in myeloid cells, including macrophages, granulocytes, myeloid dendritic cells, mast cells and their precursors, including hematopoietic stem cells (Subramanian, Boder, and Discher 2007). SIRPα has three immunoglobulin-like domains in its extracellular region and two immunoreceptor tyrosine-based inhibition motifs (ITIM) in its cytoplasmic region (Figure 7)

9.2.1 CD47 and SIRPα regulate phagocytosis

APCs express receptors that control their activity, including phagocytosis, as in the case of SIRPα, which recognizes CD47 on the surface of the cell as a "don't eat me" signal (Navarro-Alvarez and Yang 2011). This signal involves the activation of SIRPα-ITIMs and the recruitment of the phosphatases SHP-1 and -2, resulting in the inhibition of phagocytosis. In addition, this interaction allows macrophages to discern between a viable and an apoptotic cell (Martínez-Torres, 2013).

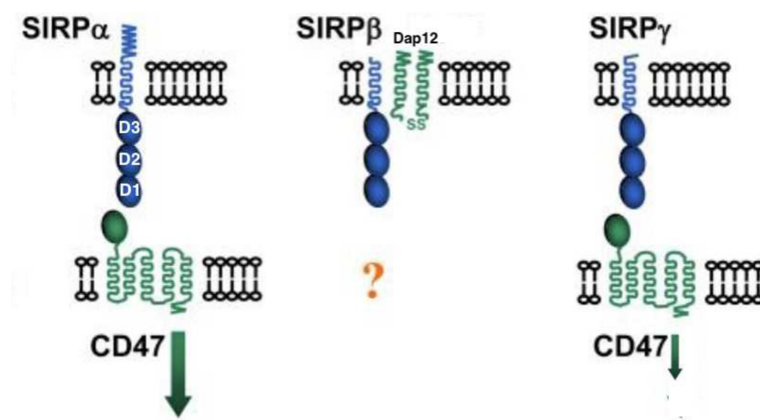

Figure 7. Interactions of SIRPs with CD47. The forms of SIRP have three Ig-like domains (blue ovals), of which only the D1 domain of SIRPα and SIRPγ interact with the Ig domain (green oval) of CD47, inducing signals of activation (green arrow). SIRPβ its activation only in the presence of the adapter protein Dap12 but doesn't bind to CD47. Obtained from (Barclay 2009).

9.3 Thrombospondin-1

Thrombospondin (TSP) belongs to a family of five multifunctional proteins that can be divided into two subgroups: first, TSP-1 and TSP-2, homotrimers with a region of procollagen type and repetitive regions, and a second group consisting of TSP-3, TSP-4 and TSP-5, which conform homopentamers (Martínez-Torres, 2013).

TSP-1 is a protein that is expressed mainly on the cell surface, transiently in the extracellular matrix in a soluble manner. It modulates cellular functions in a specific way by involving cell surface receptors and other components of the extracellular matrix. In adults, the TSP-1 expression is limited to tissue remodeling sites where it resides in the extracellular space and determines the phenotype, structure and composition of the extracellular matrix. TSP-1

promotes the migration of vascular smooth muscle cells but suppresses the chemotaxis and motility of endothelial cells with the same potency (Mirochnik, Kwiatek, and Volpert 2008). The subfamily TSP-1 and TSP-2 present anti-angiogenic activities and have been shown to be capable of blocking tumor growth (Isenberg et al. 2009).

In addition, TSP-1 is the main soluble ligand of CD47, that after its interaction transduces signals that alter cellular calcium and cyclic nucleotides signaling, integrins and growth factors, controls cell fate, its viability and resistance to stress (Roberts et al. 2012).

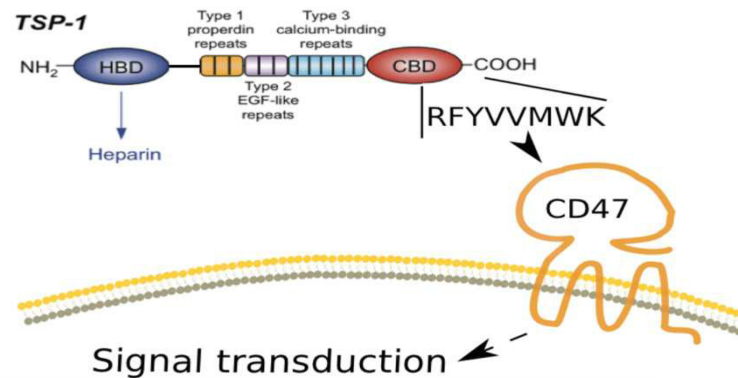

Figure 8. Thrombospondin-1 (TSP-1). The figure shows that TSP1 is composed of a globular N-terminal heparin binding domain (HBD), three chains that contain repeats of type properdin (Type 1), epidermal growth factor like repeats (EGF, Type 2), and calcium binding (Type 3), joined by disulfide bonds, and another globular region in its C-terminal domain (CBD) (Gene ID: 7057), which is the one that interacts with CD47. Adapted from Martínez-Torres, 2013.

As shown in Fig 8, the VVM motif in the carboxyl terminal domain of TSP-1 that binds to CD47 is conserved among the five families, suggesting that the binding to CD47 could be a universal characteristic of these families (Isenberg et al. 2009).

The interaction of CD47/TSP-1 is involved in different biological processes. This interaction mediates the inhibitory signal of nitric oxide (NO), having an impact on the cardiovascular system, since it decreases the amount of adhesion molecules in platelets, and alters the calcium levels in the vascular smooth muscle cells, among other mechanisms. In addition, vasodilation and chemotaxis are controlled when TSP-1 inhibits the enzyme that censes and activates NO production, soluble guanylyl cyclase (sCG) in platelets and endothelial cells (Isenberg et al. 2006). Similarly, CD47 / TSP-1 plays an important role in angiogenesis, since it has been shown that peptides derived from TSP-1 inhibit angiogenesis induced by fibroblast growth factor 2 (FGF-2) *in vitro* and *in vivo* (Kanda et al. 1999).

9.4 CD47 as a therapeutic target in cancer

According to the diverse biological functions in which CD47 is involved, on the one hand, it exerts the "don't eat me" signal that can be exploited by neoplastic cells and contribute to their survival and progression. On the other hand, it can inhibit angiogenesis by interacting with TSP-1 and induce cell death in chronic lymphocytic leukemia (Mateo et al. 1999) and breast cancer cells (Manna and Frazier 2004). The understanding of CD47, its ligands and signaling pathways, is fundamental to understanding its role as a therapeutic target.

9.5 Induction of RCD in cancer cells

It has been observed for several years that CD47 induces RCD in different cancer cell types. Monoclonal antibodies (mAbs) such as coated B6H12, soluble TSP-1, and peptides such a 4N1K have been used against CD47 to induce RCD in CLL cells (Mateo et al. 1999). Subsequently, many researchers continued to evaluate the induction of RCD in other cell types, using soluble anti-CD47 mAbs in multiple ALL (CCRF-CEM) (Uno et al. 2007), breast cancer (MCF-7) (Manna and Frazier 2004) and AML cell lines (NB4-LR1) (Saumet et al. 2005), among others. Furthermore, TSP-1, or agonist peptides such a 4N1 and 4N1K induced RCD in leukemic and breast cancer cells (Manna and Frazier 2004). In all these cases, the RCD induced by CD47 retains the following characteristics: a rapid cell death, caspase-independent, mitochondrial membrane depolarization without release of proapoptotic proteins, reactive oxygen species production, phosphatidylserine exposure, plasma membrane permeabilization, without DNA fragmentation or chromatin condensation (Martínez-Torres, 2013).

Although it was observed that peptides derived from TSP-1 activated RCD in cancer cells, the impossibility of their administration in a soluble manner limited the potential of their therapeutic use, for this reason most of the CD47 research of recent years reports the induction of RCD with mAbs through different mechanisms. Thus it was until 2015 when the CD47 agonists recovered importance when the first serum stable agonist peptide of CD47 was synthetized, called PKHB1, which shares the same amino acid sequence of 4N1K

(described above), but the natural C- and N-terminal lysines, L-amino acids were replaced by their equivalents D. PKHB1 was created with the intention of optimizing the poor solubility and serum stability of the peptides 4N1 and 4N1K, which until 2015 were the only CD47 agonist peptides that had been described (A.-C. Martinez-Torres et al. 2015). Therefore, it was demonstrated that the activation of CD47 by PKHB1 selectively induces calcium-regulated cell death in CLL cells from refractory patients, through ER changes, which leads to calcium ion overload (Ca^{2+}) in the cytosol, damaging lethally the mitochondria and inducing the proteases activation (serpases) (A.-C. Martinez-Torres et al. 2015).

On the other hand, the growth of CLL cell tumors (human cells) in xenotransplanted NSG (immunodeficient) mice was significantly reduced when treated with PKHB1 (A.-C. Martinez-Torres et al. 2015). In conjunction with such findings, Dènefle et al. demonstrated that PKHB1 is also able to induce cell death of at least lung, colon and breast cancer cell lines, without damaging healthy B and T cells (Denèfle et al. 2016a). In addition, recent results show that PKHB1 selectively kills different types of leukemia cell lines (Jurkat, CEM, HL-60 and K562) and a murine T-cell lymphoblastic cell line (L5178Y-R), but not primary cultures of non-human cancerous or murine cells (Gomez-Morales, 2017, Uscanga-Palomeque 2018, thesis works). Cell death in different types of leukemia apparently occurs through a mechanism similar to that of CLL, since it depends on the influx of calcium (Gomez-Morales, 2017, Calvillo Rodríguez 2017, Uscanga-Palomeque 2018).

9.6 Possible implications of CD47 agonist peptides in the induction of immunogenic cell death

It is clear that the efficacy of current treatments against cancer, such as different chemotherapies and radiotherapies, is fundamentally based on their ability to awaken innate and adaptive immune responses, which depend on the immunogenicity and the adjuvant capacity of the tumor (Lorenzo Galluzzi et al. 2017). An interesting feature observed in CLL cells is that PKHB1 caused exposure of CRT to the cell surface (A.-C. Martinez-Torres et al. 2015), an ER chaperone protein that is exposed to the cell membrane as a "eat me" signal

after ER stress, and which is deeply involved with the immunogenicity of cell death and favors the activation of immunological memory (Obeid et al. 2007).

In addition, CD47 plays critical roles in the immune system, regulates inflammatory responses (Gutierrez, Lopez-Dee, and Pidcock 2011; Sarfati et al. 2008), by mediating the migration of leukocytes to sites of infection (Martinelli et al. 2013) or the resolution of inflammation by the elimination of macrophages (Calippe et al. 2017). In addition, recent findings show that chronic ER stress caused by the unfolded protein response (UPR) modulates antitumor immunity in cancer and regulates the expression of calreticulin and CD47 (Cook and Soto-Pantoja 2017). Finally, in our laboratory, we used PKHB1 to study its effects on an immunocompetent murine model with a syngeneic tumor of L5178Y-R cells, and we found that treatment with PKHB1 not only reduces the tumor burden in immunocompetent mice, but induces complete regression in most cases (Uscanga-Palomeque et al. 2018a), suggesting that the immune system could play a role in the antitumor activity of the CD47 agonist peptides. So collectively, the available data highlighted the potential of CD47 agonist peptides to induce non-apoptotic immunogenic cell death of cancer cells, which was tested in this thesis.

10. JUSTIFICATION

Cancer is one of the most important public health problems worldwide. Despite the efforts to treat it, innate and acquired treatment-resistance of cancer cells to current therapies remain one of the principal problems to solve in clinical care. Immunogenic cell death (ICD) recently has been recognized as a critical determinant for the efficiency of cancer therapies. Due to this, the current treatments seek to induce ICD in cancer cells through conventional chemotherapeutics, targeted antitumor agents, and other biological and physicochemical therapies. Unfortunately, most treatments are highly nonspecific and cause serious side effects that limit their use.

From this perspective, the activation of CD47 is a promising approach in cancer therapy, since its activation through TSP-1 or mimetic agonist peptides, such as PKHB1, induces selective regulated cell death (RCD) of cancer cells. CD47-RCD is characterized by endoplasmic reticulum alterations and CRT exposure, one of the main DAMPs involved in ICD.

Therefore, this study was conceived to expand the knowledge of the immunogenicity of this type of cell death and its effect on the establishment of an antitumor immune response. This will might extend its potential application to other types of cancer, and to evidence its possible advantages over the current ICD inducers.

11.HYPOTHESIS

CD47 activation induces immunogenic cell death in cancer cells.

12.OBJECTIVES

General Objective

To evaluate the immunogenic cell death induced by PKHB1 in cancer cells

Specific Objectives

- To evaluate the cell death induced by PKHB1 in tumor cells.
- To analyze the exposure or release of DAMPs from tumor cells after treatment with PKHB1.
- To assess the anti-tumor immune responses induced by dead tumor cells treated with PKHB1.
- To evaluate the anti-tumor effect of prophylactic and therapeutic vaccinations with dead cancer cells treated with PKHB1 on syngeneic mice bearing cancer cells.
- To analyze the long-term antitumor memory induced by therapeutic vaccinations with dead cancer cells treated with PKHB1 on syngeneic mice bearing cancer cells.

13.MATERIALS AND METHODS

13.1 Cell culture

L5178Y-R (ATCC CRL-1722, murine lymphoblastic T-cell line), MDA-MB-231 and MCF-7 (ATCC HTB-26 and ATCC HTB-22, human breast cancer cells), and 4T1 (ATCC CRL-2539, murine breast cancer cell line) were obtained from the ATCC. Cells were maintained in RPMI-1640 (lymphoid cells) or DMEM-F12 (breast cancer cells) medium supplemented with 10% FBS, 2 mmol/L l-glutamine, 100 U/mL penicillin-streptomycin (GIBCO by Life Technologies, Grand Island, NY, USA), and incubated at 37°C in a controlled humidified atmosphere with 5% CO_2. Cell count was carried out using Trypan blue (0.4% Sigma-Aldrich), a Neubauer chamber and an optic microscope (Zeiss Primo Star) as proposed by the ATCC's standard protocols.

13.2 Cell death analysis

$1X10^6$ cells/mL were seeded and left untreated or treated for 2 h with PKHB1 (as indicated, with 100, 200 and 300 µM) to obtain the cytotoxic concentration that killed 50% of the cells (CC_{50}). Then, annexin-V-allophycocyanin (Ann-V-APC 0.1µg/ml; BD Pharmingen, San Jose CA, USA), and propidium iodide (PI, 0.5µg/ml Sigma-Aldrich) were used to assess phosphatidylserine exposure, cell death, and cell viability quantification, respectively, in a BD AccuryC6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) (total population 10,000 cells). Data was analyzed using FlowJo software (LLC, Ashland, OR, USA).

13.3 Calreticulin exposure

$1x10^6$ cells were plated, and then left untreated (control) or treated with PKHB1(CC_{50}), and incubated for 2 h (for L5178Y-R cells, poly-l-lysine was added to sterile coverslips placed inside a six-well plate for 24 h, then washed with PBS and $1x10^6$ cells/mL were seeded). Then, the cells were washed with PBS and stained with Calreticulin-PE antibody (2µg/ml) and Hoechst 33342 (0.5µg/ml) (Thermo Scientific Pierce, Rockford, IL, USA), incubated for 1h in FACS buffer, and assessed by confocal microscopy.

13.4 Western blot

In a serum-free culture medium, $1x10^6$ cells/mL were seeded and treated with PKHB1 (CC_{50} for each cell line) or left alone (control) for 2 h. Supernatants were recovered and the cells were lysed with lysis buffer (20 mmol/L Tris pH 6.8, 2 mmol/L EDTA, 300 mmol/L NaCl and SDS 2%) at 98ºC for 30 minutes. Protein concentration was measured using the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA) and 50 µg protein was loaded into SDS-PAGE gels. After blotting, nitrocellulose filters were probed with primary antibodies HSP90 (F-8: SC-13119) and Calreticulin (F-4: sc373863). Anti-mouse or anti-rabbit-HRP served as secondary antibodies (Santa Cruz Biotechnology). Visualization was carried out with ECL substrate system (Thermo Scientific, Waltham, MA, USA).

13.5 ATP release assay

$1x10^6$ cells/mL were treated with PKHB1 (CC_{50} for each cell line) for 2 h. Supernatants were used to assess extracellular ATP by a luciferase assay (ENLITEN kit, Promega, Madison, WI, USA) following the manufacturer's instructions. Bioluminescence was assessed in a microplate reader (Synergy HT, Software Gen5; BioTek, Winooski, VT, USA) at 560 nm.

13.6 High-mobility group box 1 release assay

Supernatants of untreated and treated cells (PKHB1 CC_{50} for each cell line and CC_{100} only for L5178Y-R) cells ($1x10^6$ cells/mL) were used to measure extracellular HMGB1 using the HMGB1 ELISA kit for MDA-MB, MCF-7 or L5178Y-R and 4T1 cells (BioAssay ELISA kit human or mouse, respectively; US Biological Life Science Salem, MA, USA), following the manufacturer's instructions. Absorbance was assessed at 450 nm.

13.7 T-cells and dendritic cells (DCs)

This study was approved by the Animal Ethical Committee (CEIBA), of the School of Biological Sciences Number: CEIBA-2018-003. All experiments were conducted according to Mexican regulation NOM-062-ZOO-1999.

The blood from sacrificed BALB/c mice was obtained by cardiac puncture. Peripheral blood mononuclear cells (PBMCs) isolation was performed by density gradient centrifugation using Ficoll-Hypaque-1119 (Sigma-Aldrich, St Louis, MO, USA). Murine CD3+ cells were isolated from total PBMCs by positive selection using magnetic-activated cell sorting (MACS) microbead technology with anti-CD3ε-biotin and anti-biotin microbeads (Miltenyi Biotech; >98% purity and >98% viability), as stated by manufacturer's instructions.
To obtain bone marrow-derived dendritic cells (DCs), after sacrifice, mice bone marrow was removed from femur and tibia of female BALB/c mice by flushing into RPMI-1640. Eluted cells were cultured for 5 days with 20 ng/mL of IL-4 and GM-CSF (R&D Systems, Minneapolis, MN, USA) until approximately 70% of the cells were CD11c+.

13.8 DCs markers

DCs ($1x10^6$ cells/mL) were stained in 100µL of FACS buffer with the indicated antibodies at RT for 30 minutes and then washed twice with PBS. The cell surface markers were evaluated by flow cytometry with the fluorescent label-conjugated antibodies, antiCD11c-Alexa-fluor 488 (R&D Systems), antiCD80-FITC and antiCD86-APC, from BD Biosciences (San Jose, CA, USA).

13.9 Co-cultures

DCs-PKHB1 tumor cell lysate: DCs were resuspended in fresh medium at a concentration of $1x10^6$ cells/mL. DCs were left untreated (control) or PKHB1-Treated tumor cells were added at a concentration of 3×10^6 cells/mL (L5178Y-R or 4T1 cells) to give a range of 1:3 DCs to PKHB1-treated tumor cells ratios. Co-culture was left for 24 hours. Then the supernatant was removed, and the well was washed twice with PBS before doing the next co-culture (with the addition of 4T1 or L5178Y-R cells respectively).
DCs-T-lymphocytes: Control DCs or DCs previously co-cultured with PKHB1-TCL were maintained in fresh medium at a concentration of $1x10^6$ cells/mL. Then, allogeneic BALB/c mCD3+ cells were added to each well at $3x10^6$ cells/mL to give a range of 1:3 DC to CD3+ cells ratios. Coculture was left for 96 hours. Then, the lymphocytes were collected (by

obtaining the supernatant), washed with PBS, and resuspended in fresh medium at a concentration of $5x10^6$ cells /mL for their use in the next co-culture (T- lymphocytes with cancer cells).

T-Lymphocytes-Tumoral cells cells: viable 4T1 or L5178Y-R cells were plated at a concentration of $1x10^5$ cells/mL. Then, unprimed (previously co-cultured with control DCs) or primed (previously co-cultured with DCs-PKHB1-TCL) allogeneic BALB/c mCD3+ cells were added to each well at 5×10^5 cells/mL to give a range of 1:5 tumor to effector ratios. Co-culture was left for 24 hours, before cytokine or calcein assessment

13.10 Cytokine release assay

The supernatants from the indicated cultures were collected for IL-2, IL-4, IL5, and TNFα assessment (BD, CBA Mouse Th1/Th2 Cytokine Kit, San Jose, CA, USA) by flow cytometry following manufacturer's instructions. IFNγ was assessed using an ELISA kit (Sigma-Aldrich) and using the Synergy HTTM (BioTek Instruments, Inc., Winooski, VT, USA) plate reader at 570 nm wavelength, following manufacturer's instructions.

13.11 Calcein assay

L5178Y-R or 4T1 cells ($1x10^6$ cells/mL) were stained with (0.1mL/mL) of Calcein-AM from BD Biosciences (San Jose, CA) for 30 minutes, then washed twice with PBS. After this, T cells previously primed with DCs pulsed with PKHB1-TCL or with unpulsed DCs were added in a 1:5 ratio. The co-culture was incubated at 37 °C and 5% CO_2 for 24 h. Finally, calcein positive or negative cells were assessed in a BD AccuryC6 flow cytometer (BD Biosciences) (total population 10,000 cells). Data was then analyzed using FlowJo software.

13.12 *In vivo* model

Six-to-eight-week-old BALB/c female mice were maintained in controlled environmental conditions (25ºC and 12 h light/dark cycle) and were supplied with rodent food (LabDiet, St. Louis, MO, USA) and water *ad libitum*.

13.13 Prophylactic vaccinations

L5178Y-R cells ($5x10^6$) or 4T1 cells ($3x10^6$) were treated with 350 or 400 μM PKHB1 (CC_{100}) respectively in serum-free medium for 2 h. Cell death was confirmed using Trypan blue staining and flow cytometry. Treatment was carried out as follows: PKHB1-TCL was inoculated s.c. in 100 μL of serum free medium into the left hind leg (day -7), seven days later viable (2×10^6) L5178Y-R or (5×10^5) 4T1 cells were inoculated into the right hind leg (day 0). Tumor volume and weight were measured three times per week using a caliper (Digimatic Caliper; Mitutoyo Corporation, Kawasaki, Japan) and a digital scale (AWS-600-BLK American Weigh Scales Inc. Norcross, GA, USA). Tumor volume was determined with the formula: tumor volume (mm3) = 4π/3 * A * B * C where 4π / 3 is a mathematical constant, A= width, B= high, and C= depth.

13.14 Therapeutic vaccinations

Tumor was established by subcutaneous injection of $2x10^6$ L5178Y-R or $5x10^5$ 4T1 cells in 100 μL PBS, in the left hind. Tumor volume and mice weight were measured how was described above. When the tumor reached 100 mm^3 the first therapeutic-vaccine of PKHB1-tumor cell lysate (PKHB1-TCL) was applied as follows; L5178Y-R cells ($5x10^6$) or 4T1($3x10^6$) cells were treated *in vitro* with 300 or 400 μM PKHB1 for 2 h (CC_{100}) in serum-free RPMI medium. Cell death was confirmed as previously indicated. Treated cells were inoculated subcutaneously in 100 µl serum free media, in the right hind, twice a week. Controls were treated with 100 µl serum free media.

13.15 Long-term memory assay

Mice in complete remission after prophylactic or therapeutic vaccination treatments were re-challenged with $2x10^6$ L5178Y-R or $5x10^5$ 4T1 viable cells in 100 μL PBS into the opposite limb, and tumor volume was measured as described above.

13.16 Statistical analysis

Mice were randomly assigned to different groups for all *in vivo* studies. Independent experiments were repeated three independent times. Mann-Whitney tests and two-tailed

unpaired Student's *t*-tests were performed using GraphPad Prism Software (San Diego CA, USA) and presented as mean values ±SD. The *p* values were considered significant as follows: $p<0.05$; $p<0.01$ and $p<0.001$.

14 RESULTS

14.1 CD47 agonist peptide, PKHB1, induces cell death in tumor cells

The thrombospondin-1 mimetic peptide PKHB1 has shown cytotoxicity in several neoplastic cell lines, mostly in hematopoietic malignances (A.-C. Martinez-Torres et al. 2015; Denèfle et al. 2016a) . In this thesis the objective was to evaluate its effects in a murine T-cell lymphoblastic tumor cell line, L5178Y-R, and in human breast cancer cell lines, MCF-7 and MDA-MB-231, as well as on the murine homologous, 4T1 cell line. PKHB1 induces cell death in a concentration-dependent way, because the cells incubated for 2 h with increasing concentrations (100, 200 and 300 μM) of PKHB1 showed an increase number of Ann-V-APC/PI positive L5178Y-R (Figure 9A), 4T1 (Figure 9B), MDA-MB-231 (Figure 9C) and MCF-7 (Figure 9D) cells. The cytotoxic concentration that induces approximately 50% of cell death (CC_{50}) in L5178Y-R is 200 μM, in 4T1 is 300 μM, in MDA-MB-231 is 200 μM and in MCF-7 is 300 μM.

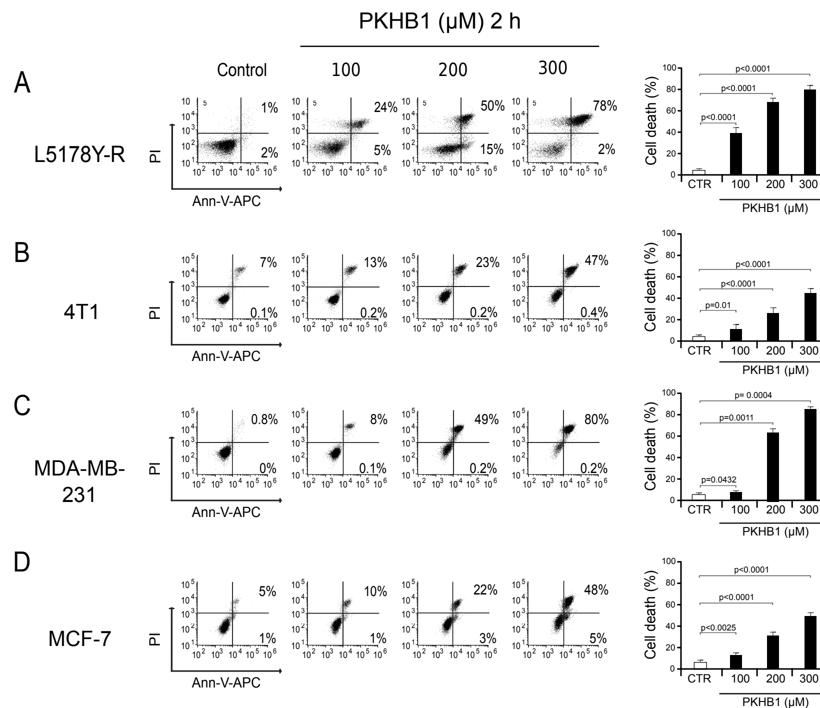

FIGURE 9. PKHB1 induces cell death in different cancer cell lines. Cell death was measured by Annexin-V- allophycocyanin (Annexin-V-APC) and propidium iodide (PI) staining and graphed. Dot plots of (A) L5178Y-R murine lymphoblastic T-cell line, (B) 4T1 murine breast cancer cells, (C) MDA-MB-231 and (D) MCF-7 human breast cancer cell lines, without treatment (Control) and treated with 100, 200 and 300 μM of PKHB1 for 2 h. Graphs represent the means (± SD) of triplicates of at least three independent experiments (right side for each cell line).

14.2 PKHB1 treatment induces calreticulin exposure

To assess the immunogenicity of the cell death induced by the activation of CD47 by PKHB1 in cancer cells, first the exposure of CRT was evaluated, since this protein is one of the principal DAMPs necessary for the correct maturation of DCs and antigen presentation on ICD (Obeid et al. 2007; Garg et al. 2015). Additionally, previous results using PKHB1 displayed CRT exposure in CLL (A.-C. Martinez-Torres et al. 2015). The results in Figure 10 show that PKHB1 treatment is able to induce CRT exposure in all the cell lines tested.

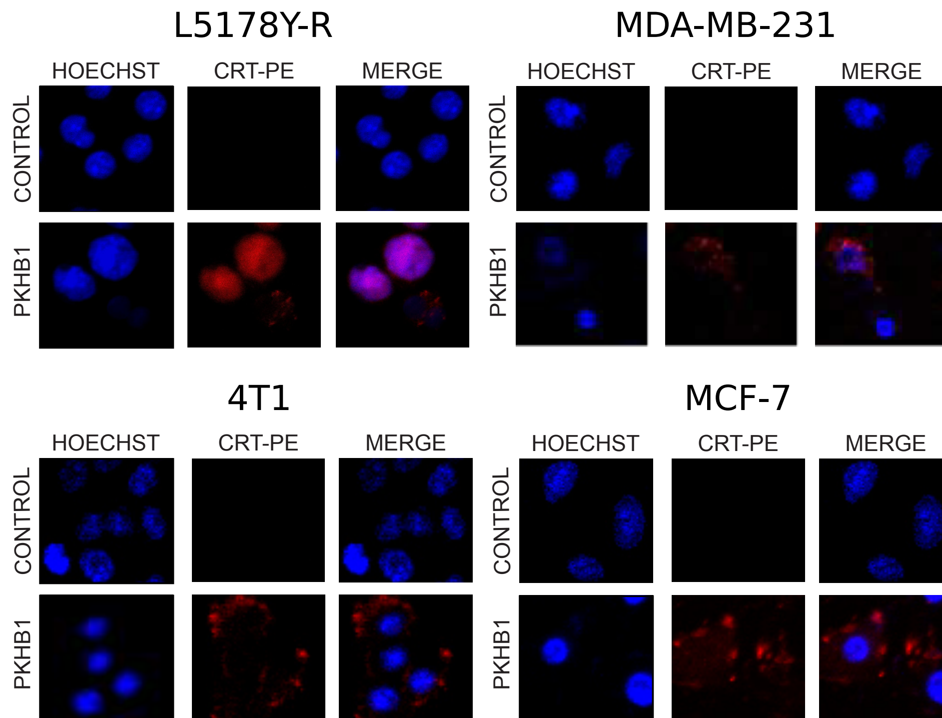

FIGURE 10. PKHB1 treatment induces calreticulin exposure. ECTO-CRT was observed by confocal microscopy 40× (Mechanical zoom 7, Olympus X70; Olympus, Tokyo, Japan) in the cells left untreated (Control) or treated with PKHB1, using CRT-PE staining and Hoechst 33342 (for nuclear staining). Results shown are representative of triplicates of three independent experiments. CRT-PE= Calreticulin-PhycoErythrin.

14.3 Expression and release of HSP90 and CRT in tumor cells and supernatants

Different ICD inductors can lead to DAMPs release by cancer cells (Krysko et al. 2012; Garg et al. 2015). For this reason, once confirmed that PKHB1 treatment induce CRT exposure in cancer cells, the presence of DAMPs was evaluated in cellular lysates and supernatants of untreated and PKHB1-treated cells (CC_{50} for each cell line), by Western blot.

Figure 11A shows the decrease in the expression of HSP90 and CRT in cellular lysates of cells treated with PKHB1. Conversely, the increase of these DAMPs was observed in PKHB1-treated cells' supernatants compared with the untreated cells by western blot (Figure11). These results indicate that PKHB1 treatment prompts the release of HSP90 and CRT to the extracellular medium.

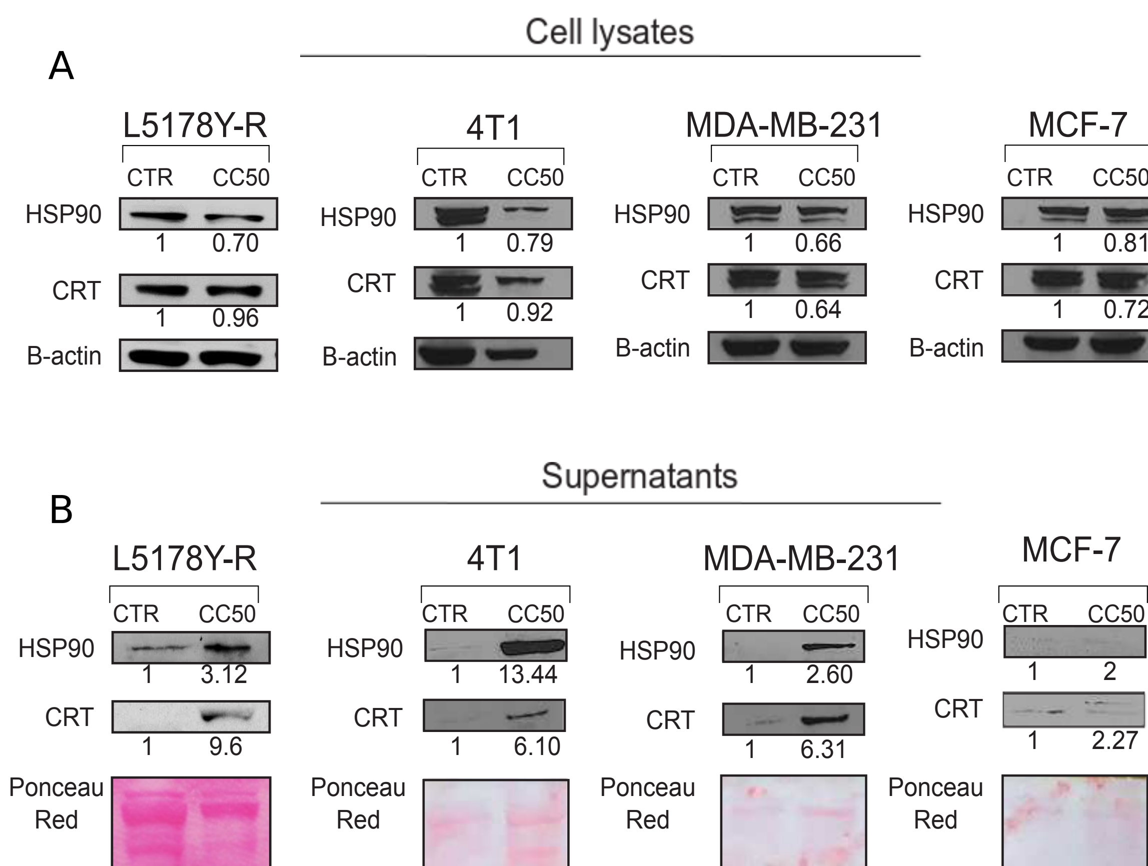

FIGURE 11. Heat shock proteins (HSP90, HSP70) and calreticulin (CRT) expression and release in response to PKHB1 treatment. Western blot was carried out using cellular lysates (A) or supernatants (B) of L5178Y-R, 4T1, MDA-MB-231 and MCF-7 cells untreated (CTR) and treated with PKHB1 (CC_{50}) for 2 h. β-actin and Ponceau red were used and loading controls. Results shown are representative of triplicates of at least three independent experiments.

14.4 HMGB1 and ATP release by PKHB1 treatment in cancer cells

Another important indicator of ICD is the release of HMGB1 and ATP by the dying cancer cells (Krysko et al. 2012; Garg et al. 2015). Therefore, the presence of HMGB1 and ATP in the supernatants of treated and untreated cancer cells was assessed by ELISA or

bioluminescence detection (respectively). Results show a significant release of HMGB1 and ATP in PKHB1-treated cells (Figure 12A and B), when compared with untreated cells. These results indicate that PKHB1 treatment induces the release of HMGB1 and ATP in all the cancer cells tested.

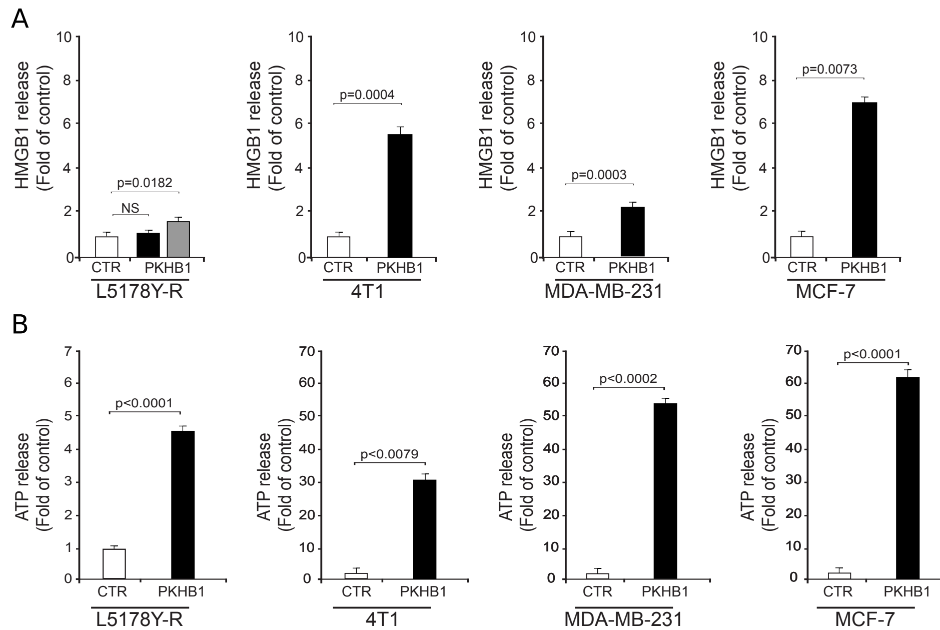

Figure 12. PKHB1 treatment induces high-mobility group box1 (HMGB1) and ATP release. Cells were treated with PKHB1 (CC_{50} or CC_{100}) for 2 h, then 100 µL supernatant of each sample was taken to measure HMGB1 release by ELISA (A) or ATP release through bioluminescence detection (B). Graphs shown are means (± SD) of triplicates of three independent experiments. NS= Not significant.

14.5 PKHB1-TCL induces maturation of bone marrow-derived DCs

As previously indicated, DAMPS release is not determinant for ICD, and *ex vivo* and *in vivo* analyses are necessary to confirm ICD. To assess the antitumor immune response induced by PKHB1, a tumor cell lysate through PKHB1 CC_{100} treatment was obtained (PKHB1-TCL). Then, it was determined if the PKHB1-TCL was able to induce DCs maturation, through the evaluation of the expression of CD80, CD86, and the release of TNFα, indicators of a mature DCs phenotype (Dudek et al. 2013). Bone marrow-derived murine DCs were left untreated (Control) or pulsed for 24 h with the previously obtained PKHB1-TCL. After co-culture with PKHB1-TCL, DCs show a significant increase in the expression of co-stimulatory molecules CD80 and CD86 passing from 50% to 78% and 83%, respectively in L5178Y-R-PKHB1-TCL, and from 50% to 60% and 90%, respectively in 4T1-PKHB1-

TCL, while maintaining the expression of the DCs marker CD11c in both cases (Figure 13 A, B). Furthermore, only DCs pulsed with PKHB1-TCL show a significant increase in TNFα release in comparison with unstimulated DCs (Figure 13 C, D). These results show that PKHB1-induced cell death is able to promote DCs maturation.

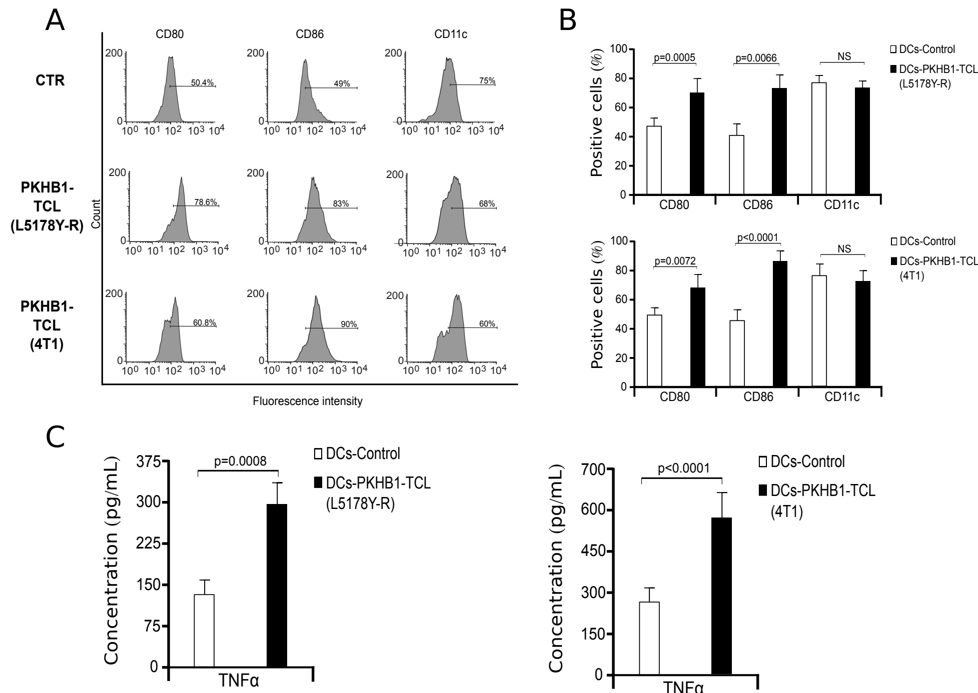

Figure 13. PKHB1-Tumor cell lysate induces the maturation of bone marrow-derived dendritic cells. (A) Bone marrow-derived murine DCs were left only with medium (Control) or pulsed during 24 h with a PKHB1-TCL. DCs were then stained to assess cell surface markers (CD11c, CD80, or CD86) by FACS, and representative histograms are shown. (B) The means obtained by FACS were graphed for each TCL. (C) DCs were treated as in A and the supernatants were collected to quantify TNFα release, by FACS. Graphs represent the means (± SD) of triplicates of at least three independent experiments.

14.6 PKHB1-TCL induces anti-tumor T cell responses

Once determined that PKHB1-TCL was able to induce DCs maturation, it was assessed if the pulsed DCs (DCs-PKHB1-TCL) were able to prime T cells. First, CD3+ cells were co-cultured for four days with pulsed or unpulsed DCs and the release of TNFα, IFNγ, IL-5, IL-4, and IL-2 was assessed. Table 1 shows that co-culture of pulsed DCs with primary T lymphocytes induces the release of TNFα, IFNγ, and IL-2, while IL-5 and IL-4 release were not detected.

	TNFα (pg/mL)	IFNγ (pg/mL)	IL-5 (pg/mL)	IL-4 (pg/mL)	IL-2 (pg/mL)
DCs-Control + T-lymphocytes	38.9 ± 14	0 ± 0	0 ± 0	0 ± 0	0.2 ± 0.2
DCs-PKHB1-TCL (L5178Y-R) + T-lymphocytes	479.6** ± 156	974.33*** ± 115	0 ± 0	0 ± 0	3.5*** ± 0.7
DCs-PKHB1-TCL (4T1) + T-lymphocytes	335.17*** ± 33.64	698.95 ** ± 322.54	0 ± 0	0 ± 0	1.83* ± 1.4

Table 3. TNFα, IFNγ, IL-5, IL-4, and IL-2 cytokine release in co-cultures of T lymphocytes with control or pulsed DCs. Bone marrow-derived murine DCs were left with medium alone (DCs-Control) or pulsed (DCs-PKHB1-TCL) during 24h with a PKHB1-TCL. Then, DCs were co-cultured during 4 days with T-lymphocytes, and the supernatants were collected to quantify TNFα, IFNγ, IL-5, IL-4, and IL-2 release, by FACS. **p<0.01; ***p<0.001.

Next, primed (co-cultured with pulsed DCs-PKHB1-TCL) or unprimed (co-cultured with unpulsed DCs) T-lymphocytes were collected and co-cultured during 24h with L5178Y-R or 4T1 cells (previously stained with calcein-AM). Then, supernatants were obtained and IFNγ, IL-4, and IL-2 cytokine release was assessed. A significant increase of IFN-γ release was observed in the supernatants of primed T-lymphocytes when being co-cultured with L5178Y-R cells (Figure 14 A) or 4T1 cells (Figure 14 B), similarly, a significant increase of IL-2 release was observed (Figure 14 C, D) while no difference was observed in IL-4 release (Figure 14 C,D).

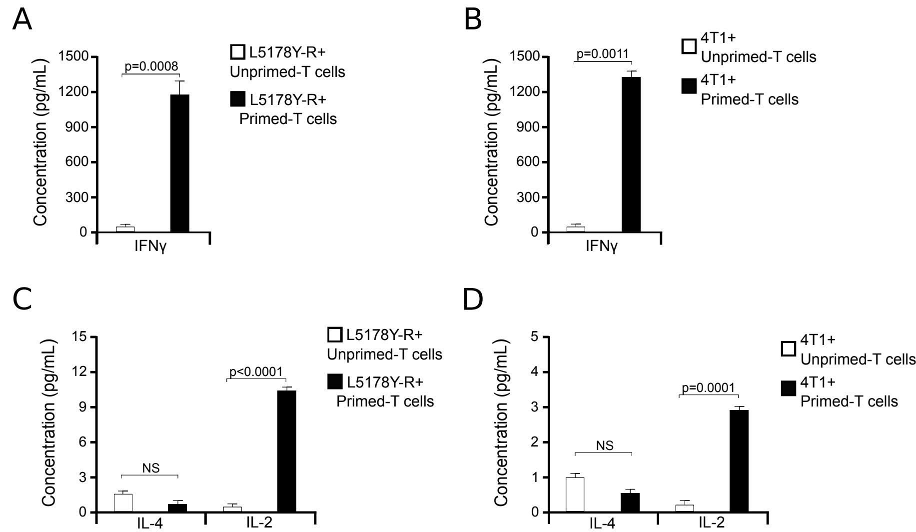

Figure 14. IFN-γ, IL-4 and IL-2 secretion by unprimed or primed T cells co-cultured with murine tumor cells. L5178Y-R or 4T1 cells were co-cultured with unprimed T lymphocytes (previously co-cultured with unstimulated DCs-Control) or primed T-lymphocytes (previously co-cultured with pulsed DCs-PKHB1-TCL) in a 1:5 tumor to effector ratio, for 24 h and the supernatants were collected and assayed for IFN-γ release by ELISA (A,B) and IL-4 and IL-2 release by FACS (B,C).

Once we observed that PKHB1-TCL induced IFNγ and IL-2 release, suggesting Th1 responses (Viallard et al. 1999), we assessed antitumor cell cytotoxicity. For this purpose, we evaluated the decrease in calcein stained L5178Y-R or 4T1 cells after co-culture with primed or unprimed T-lymphocytes. Results show that only T-lymphocytes co-cultured with pulsed DCs-PKHB1-TCL induce a significant decrease in calcein stained L5178Y-R (Figure 15 A) and 4T1 (Figure 15 B) cells, in comparison with the L5178Y-R and 4T1 cells cocultured with unprimed T lymphocytes (Figure 15 A, B respectively). This confirms the correct antigen presentation by DCs-PKHB1-TCL and the T cell cytotoxicity against L5178Y-R and 4T1 cancer cells.

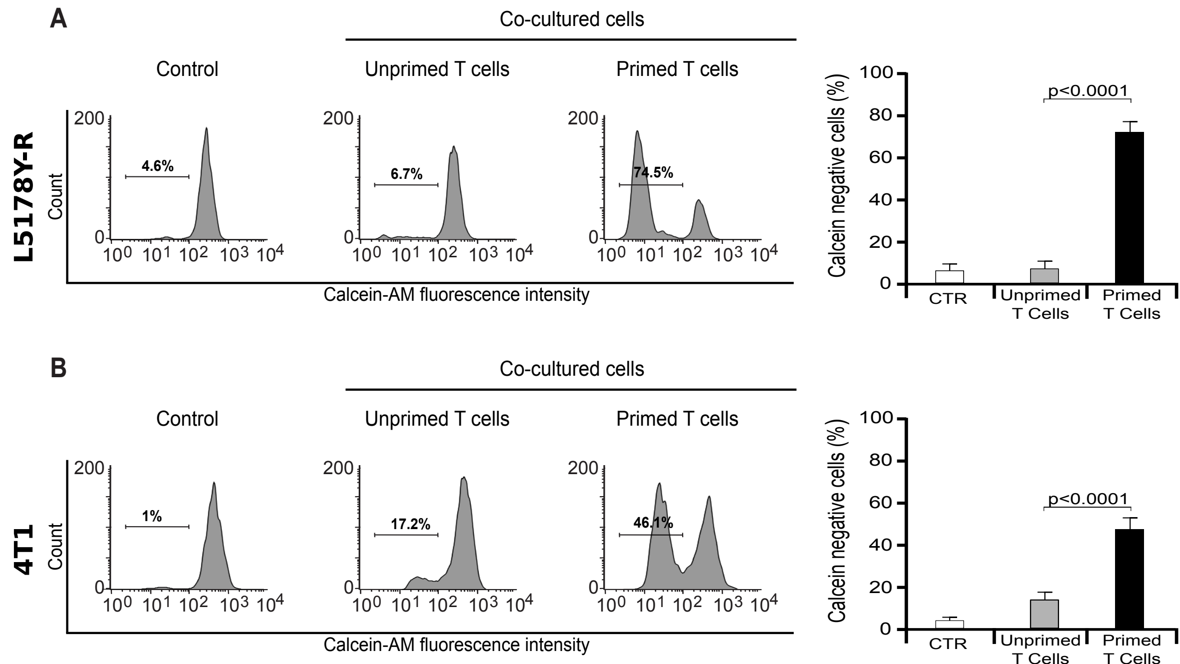

Figure 15. Primed T cells' cytotoxicity in murine cancer cells. (A) L5178Y-R or (B) 4T1 cells were stained with calcein-AM and co-cultured with unprimed T lymphocytes (previously co-cultured with unstimulated DCs-Control) or primed T-lymphocytes (previously co-cultured with pulsed DCs-PKHB1-TCL) in a 1:5 tumor to effector ratio for 24h. The percentage of L5178Y-R or 4T1 calcein negative cells was assessed by FACS; representative histograms are shown. Graphs at the right represent the means (± SD) of triplicates of at least three independent experiments.

14.7 The prophylactic vaccine with PKHB1-treated cells prevented the tumor establishment of L5178Y-R and 4T1 cells

Considering that PKHB1 treatment induces DAMPs release and an antitumor immune response *ex vivo*, the next step was to carry out a prophylactic vaccination, to confirm ICD. The vaccine is based in the use of PKHB1-TCL of L5178YR or 4T1 cells. Two groups of mice were used as follows for every tumor (L5178Y-R or 4T1) model: (I) control group without vaccine; (II) vaccine group, with $5x10^6$ of L5178Y-R or $3x10^6$ 4T1 PKHB1-treated cells. Results showed that vaccination containing PKHB1-treated cells prevented the establishment of Lymphoma (L5178Y-R) and Breast cancer (4T1) tumor models (Figure 16 A, B). In the control group of L5178Y-R, six out of six mice (100%) developed tumor after inoculation with viable cells, whereas none of the mice (0%) in the 5M vaccine group developed the tumor (Figure 16A). In the control group of 4T1, eight out of eight mice (100%) developed tumor after inoculation with viable cells, whereas two of ten mice (20%) in the 3M vaccine group developed the tumor (Figure 16 B). The 60-day survival rates of

mice in each group were consistent with tumor growth, being 100% in the 5M vaccine group for lymphoma model (L5178Y-R) and 80% in the breast cancer model (4T1) (Figure 16 C, D).

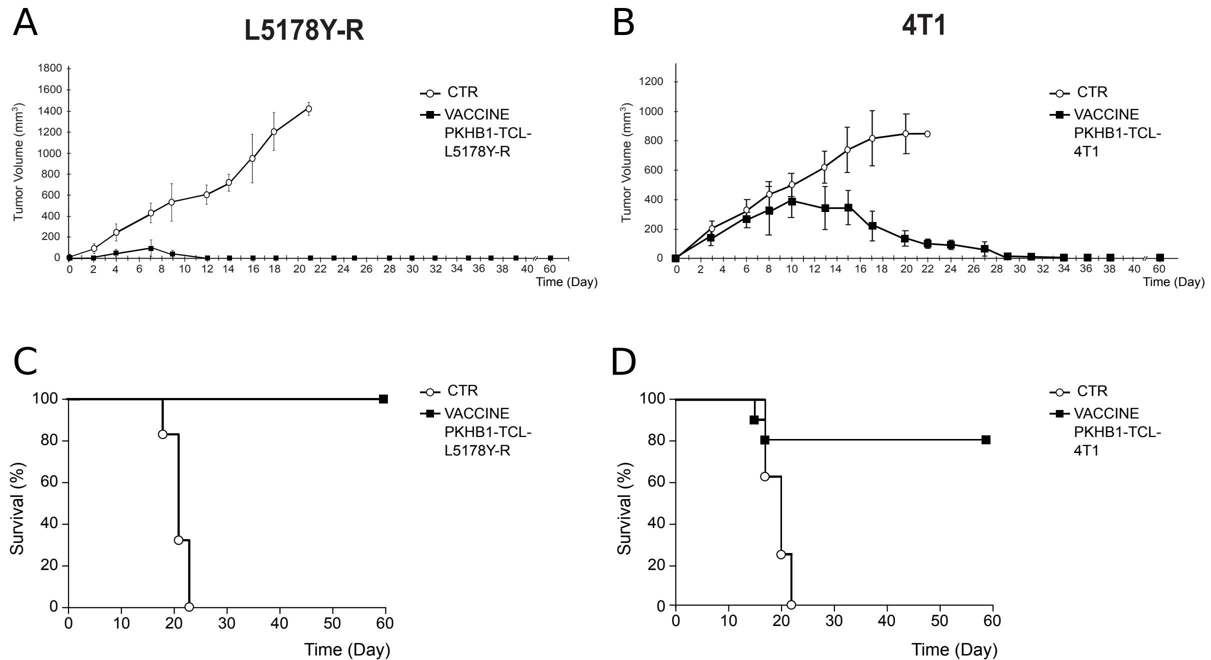

Figure 16. PKHB1-TCL prevents the tumor establishment through a single prophylactic vaccination. (A) Graph indicates tumor growth in unvaccinated mice of L5178Y-R (Control; n = 6) or vaccinated with $5x10^6$ (PKHB1-TCL-L5178Y-R, n= 6) CC_{100} PKHB1-treated L5178Y-R cells and re-challenged with $2x10^6$ L5178Y-R cells. Each line represents the mean of the tumor volume per group. (B) Graph indicates tumor growth in unvaccinated mice of 4T1 (C, Control; n = 8) or vaccinated with $3X10^6$ (PKHB1-TCL-4T1, n=10) CC_{100} PKHB1-treated 4T1 cells and re-challenged with $5X10^5$ 4T1 cells. Each line represents the mean of the tumor volume per group. (C, D) Kaplan Meier survival graph of mice treated as in A and B over time

14.8 PKHB1-TCL induces tumor regression

After the *ex-vivo* and *in vivo* results, then it was evaluated if the PKHB1-TCL was able to diminish tumor growth and improve overall survival in syngeneic mice transplanted with L5178Y-R or 4T1 cells. First, viable cells were inoculated in BALB/c mice, and when the tumor reached 100 mm^3, a mice control-group was left without treatment, and a second group was treated with PKHB1-TCL two times per week for a total of four injections (Figure 17 A). Tumor growth measurements show that PKHB1-TCL-treated mice had a significantly diminished tumor growth after day 10 (7 days after the first treatment), which continued to decrease until no tumor was detected by day 30, for both lymphoma and breast cancer models (Figure 17 B, C). Tumor growth diminution was reflected in overall mice survival, as

L5178Y-R-PKHB1-TCL-treated mice presented an 80% of survival over time, while all control mice perished by day 11 (Figure 17 D). In the breast cancer model as 4T1-PKHB1-TCL-treated mice presented an 80% of survival over time, while all control mice perished by day 24 (Figure 17 E).

When observing that PKHB1-TCL induces tumor remission in two completely different tumor models (lymphoma and breast cancer), the strength of the immunogenicity of death induced by CD47 activation is evidenced.

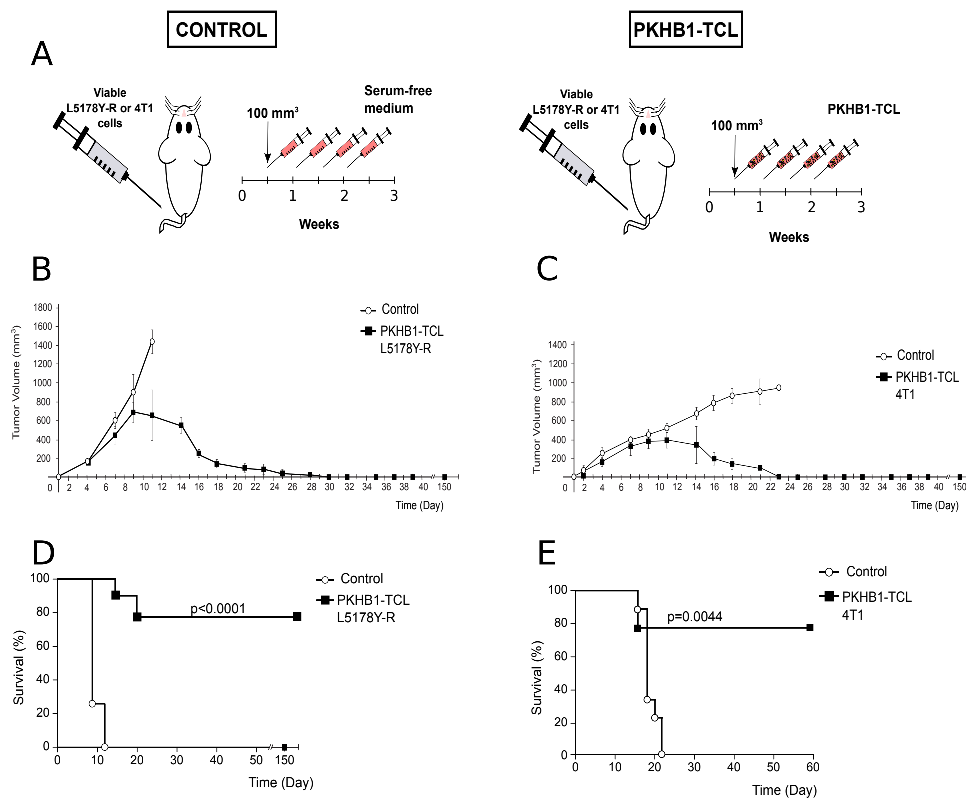

Figure 17. PKHB1-TCL treatment induces tumor regression, increasing mice's survival over time. (A) Schema of PKHB1-TCL (5×10^6 or $3x10^6$ PKHB1-treated L5178Y-R or 4T1 cells respectively) treatment started when tumor reached 100 mm^3, then PKHB1-TCL was administrated every 3 days for two weeks (for a total of four injections). (A) Graph indicates tumor growth in unvaccinated mice of L5178Y-R (Control; n = 7) or vaccinated with $5x10^6$ (PKHB1-TCL-L5178Y-R, n= 9) CC_{100} PKHB1-treated L5178Y-R cells. Each line represents the mean of the tumor volume per group. (B) Graph indicates tumor growth in unvaccinated mice of 4T1 (C, Control; n = 9) or vaccinated with $3X10^6$ (PKHB1-TCL-4T1, n=9) CC_{100} PKHB1-treated 4T1 cells. Each line represents the mean of the tumor volume per group. (C, D) Kaplan Meier survival graph of mice treated as in A and B over time.

14.9 PKHB1-TCL therapeutic vaccination induces long-term antitumor memory

To assess the immunological memory against tumor antigens induced by PKHB1-TCL-treatment, mice in complete remission (tumor free >60 days) were re-challenged with living L5178YR or 4T1 cells. Compared to naïve mice (Control), in which a primary L5178YR cells challenge resulted in a 0% of survival and all control mice perished by day 22, those that were in remission were completely resistant to a re-challenge of L5178YR cells resulted in a 100% of survival (Figure 18 A). In the breast cancer model, control mice after primary 4T1 cells challenge resulted in a 0% of survival in which all control mice perished by day 24, and those that were in remission were completely resistant to a re-challenge of 4T1 cells resulted in a 100% of survival (Figure 18 B).

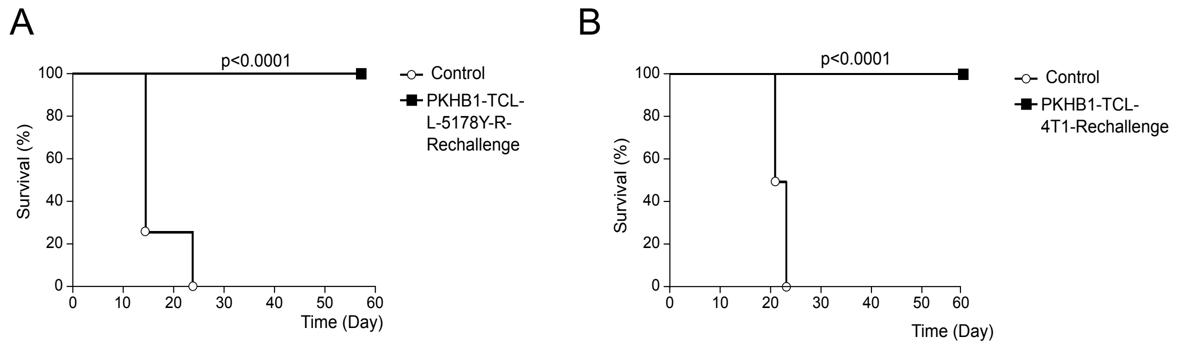

Figure 18. PKHB1-TCL therapeutic vaccination induces long-term antitumor memory. Mice in remission after therapeutic vaccinations were re-challenged with $3x10^6$ L5178Y-R (A) or $5x10^5$ 4T1 (B) viable cells. Survival is represented by the Kaplan-Meier graph. (A) Control n = 9, PKHB1-TCL n = 9. (B) Control n = 8, PKHB1-TCL n = 7.

15.DISCUSSION

"The danger theory", proposed by Polly Matzinger (Matzinger 1994), establishes that the main trigger for the induction of an immune response are the "danger signals" recognized by the immune system cells. These signals were later called damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs), and it is now known that the absence of these signals results in immune-tolerance. One of the applications of this theory is in the context of antitumor therapy, especially after the description of the immunogenic types of cell death. ICD is a type of cell death that is able to activate antitumor immune responses through the release or exposure of DAMPs (Garg et al. 2015). Currently, this type of cell death is a critical determinant of the efficacy of antitumor treatments, since its execution may lead to the elimination of chemo-resistant cells with a high recurrence rate (Garg et al. 2015). For this reason, current therapeutic strategies search the induction of ICD. In this context, our research group previously demonstrated that the activation of CD47 by agonist peptides derived from TSP-1 such as PKHB1 induces regulated cell death, causing calreticulin exposure and morphological changes of ER in CLL, two characteristics intimately related to ICD. Therefore, within this thesis it was explored the immunogenicity of cell death induced by the activation of CD47 in murine lymphoma cells L5178Y-R (T-cell lymphoblasts), breast cancer cells from mice, 4T1 (triple negative), and human, MDA-MB-231 (triple negative) and MCF-7 (Luminal A).

In summary, this thesis describes the immunogenicity of the cell death induced by the activation of CD47. Here I will discuss the five main aspects of my thesis' results:

1. Molecular characteristics of the ICD induced by the activation of CD47
2. Effect of CD47-ICD on the immune response
3. PKHB1-TCL from the proof of concept to therapeutic potential
4. CD47-ICD and immunological memory
5. CD47 activation by agonist peptides: an ideal ICD strategy?

Through the following pages it will be discussed the relevance, scope and perspectives of the results obtained.

1. Molecular characteristics of the ICD induced by the activation of CD47

The RCD induced by CD47-agonist peptides has been reported in different types of cancer, including lung, colon, breast and leukemia cancers (Manna and Frazier 2004; A.-C. Martinez-Torres et al. 2015; Denèfle et al. 2016a). In all cases, peptides such as 4N1K and PKHB1 induce a dose-dependent cell death (Manna and Frazier 2004; A.-C. Martinez-Torres et al. 2015; Denèfle et al. 2016a). As previously reported, here it was found that PKHB1 induces a dose-dependent cell death, because as the concentration of PKHB1 increases (100, 200 and 300uM) the percentage of cell death increases. In addition, it was observed that the mean cytotoxic concentration (CC_{50}) of PKHB1 was: 200μM for L5178Y-R and MDA-MB-231 cells and 300μM for 4T1 and MCF-7 cells.

The mechanism of cell death induced by CD47-agonist peptides, is characterized by being calcium-dependent and dependent of the phosphorylation of PLCγ1 in CLL cells (A.-C. Martinez-Torres et al. 2015). Thus, it is logical to think that a higher expression of proteins involved in calcium signaling can confer to the cancer cells a greater susceptibility to this type of RCD. In this sense, the most sensitive cells to CD47-RCD were the L5178Y-R and MDA-MB-231 cells. It is important to note that the L5178Y-R cells are tumor T lymphoblasts, which highly express PLCγ1 (Irvin et al. 2002), while MDA-MB-231 cells have been reported to overexpress proteins involved in the regulation of calcium, such as PLCγ1 and ORAI1, when compared to MCF-7 (McAndrew et al. 2011; Abalsamo et al. 2012). These molecular characteristics may confer an increased susceptibility to CD47-RCD in L5178Y-R and MDA-MB-231 cells.

On the other hand, the 4T1 cell line is a murine model of breast cancer that has been widely used in research due to its tumorigenic and invasive capacity, and which has a great similarity with the MDA-MB-231 cell line, sharing characteristics such as lack of estrogen, progesterone, and growth factor receptors, in addition to resembling a late stage of breast cancer *in vivo* (Pulaski and Ostrand-Rosenberg 2001). Despite the similarities found between these two cell lines, I observed a lower susceptibility to CD47-RCD in 4T1 cells, indicating

that these could have different molecular characteristics than MDA-MB-231 cells that have not been reported until today.

The general, resistance of cancer cells against immunosurveillance and antitumor immunity is achieved by mechanisms including: low immunogenicity, the ability to induce immunosuppression, and resistance to death by immune cells (Dunn et al. 2002; Kroemer et al. 2013; Garg et al. 2015). The low immunogenicity of tumor cells is the main obstacle in the reactivation of the antitumor immune response, therefore it was proposed that a way to reactivate potent antitumor immune responses is accentuating the immunogenicity of cancer cells (Casares et al. 2005) by the emission of danger signals or damage associated molecular patterns (DAMPs) (Kepp et al. 2014). Recently, it was demonstrated that the induction of a cancer cell death pathway called "immunogenic cell death" is defined as a functionally distinct form of regulated cell death that facilitates the establishment of an adaptive immune response, thanks to the emission of DAMPs and neoantigens, increasing the immunogenicity of moribund cells (Kepp et al. 2014; Garg et al. 2017).

Therefore, once determined that PKHB1 is cytotoxic in the cell lines proved, the immunogenicity of cell death was evaluated, determining the exposure or release of DAMPs involved with ICD, which are calreticulin (CRT) , heat shock proteins (HSP90), HMGB1, and ATP (Garg et al. 2015).

The first DAMP to be evaluated was CRT, because it is one of the main molecules necessary to determine that cell death is immunogenic (Obeid et al. 2007; Bezu et al. 2015), and its exposure in the cell membrane has been continuously reported as one of the main DAMPs necessary for the correct maturation of DCs and antigen presentation (Obeid et al. 2007; Garg et al. 2015). In addition, it was previously reported that the activation of CD47 by PKHB1 induces CRT exposure in CLL cells (A.-C. Martinez-Torres et al. 2015). Here it was demonstrated that PKHB1 is capable of inducing CRT exposure in L5178Y-R, 4T1, MDA-MB-231 and MCF-7 cells. With this, it is confirmed that PKHB1 is able to induce the CRT exposure in other types of cancer cells, this being the first report of CRT exposure by treatment with PKHB1 in breast cancer cells.

There is a close correlation between the expression of CRT and CD47 in cancer cells. It has been observed that the expression of CRT is an early event in newly arising neoplasms and only cells that increase the expression of CD47 can avoid phagocytosis (Chao et al. 2010). Contrary to the "don´t eat me" signal that generates CD47, it has been observed that the peptide 4N1K, derived from TSP-1, when bound to CD47, is capable of promoting phagocytosis of the target cell, depending on the conformation of CD47 (Burger et al. 2012). With this, the use of CD47 as a target for the induction of cell death becomes more attractive, since in addition to taking advantage of its over-expression in most types of cancers reported, peptides such as PKHB1 could also promote the phagocytosis of cancer cells. However, there is a lack of evidence to determine what happens after PKHB1 activates CD47 and induces cell death. CD47 is internalized? PKHB1 dissociates and continues to activate other CD47`s? does PKHB1 promotes the formation of CD47 clusters? for example.

In addition to CRT exposure, different ICD inductors can promote the release of others DAMPs and induce the activation of a potent antitumor response (Krysko et al. 2012; Kepp et al. 2014). Therefore, additionally to determining the exposure of CRT, its release was also evaluated, as well as that of other DAMPs: HSP90, HMGB1 and ATP.

These results demonstrate that PKHB1 is able to induce the release of CRT, HSP90 and ATP in all tumor cells assessed and only in L5178Y-R cells the release of HMGB1 was not detectable in the conditions it was assessed, however, recently we found that the CC_{100} of PKHB1 effectively induce the HMGB1 release (Uscanga-Palomeque et al. 2018a). It has been observed that the presence of all DAMPs is not necessary for cell death to be defined as immunogenic (Bezu et al. 2015), additionally neither the presence of all DAMPs is sufficient evidence to ensure the induction of MCI. Therefore, the analysis of molecular determinants of ICD is not sufficient to determine that ICD is taking place. In fact, the analysis of DAMPs should be considered complementary to the analysis of the immune response *ex vivo* and *in vivo* to conclude that an agent / strategy is an ICD inductor (Garg et al. 2015). This is why our next step was to evaluate these parameters after cell death induction by PKHB1.

2. Effect of CD47-ICD on the immune response

It is important to know the effect of the DAMPs released or exposed after the CD47-ICD induction in the activation of the immune response. CRT, HSP90, ATP and HMGB1 are recognized by a wide range of receptors among which are: CD91, purinergic receptors (P2Y2 and P2X7), toll-like receptors (TLR2 and 4) and Scavenger receptors (SREC1 and FEEL1) among others, which promote the phagocytosis of cellular debris, induce the migration of phagocytic cells, promote the antigen presentation and induce the cytokines' release, culminating in the activation of the specific antitumor immune responses (Krysko et al. 2012; Garg et al. 2015).

Due to the importance of DAMPs in the activation of the antitumor immune response, it was evaluated whether the dead tumor cells treated with PKHB1 (PKHB1-TCL) promote the establishment of the antitumor immune response, through the maturation of dendritic cells and consequent activation of T lymphocytes.

The phenotype of mature DCs is characterized by the expression of the CD11c differentiation receptor (Laursen et al. 2018), and a greater expression of CD80 and CD86 co-stimulatory molecules (Dudek et al. 2013). Here it was demonstrated that cell death induced by PKHB1 in L5178Y-R (PKHB1-TCL-L5178Y-R) and 4T1 (PKHB1-TCL-4T1) cells is able to promote the maturation of DCs since this induced a significant increase in the expression of CD80 and CD86 co-stimulatory molecules, as well as the significant release of TNFα. A slight (non-significant) decrease in the expression of CD11c was also observed, which is a marker of differentiation that can be regulated downwards by dendritic cells after being activated by TLR agonists (Singh-Jasuja et al. 2013).

The secretion of TNFα has been associated with a mature phenotype of DCs, since it acts as an autocrine maturation factor for DCs (Dudek et al. 2013). Several TCLs can induce their secretion in different degrees, from 20 pg / mL to 250 pg / mL (Chiarella et al. 2008; Chiang et al. 2013). In this work it was observed that the DCs pulsed with the PKHB1-TCL-L5178Y-R induced the secretion of TNFα at a concentration of 270 pg / ml and the PKHB1-TCL-4T1

at a concentration of 600 pg/ml, in both cases was a greater release than most of the reports where they use TCL, which indicates the effective maturation of the DCs and the high immunogenicity of the PKHB1-TCL.

On the other hand, different types of TCL can induce the DCs maturation in different degrees (Chiang, Coukos, and Kandalaft 2015); however, most of them use LPS (Chen et al. 2012) or other adjuvants such as phyto-extracts (Chang et al. 2013) and bacterial ghosts (Dobrovolskienė et al. 2018) in combination with TCL. The results obtained in this thesis demonstrate that cell death induced by PKHB1 is able to promote DCs maturation and TNFα secretion, even in the absence of other immunological stimulants. In this way, we can confirm that DAMPs and neo-antigens released by the treatment with PKHB1 in the cell lines used promote the efficient maturation of DCs.

3. PKHB1-TCL from the proof of concept to therapeutic potential

As described above, the simultaneous detection of cell death, exposure and release of CRT, the release of HSP90, ATP and HMGB1 (*in vitro*), in addition to the evaluation of ICD in the establishment of the antitumor immune response (*ex vivo*) are useful for the identification of possible inductors of ICD. However, vaccination assays involving immunocompetent mice and syngeneic cancer cells are the gold standard to formally identify ICD inducers, because this demonstrates the tumor rejection capacity of the immunized host (Kepp et al. 2014; Garg et al. 2015).

When assessing the gold standard, here it was demonstrated that the death induced by PKHB1 activates the antitumor immune response against lymphoma cells (L5178Y-R) and breast cancer cells (4T1), since it achieved to prevent tumor establishment and increase survival in 100% and 80% of the mice respectively.

This has also been reported with different agents that induce ICD and in different tumor models. Agents such as doxorubicin, iadurobicin and mitoxantrone in a model of colorectal carcinoma presented between 90 and 80% of tumor-free mice (Obeid et al. 2007). Also, in a

colorectal carcinoma model (MC-38), specific forms of radiotherapy induced 90% survival of mice (Gorin et al. 2014). The alkylating agent melphalan induced 40% of tumor-free mice in a melanoma model (Dudek-Perić et al. 2015). In addition, the antibody 7A7 (anti-EGFR) induced 50% of survival in a lung carcinoma model (D-122) (Garrido et al. 2011).

However, despite the existence of the gold standard, multiple publications and reviews (Bezu et al. 2015) classify different ICD-inducing agents even though their direct effectiveness in prophylactic vaccination trials has not been proven. For example, bleomycin is considered as an ICD inductor (Bezu et al. 2015), however when it was tested *in vivo*, no prophylactic vaccination trials were performed and the *ex vivo* assays did not demonstrate a clear activation of the antitumor immune response (Bugaut et al. 2013). Therefore, it is necessary to maintain rigorous standards to confirm that an agent induces ICD to eliminate the possibility of reporting "false positives" (Garg et al. 2015) since this can be prejudicial in clinical practice.

Also, it must be taken into account that the ability of an agent to cause ICD can´t be predicted even from its chemical or structural properties, since molecules as similar to each other as oxaliplatin and cisplatin don´t share this functional profile (Tesniere et al. 2010; Martins et al. 2011).

3.1 Therapeutic application of ICD

Overall, the results obtained in this work, which showed a powerful *ex vivo* and *in vivo* antitumor immune response induced by PKHB1-TCL in two tumor models with different characteristics such as murine lymphoma (L5178Y-R) and breast cancer (4T1) encouraged the idea of evaluating the treatment with PKHB1-TCL on a previously established tumor. This strategy can be used as a confirmatory trial of ICD inductors, to evaluate their ability to mediate therapeutic effects dependent on the immune system against established neoplasms (Kepp et al. 2014).

In clinical trials, TCLs have been tested in melanoma, prostate, mesothelioma, ovarian and colorectal cancer (Chiang, Coukos, and Kandalaft 2015). These TCLs are usually produced by radiation, repeated freezing and thawing, among others. In this thesis work it was demonstrated that the activation of CD47 by PKHB1, which has been reported to effectively induce cell death in different types of cancer (A.-C. Martinez-Torres et al. 2015; Denèfle et al. 2016a), including cells from refractory patients (A.-C. Martinez-Torres et al. 2015; Denèfle et al. 2016a), can provide an immunogenic TCL that is capable of promoting an antitumor immune response, even in the absence of adjuvants.

Interestingly, when using PKHB1-TCL on established tumors of murine lymphoma and breast cancer, it was observed that tumor volume began to decrease 7 days after the first administration of PKHB1-TCL both in the lymphoma model and for the breast cancer model, reaching tumor regression on day 28 and 23 respectively. This waiting time corresponds to the time necessary for the T cells to expand and activate an antitumor immune response (Kaech, Wherry, and Ahmed 2002). Other types of TCL have been able to induce tumor regression, increasing the survival rate in patients with melanoma and prostate cancer (Chiang, Coukos, and Kandalaft 2015); however, they do so only in combination with adjuvants, as in the case of CpG oligodeoxynucleotides, which are TLR9 agonists (Anhua Wu 2007). It is important to note that the immunogenicity of PKHB1-TCL was found even in the absence of adjuvants, which have also been proved to be important to promote immunological memory.

4. CD47-ICD and immunological memory

The perspectives for cell therapy against cancer are based on the development of T cell responses, resulting in effective rejection of the tumor and long-term protection (Henry et al. 1999; Farkona, Diamandis, and Blasutig 2016). From this fact, the induction of ICD is a potential strategy capable of inducing a long-term protection since it induces the activation of the adaptive immune response, through the activation of T lymphocytes.

Recently, it was shown by our group that PKHB1 treatment in tumor-bearing mice induced long-term tumor prevention in 90% of the mice that had complete tumor regression (Uscanga-Palomeque et al. 2018a). Here it was shown that PKHB1-TCL-treatment induces long-term immunological memory against murine lymphoma and breast cancer cells, as 100% of mice in remission after treatment with PKHB1-TCL survived after re-challenge with L5178YR or 4T1 cells, respectively. This underlines the immunogenicity of cell death mediated by CD47, when a CD47 agonist peptide or CD47-dead cells are administered. This long-lasting immunological memory has also been promoted by a TCL obtained by repeated freezing and thawing and glioma cells treated with radiation, where almost 100% survival was observed (Belmans et al. 2017).

Although immunotherapy with pulsed DCs, primed T lymphocytes or CAR-T cells is the main approach used to stimulate antitumor immune responses, it was shown here that the crude TCL obtained from treatment with PKHB1 is capable of inducing tumor regression in the 80 % of the mice, while preventing the establishment of the tumor in 100% of the re-challenged mice that survived after treatment with PKHB1-TCL. Interestingly all of these occurs similarly in two clearly different tumor models (L5178Y-R and 4T1).

5. CD47 activation by agonist peptides: an ideal ICD strategy?

According to the different trends in the fields of ICD, immunology, immunosurveillance, inflammation and cancer therapy in the last decade, a list of properties of an ideal ICD inductor were proposed, which enhance its application in clinical practice (Krysko et al. 2012). Therefore, the evaluation of these characteristics in CD47-ICD, is important to determine which properties it possess, and which ones are still to be determined.

First, an ideal ICD inductor must be an efficient inducer of apoptosis or other types of cell death, in doses that can be used preclinically or clinically without substantial toxicities or side effects. In this regard, it has been shown that PKHB1 induces regulated cell death in different types of cancer cells (A.-C. Martinez-Torres et al. 2015; Denèfle et al. 2016b; Uscanga-Palomeque et al. 2018b), and it has been determined that PKHB1 treatment does

not induce side effects in immunodeficient mice (A. C. Martinez-Torres et al. 2015), in the same way, no damage is observed in different vital organs, and does not affect the percentage of peripheral blood leukocytes in immunocompetent mice (Uscanga-Palomeque et al. 2018b).

Second, an ideal ICD inductor must be capable of inducing the emission of multiple types of DAMPs, which ensure the immune system sensitization, promoting the establishment of the antitumor immune response. In this work, it was determined that PKHB1 effectively induces the exposure and release of different DAMPs, such as CRT, HSP90, ATP and HMGB1, and that CD47-ICD also activates the antitumor immune response (*ex vivo*).

Third, an ideal ICD inductor should not be susceptible to drug efflux channels, in this sense, the PKHB1 peptide is not sensitive to these resistance mechanisms because it depends on the binding with CD47 and not on its entry into the cell.

In addition, since ER stress is fundamental for the ICD induction, as it activates robust pro-death signaling, an ideal ICD inductor must be able to induce ER stress, which would improve the traffic of DAMPs and increase its emission. One of the main ways to induce ER stress is the depletion of Ca^{2+} reserves, which can initiate stress responses such as the unfolded protein response (UPR), which involves the activation of different pathways, by phosphorylation of PERK, IRE1α, and the transcription initiator factor eIF2α as well as the cleavage of ATF-6 (Bravo et al. 2013).
In this sense, it has been observed that PKHB1 treatment in CLL cells induces a sustained Ca^{2+} release and ER morphological changes (A. C. Martinez-Torres et al. 2015). However, other characteristics ares still to be elucidated, such as the activation of the UPR that confirm the ER stress induced by the PKHB1 treatment.

On the other hand, during cancer microevolution, cancer cells can acquire mutations such as caspase and BAX deletion among others, that could prevent the activation of signaling pathways of danger (Panaretakis et al. 2009; Krysko et al. 2012). Therefore, an ideal ICD inducer must overcome as many of these mutations as possible.

It has been reported that PKHB1 is able to induce RCD in cells with dysfunctional p53 and resistant to conventional treatments (A. C. Martinez-Torres et al. 2015), also in this work was observed that PKHB1 is able to induce ICD in MCF-7 cells which present a deletion of caspase 3. Interestingly, the RCD induction by CD47-activation has been reported in different cancer cell lines as a caspase-independent mechanism (A. C. Martinez-Torres et al. 2015), this could overcome mechanisms of immunogenic cell death resistance by cancer cells (Krysko et al. 2012). However, it is necessary to know the mechanism of DAMPs release in the death induced by PKHB1 in order to predict its effect in cells with mutations in this ICD pathway.

Finally, an ideal ICD inductor must be capable of inhibiting the activation of proinflammatory transcription factors in order to induce tumor regression. At the same time, an ideal inducer of ICD should not have suppressive or inhibitory effects on immune cells (Th1 cells, cytotoxic T cells, DCs, among others) since they are necessary for the antitumor immune response establishment. Conversely, it is crucial for an ideal ICD inducer to be able to inhibit immunosuppressive responses, such as those mediated by tumor-associated macrophages (TAM, mainly M2 phenotype), myeloid-derived suppressor cells (MDSC) and regulatory T cells, among others (Krysko et al. 2012).
From this point of view, it has been reported that PKHB1 induces selective RCD of tumor cells, since it does not affect non-tumor B cells of patients with CLL (A. C. Martinez-Torres et al. 2015), in addition it has been observed that it is not cytotoxic to human or murine PBMCs, nor to cells of primary and secondary murine lymphoid organs.

As for the functions of CD47 within the immune system, in addition to functioning as a "don´t eat me" signal (Matozaki et al. 2009), CD47 has been reported as a key piece in the contraction process and resolution of the immune response in humans and mice, and in the differentiation of memory lymphocytes (Van et al. 2012). In addition, it has been reported that CD47 may be involved in the activation signals of T lymphocytes (Reinhold et al. 2002; Rebres et al. 2001) and in the homeostasis of regulatory T lymphocytes (Pandiyan and Lenardo 2008).

That is why the use of CD47 agonist peptides for the induction of ICD is a promising therapeutic strategy, since it presents most of the characteristics of an ideal ICD-inductor agent.

In summary;

- PKHB1 induces an atypical type of RCD in a large group of tumor cells, even in cells with mutations that make them resistant to conventional therapies.
- CD47-ICD causes the exposure and release of different DAMPs, which induce the establishment of the specific antitumor immune response.
- PKHB1 is not susceptible to drug efflux channels, since it binds to a transmembrane receptor, usually overexpressed by cancer cells, to induce ICD.
- PKHB1 does not seem to affect non-tumor cells, as it has been demonstrated to spare immune system cells and to maintain vital organs in mice terated with this agent.

For all of the above, this work evidences the promising use of CD47-activation for the induction of ICD. Furthermore, the results obtained from this thesis support the CD47 point of view that has been poorly exploited, where instead of seeing it as an enemy, we can begin to see it as a good friend.

16.CONCLUSIONS

From the results obtained in the present thesis work we can conclude that the treatment with PKHB1 induces cell death and DAMPs' release in lymphoma and breast cancer cells. Also, CD47-ICD is able to induce the antitumor immune system activation *ex vivo* through the maturation of dendritic cells and the activation of specific T cells responses. Furthermore, the gold standard was confirmed for CD47-ICD in lymphoma and breast cancer tumor models. Additionally, it was observed that CD47-ICD induces the tumor regression in both tumor models as a therapeutic antitumor strategy. Finally, it was observed for first time that CD47-ICD is able to induce a long-lasting antitumor immunity.

The results obtained in this work provide the evidence of a new ICD inductor for lymphoma and breast cancer cells and highlight a new approach of CD47 agonist peptides, which could induce ICD in others cancer cells.

17.PERSPECTIVES

- To assess the effect of the treatment with PKHB1 in a murine model of breast cancer (4T1).
- To determine which cellular organelles are involved in the cell death induced by CD47 activation.
- To determine the mechanism of DAMPs' release by PKHB1 treatment in cancer cells.
- To determine the molecular characteristics of the cell death induced by PKHB1, since it seems to induce a conserved immunogenic death.
- To analyze the combinatorial effect of PKHB1 and chemotherapies *in vitro* and *in vivo*.
- To evaluate the importance of CD47 in the cancer biology.
- To evaluate the effect of CD47 agonist peptides on the immune system cells (cell death, activation, migration, etc.).

18.BIBLIOGRAPHY

Abalsamo, Laura, Francesca Spadaro, Giuseppina Bozzuto, Luisa Paris, Serena Cecchetti, Luana Lugini, Egidio Iorio, Agnese Molinari, Carlo Ramoni, and Franca Podo. 2012. "Inhibition of Phosphatidylcholine-Specific Phospholipase C Results in Loss of Mesenchymal Traits in Metastatic Breast Cancer Cells." *Breast Cancer Research*. https://doi.org/10.1186/bcr3151.

Anhua Wu, Seunguk Oh, Soheila Gharagozlou, Raji N. Vedi, Katya Ericson, Walter C. Low, Wei Chen, John R. Ohlfest. 2007. "*In Vivo* Vaccination With Tumor Cell Lysate Plus Cpg Oligodeoxynucleotides Eradicates Murine Glioblastoma." *Journal of Immunotherapy* 30 (8): 789–97. https://doi.org/10.1097/cji.0b013e318155a0f6.

Barclay, A. Neil. 2009. "Signal Regulatory Protein Alpha (SIRPα)/CD47 Interaction and Function." *Current Opinion in Immunology*. https://doi.org/10.1016/j.coi.2009.01.008.

Belmans, Jochen, Matthias Van Woensel, Brecht Creyns, Joost Dejaegher, Dominique M. Bullens, and Stefaan W. Van Gool. 2017. "Immunotherapy with Subcutaneous Immunogenic Autologous Tumor Lysate Increases Murine Glioblastoma Survival." *Scientific Reports* 7 (1): 13902. https://doi.org/10.1038/s41598-017-12584-0.

Bezu, Lucillia, Ligia C. Gomes-da-Silva, Heleen Dewitte, Karine Breckpot, Jitka Fucikova, Radek Spisek, Lorenzo Galluzzi, Oliver Kepp, and Guido Kroemer. 2015. "Combinatorial Strategies for the Induction of Immunogenic Cell Death." *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2015.00187.

Bravo, Roberto, Valentina Parra, Damián Gatica, Andrea E. Rodriguez, Natalia Torrealba, Felipe Paredes, Zhao V. Wang, et al. 2013. "Endoplasmic Reticulum and the Unfolded Protein Response. Dynamics and Metabolic Integration." In *International Review of Cell and Molecular Biology*. https://doi.org/10.1016/B978-0-12-407704-1.00005-1.

Bugaut, Hélène, Mélanie Bruchard, Hélène Berger, Valentin Derangère, Ludivine Odoul, Romain Euvrard, Sylvain Ladoire, et al. 2013. "Bleomycin Exerts Ambivalent Antitumor Immune Effect by Triggering Both Immunogenic Cell Death and Proliferation of Regulatory T Cells." *PLoS ONE*. https://doi.org/10.1371/journal.pone.0065181.

Burger, Patrick, Petra Hilarius-Stokman, Dirk De Korte, Timo K. Van Den Berg, and Robin Van Bruggen. 2012. "CD47 Functions as a Molecular Switch for Erythrocyte Phagocytosis." *Blood*. https://doi.org/10.1182/blood-2011-10-386805.

Calippe, Bertrand, Sebastien Augustin, Fanny Beguier, Hugo Charles-Messance, Lucie Poupel, Jean Baptiste Conart, Shulong J. Hu, et al. 2017. "Complement Factor H Inhibits CD47-Mediated Resolution of Inflammation." *Immunity*. https://doi.org/10.1016/j.immuni.2017.01.006.

Casares, Noelia, Marie O Pequignot, Antoine Tesniere, François Ghiringhelli, Stéphan Roux, Nathalie Chaput, Elise Schmitt, et al. 2005. "Caspase-Dependent Immunogenicity of Doxorubicin-Induced Tumor Cell Death." *The Journal of Experimental Medicine* 202 (12): 1691–1701. https://doi.org/10.1084/jem.20050915.

Chang, Wei Ting, Hui Ming Chen, Shu Yi Yin, Yung Hsiang Chen, Chih Chun Wen, Wen Chi Wei, Phoency Lai, Cheng Hsin Wang, and Ning Sun Yang. 2013. "Specific Dioscorea Phytoextracts Enhance Potency of TCL-Loaded DC-Based Cancer Vaccines." *Evidence-Based Complementary and Alternative Medicine*. https://doi.org/10.1155/2013/932040.

Chao, Mark P., Siddhartha Jaiswal, Rachel Weissman-Tsukamoto, Ash A. Alizadeh, Andrew J. Gentles, Jens Volkmer, Kipp Weiskopf, et al. 2010. "Calreticulin Is the Dominant Pro-Phagocytic Signal on Multiple Human Cancers and Is Counterbalanced by CD47." *Science Translational Medicine*. https://doi.org/10.1126/scitranslmed.3001375.

Chao, Mark P., Irving L. Weissman, and Ravindra Majeti. 2012. "The CD47-SIRPα Pathway in Cancer Immune Evasion and Potential Therapeutic Implications." *Current Opinion in Immunology*. https://doi.org/10.1016/j.coi.2012.01.010.

Chen, Hui-Ming, Pi-Hsueh Wang, Swey-Shen Chen, Chih-Chun Wen, Yun-Hsiang Chen, Wen-Chin Yang, and Ning-Sun Yang. 2012. "Shikonin Induces Immunogenic Cell Death in Tumor Cells and Enhances Dendritic Cell-Based Cancer Vaccine." *Cancer Immunology, Immunotherapy* 61 (11):

1989–2002. https://doi.org/10.1007/s00262-012-1258-9.
Chiang, Cheryl Lai-Lai, George Coukos, and Lana E Kandalaft. 2015. “Whole Tumor Antigen Vaccines: Where Are We?” *Vaccines* 3 (2): 344–72. https://doi.org/10.3390/vaccines3020344.
Chiang, Cheryl Lai-Lai, Lana E Kandalaft, Janos Tanyi, Andrea R Hagemann, Gregory T Motz, Nikolaos Svoronos, Kathleen Montone, et al. 2013. “A Dendritic Cell Vaccine Pulsed with Autologous Hypochlorous Acid-Oxidized Ovarian Cancer Lysate Primes Effective Broad Antitumor Immunity: From Bench to Bedside.” *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 19 (17): 4801–15. https://doi.org/10.1158/1078-0432.CCR-13-1185.
Chiarella, Paula, Verónica Reffo, Juan Bruzzo, Oscar D. Bustuoabad, and Raúl A. Ruggiero. 2008. “Therapeutic Anti-Tumor Vaccines: From Tumor Inhibition to Enhancement.” *Clinical Medicine. Oncology* 2 (January): CMO.S538. https://doi.org/10.4137/CMO.S538.
Cook, Katherine L., and David R. Soto-Pantoja. 2017. “‘UPRegulation’ of CD47 by the Endoplasmic Reticulum Stress Pathway Controls Anti-Tumor Immune Responses.” *Biomarker Research*. https://doi.org/10.1186/s40364-017-0105-8.
Denèfle, Thomas, Héloise Boullet, Linda Herbi, Clara Newton, Ana-Carolina Martinez-Torres, Alexandre Guez, Elodie Pramil, et al. 2016a. “Thrombospondin-1 Mimetic Agonist Peptides Induce Selective Death in Tumor Cells: Design, Synthesis, and Structure–Activity Relationship Studies.” *Journal of Medicinal Chemistry* 59 (18): 8412–21. https://doi.org/10.1021/acs.jmedchem.6b00781.
Dobrovolskienė, N., V. Pašukonienė, A. Darinskas, J.A. Kraśko, K. Žilionytė, A. Mlynska, Ž. Gudlevičienė, et al. 2018. “Tumor Lysate-Loaded Bacterial Ghosts as a Tool for Optimized Production of Therapeutic Dendritic Cell-Based Cancer Vaccines.” *Vaccine* 36 (29): 4171–80. https://doi.org/10.1016/J.VACCINE.2018.06.016.
Dudek-Perić, Aleksandra M., Gabriela B. Ferreira, Angelika Muchowicz, Jasper Wouters, Nicole Prada, Shaun Martin, Santeri Kiviluoto, et al. 2015. “Antitumor Immunity Triggered by Melphalan Is Potentiated by Melanoma Cell Surface-Associated Calreticulin.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-14-2089.
Dudek, Aleksandra M, Shaun Martin, Abhishek D Garg, and Patrizia Agostinis. 2013. “Immature, Semi-Mature, and Fully Mature Dendritic Cells: Toward a DC-Cancer Cells Interface That Augments Anticancer Immunity.” *Frontiers in Immunology* 4 (December): 438. https://doi.org/10.3389/fimmu.2013.00438.
Dunn, Gavin P, Allen T Bruce, Hiroaki Ikeda, Lloyd J Old, and Robert D Schreiber. 2002. “Cancer Immunoediting: From Immunosurveillance to Tumor Escape.” *Nature Immunology*. https://doi.org/10.1038/ni1102-991.
Elliott, Michael R., Faraaz B. Chekeni, Paul C. Trampont, Eduardo R. Lazarowski, Alexandra Kadl, Scott F. Walk, Daeho Park, et al. 2009. “Nucleotides Released by Apoptotic Cells Act as a Find-Me Signal to Promote Phagocytic Clearance.” *Nature*. https://doi.org/10.1038/nature08296.
Farkona, Sofia, Eleftherios P. Diamandis, and Ivan M. Blasutig. 2016. “Cancer Immunotherapy: The Beginning of the End of Cancer?” *BMC Medicine*. https://doi.org/10.1186/s12916-016-0623-5.
Galluzzi, L, I Vitale, J M Abrams, E S Alnemri, E H Baehrecke, M V Blagosklonny, T M Dawson, et al. 2012. “Molecular Definitions of Cell Death Subroutines: Recommendations of the Nomenclature Committee on Cell Death 2012.” *Cell Death and Differentiation*. https://doi.org/10.1038/cdd.2011.96.
Galluzzi, Lorenzo, Aitziber Buqué, Oliver Kepp, Laurence Zitvogel, and Guido Kroemer. 2017. “Immunogenic Cell Death in Cancer and Infectious Disease.” *Nature Reviews Immunology*. https://doi.org/10.1038/nri.2016.107.
Garg, Abhishek D., Sanket More, Nicole Rufo, Odeta Mece, Maria Livia Sassano, Patrizia Agostinis, Laurence Zitvogel, Guido Kroemer, and Lorenzo Galluzzi. 2017. “Trial Watch: Immunogenic Cell Death Induction by Anticancer Chemotherapeutics.” *OncoImmunology*. https://doi.org/10.1080/2162402X.2017.1386829.
Garg, Abhishek D, Aleksandra M Dudek-Peric, Erminia Romano, and Patrizia Agostinis. 2015. “Immunogenic Cell Death.” *The International Journal of Developmental Biology* 59 (1–3): 131–40. https://doi.org/10.1387/ijdb.150061pa.

Garrido, Greta, Ailem Rabasa, Belinda Sánchez, María Victoria López, Rances Blanco, Armando López, Diana Rosa Hernández, Rolando Pérez, and Luis Enrique Fernández. 2011. "Induction of Immunogenic Apoptosis by Blockade of Epidermal Growth Factor Receptor Activation with a Specific Antibody." *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.1003477.

Ghiringhelli, François, Lionel Apetoh, Antoine Tesniere, Laetitia Aymeric, Yuting Ma, Carla Ortiz, Karim Vermaelen, et al. 2009. "Activation of the NLRP3 Inflammasome in Dendritic Cells Induces IL-1B-Dependent Adaptive Immunity against Tumors." *Nature Medicine*. https://doi.org/10.1038/nm.2028.

Gorin, Jean Baptiste, Jérémie Ménager, Sébastien Gouard, Catherine Maurel, Yannick Guilloux, Alain Faivre-Chauvet, Alfred Morgenstern, et al. 2014. "Antitumor Immunity Induced after α Irradiation." *Neoplasia (United States)*. https://doi.org/10.1016/j.neo.2014.04.002.

Gutierrez, Linda S., Zenaida Lopez-Dee, and Kenneth Pidcock. 2011. "Thrombospondin-1: Multiple Paths to Inflammation." *Mediators of Inflammation*. https://doi.org/10.1155/2011/296069.

Hanahan, Douglas, and Robert A Weinberg. 2011. "Hallmarks of Cancer: The next Generation." *Cell*. https://doi.org/10.1016/j.cell.2011.02.013.

Henry, F., L. Bretaudeau, A. Hequet, I. Barbieux, B. Lieubeau, K. Meflah, and M. Grégoire. 1999. "Role of Antigen-Presenting Cells in Long-Term Antitumor Response Based on Tumor-Derived Apoptotic Body Vaccination." In *Pathobiology*. https://doi.org/10.1159/000028086.

Hou, W., Q. Zhang, Z. Yan, R. Chen, H. J. Zeh, R. Kang, M. T. Lotze, and D. Tang. 2013. "Strange Attractors: DAMPs and Autophagy Link Tumor Cell Death and Immunity." *Cell Death and Disease*. https://doi.org/10.1038/cddis.2013.493.

Inoue, H., and K. Tani. 2014. "Multimodal Immunogenic Cancer Cell Death as a Consequence of Anticancer Cytotoxic Treatments." *Cell Death and Differentiation*. https://doi.org/10.1038/cdd.2013.84.

Irvin, B. J., B. L. Williams, A. E. Nilson, H. O. Maynor, and R. T. Abraham. 2002. "Pleiotropic Contributions of Phospholipase C-Gamma 1 (PLC-Gamma 1) to T-Cell Antigen Receptor-Mediated Signaling: Reconstitution Studies of a PLC-Gamma 1-Deficient Jurkat T-Cell Line." *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.20.24.9149-9161.2000.

Isenberg, Jeff S., Douglas S. Annis, Michael L. Pendrak, Malgorzata Ptaszynska, William A. Frazier, Deane F. Mosher, and David D. Roberts. 2009. "Differential Interactions of Thrombospondin-1, -2, and -4 with CD47 and Effects on CGMP Signaling and Ischemic Injury Responses." *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M804860200.

Isenberg, Jeff S., Lisa A. Ridnour, Julie Dimitry, William A. Frazier, David A. Wink, and David D. Roberts. 2006. "CD47 Is Necessary for Inhibition of Nitric Oxide-Stimulated Vascular Cell Responses by Thrombospondin-1." *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M605040200.

Kaech, Susan M., E. John Wherry, and Rafi Ahmed. 2002. "Effector and Memory T-Cell Differentiation: Implications for Vaccine Development." *Nature Reviews Immunology* 2 (4): 251–62. https://doi.org/10.1038/nri778.

Kanda, Shigeru, Takefumi Shono, Bianca Tomasini-Johansson, Peter Klint, and Yutaka Saito. 1999. "Role of Thrombospondin-1-Derived Peptide, 4N1K, in FGF-2-Induced Angiogenesis." *Experimental Cell Research*. https://doi.org/10.1006/excr.1999.4622.

Kepp, Oliver, Lorenzo Galluzzi, Isabelle Martins, Frederic Schlemmer, Sandy Adjemian, Mickael Michaud, Abdul Qader Sukkurwala, Laurie Menger, Laurence Zitvogel, and Guido Kroemer. 2011. "Molecular Determinants of Immunogenic Cell Death Elicited by Anticancer Chemotherapy." *Cancer and Metastasis Reviews*. https://doi.org/10.1007/s10555-011-9273-4.

Kepp, Oliver, Eric Tartour, Ilio Vitale, Erika Vacchelli, Sandy Adjemian, Patrizia Agostinis, Lionel Apetoh, et al. 2014. "Consensus Guidelines for the Detection of Immunogenic Cell Death." *OncoImmunology*. https://doi.org/10.4161/21624011.2014.955691.

Kroemer, Guido, Lorenzo Galluzzi, Oliver Kepp, and Laurence Zitvogel. 2013. "Immunogenic Cell Death in Cancer Therapy." *Annual Review of Immunology*. https://doi.org/10.1146/annurev-immunol-032712-100008.

Krysko, Dmitri V., Abhishek D. Garg, Agnieszka Kaczmarek, Olga Krysko, Patrizia Agostinis, and Peter

Vandenabeele. 2012. “Immunogenic Cell Death and DAMPs in Cancer Therapy.” *Nature Reviews Cancer*. https://doi.org/10.1038/nrc3380.

Land, Walter G. 2015. “The Role of Damage-Associated Molecular Patterns (DAMPs) in Human Diseases Part II: DAMPs as Diagnostics, Prognostics and Therapeutics in Clinical Medicine.” *Sultan Qaboos University Medical Journal*.

Laursen, Marlene F., Esben Christensen, Laura L.T. Degn, Kasper Jønsson, Martin R. Jakobsen, Ralf Agger, and Emil Kofod-Olsen. 2018. “CD11c-Targeted Delivery of DNA to Dendritic Cells Leads to CGAS- A Nd STING-Dependent Maturation.” *Journal of Immunotherapy*. https://doi.org/10.1097/CJI.0000000000000195.

Manna, Partha Pratim, and William A. Frazier. 2004. “CD47 Mediates Killing of Breast Tumor Cells via Gi-Dependent Inhibition of Protein Kinase A.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-03-1708.

Martinelli, Roberta, Gail Newton, Christopher V. Carman, John Greenwood, and Francis W. Luscinskas. 2013. “Novel Role of CD47 in Rat Microvascular Endothelium: Signaling and Regulation of T-Cell Transendothelial Migration.” *Arteriosclerosis, Thrombosis, and Vascular Biology*. https://doi.org/10.1161/ATVBAHA.113.301903.

Martinez-Torres, Ana-Carolina, Claire Quiney, Tarik Attout, Heloïse Boullet, Linda Herbi, Laura Vela, Sandrine Barbier, et al. 2015. “CD47 Agonist Peptides Induce Programmed Cell Death in Refractory Chronic Lymphocytic Leukemia B Cells via PLCγ1 Activation: Evidence from Mice and Humans.” Edited by Shigekazu Nagata. *PLOS Medicine* 12 (3): e1001796. https://doi.org/10.1371/journal.pmed.1001796.

Martins, I., O. Kepp, F. Schlemmer, S. Adjemian, M. Tailler, S. Shen, M. Michaud, et al. 2011. “Restoration of the Immunogenicity of Cisplatin-Induced Cancer Cell Death by Endoplasmic Reticulum Stress.” *Oncogene*. https://doi.org/10.1038/onc.2010.500.

Mateo, V., L. Lagneaux, D. Bron, G. Biron, M. Armant, G. Delespesse, and M. Sarfati. 1999. “CD47 Ligation Induces Caspase-Independent Cell Death in Chronic Lymphocytic Leukemia.” *Nature Medicine* 5 (11): 1277–84. https://doi.org/10.1038/15233.

Matozaki, Takashi, Yoji Murata, Hideki Okazawa, and Hiroshi Ohnishi. 2009. “Functions and Molecular Mechanisms of the CD47-SIRPα Signalling Pathway.” *Trends in Cell Biology*. https://doi.org/10.1016/j.tcb.2008.12.001.

Matzinger, Polly. 1994. “TOLERANCE , DANGER , DANGER , AND AND THE THE EXTENDED FAMILY * F AMIL y * EXTENDED.” *Annu. Rev.Lmmunol*.

McAndrew, D., D. M. Grice, A. A. Peters, F. M. Davis, T. Stewart, M. Rice, C. E. Smart, et al. 2011. “ORAI1-Mediated Calcium Influx in Lactation and in Breast Cancer.” *Molecular Cancer Therapeutics*. https://doi.org/10.1158/1535-7163.MCT-10-0923.

Mirochnik, Y., A. Kwiatek, and O. Volpert. 2008. “Thrombospondin and Apoptosis: Molecular Mechanisms and Use for Design of Complementation Treatments.” *Current Drug Targets*. https://doi.org/10.2174/138945008785909347.

Navarro-Alvarez, Nalu, and Yong Guang Yang. 2011. “CD47: A New Player in Phagocytosis and Xenograft Rejection.” *Cellular and Molecular Immunology*. https://doi.org/10.1038/cmi.2010.83.

Obeid, Michel, Antoine Tesniere, François Ghiringhelli, Gian Maria Fimia, Lionel Apetoh, Jean-Luc Perfettini, Maria Castedo, et al. 2007. “Calreticulin Exposure Dictates the Immunogenicity of Cancer Cell Death.” *Nature Medicine* 13 (1): 54–61. https://doi.org/10.1038/nm1523.

Panaretakis, Theocharis, Oliver Kepp, Ulf Brockmeier, Antoine Tesniere, Ann Charlotte Bjorklund, Daniel C. Chapman, Michael Durchschlag, et al. 2009. “Mechanisms of Pre-Apoptotic Calreticulin Exposure in Immunogenic Cell Death.” *EMBO Journal*. https://doi.org/10.1038/emboj.2009.1.

Pandiyan, Pushpa, and Michael J. Lenardo. 2008. “The Control of CD4+CD25+Foxp3+ Regulatory T Cell Survival.” *Biology Direct*. https://doi.org/10.1186/1745-6150-3-6.

Pulaski, Beth A., and Suzanne Ostrand-Rosenberg. 2001. “Mouse 4T1 Breast Tumor Model.” In *Current Protocols in Immunology*. https://doi.org/10.1002/0471142735.im2002s39.

Rebres, Robert A., Louise E. Vaz, Jennifer M. Green, and Eric J. Brown. 2001. “Normal Ligand Binding and Signaling by CD47 (Integrin-Associated Protein) Requires a Long Range Disulfide Bond between the Extracellular and Membrane-Spanning Domains.” *Journal of Biological Chemistry*.

https://doi.org/10.1074/jbc.M106107200.
Reinhold, Martina I., Frederik P. Lindberg, Gilbert J. Kersh, Paul M. Allen, and Eric J. Brown. 2002. "Costimulation of T Cell Activation by Integrin-Associated Protein (CD47) Is an Adhesion-Dependent, CD28-Independent Signaling Pathway." *The Journal of Experimental Medicine*. https://doi.org/10.1084/jem.185.1.1.

Roberts, David D., Thomas W. Miller, Natasha M. Rogers, Mingyi Yao, and Jeffrey S. Isenberg. 2012. "The Matricellular Protein Thrombospondin-1 Globally Regulates Cardiovascular Function and Responses to Stress via CD47." *Matrix Biology*. https://doi.org/10.1016/j.matbio.2012.01.005.

Sarfati, Marika, Genevieve Fortin, Marianne Raymond, and Santos Susin. 2008. "CD47 in the Immune Response: Role of Thrombospondin and SIRP-α Reverse Signaling." *Current Drug Targets*. https://doi.org/10.2174/138945008785909310.

Saumet, Anne, Mouna Ben Slimane, Michel Lanotte, Jack Lawler, and Véronique Dubernard. 2005. "Type 3 Repeat/C-Terminal Domain of Thrombospondin-1 Triggers Caspase-Independent Cell Death through CD47/Avβ3 in Promyelocytic Leukemia NB4 Cells." *Blood*. https://doi.org/10.1182/blood-2004-09-3585.

Showalter, Anne, Arati Limaye, Jeremiah L. Oyer, Robert Igarashi, Christina Kittipatarin, Alicja J. Copik, and Annette R. Khaled. 2017. "Cytokines in Immunogenic Cell Death: Applications for Cancer Immunotherapy." *Cytokine*. https://doi.org/10.1016/j.cyto.2017.05.024.

Singh-Jasuja, Harpreet, Allan Thiolat, Matthieu Ribon, Marie-Christophe Boissier, Natacha Bessis, Hans-Georg Rammensee, and Patrice Decker. 2013. "The Mouse Dendritic Cell Marker CD11c Is Down-Regulated upon Cell Activation through Toll-like Receptor Triggering." *Immunobiology* 218 (1): 28–39. https://doi.org/10.1016/J.IMBIO.2012.01.021.

Sjostrom, J. 2001. "How Apoptosis Is Regulated, and What Goes Wrong in Cancer." *BMJ*. https://doi.org/10.1136/bmj.322.7301.1538.

Soto-Pantoja, David R., Sukhbir Kaur, and David D. Roberts. 2015. "CD47 Signaling Pathways Controlling Cellular Differentiation and Responses to Stress." *Critical Reviews in Biochemistry and Molecular Biology*. https://doi.org/10.3109/10409238.2015.1014024.

Subramanian, Shyamsundar, Eric T. Boder, and Dennis E. Discher. 2007. "Phylogenetic Divergence of CD47 Interactions with Human Signal Regulatory Protein α Reveals Locus of Species Specificity: Implications for the Binding Site." *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M603923200.

Tesniere, A., T. Panaretakis, O. Kepp, L. Apetoh, F. Ghiringhelli, L. Zitvogel, and G. Kroemer. 2008. "Molecular Characteristics of Immunogenic Cancer Cell Death." *Cell Death and Differentiation*. https://doi.org/10.1038/sj.cdd.4402269.

Tesniere, A., F. Schlemmer, V. Boige, O. Kepp, I. Martins, F. Ghiringhelli, L. Aymeric, et al. 2010. "Immunogenic Death of Colon Cancer Cells Treated with Oxaliplatin." *Oncogene*. https://doi.org/10.1038/onc.2009.356.

Uno, Shinsuke, Yasuko Kinoshita, Yumiko Azuma, Toshiaki Tsunenari, Yasushi Yoshimura, Shinichiro Iida, Yasufumi Kikuchi, Hisafumi Yamada-Okabe, and Naoshi Fukushima. 2007. "Antitumor Activity of a Monoclonal Antibody against CD47 in Xenograft Models of Human Leukemia." *Oncology Reports*.

Uscanga-Palomeque, Ashanti Concepción, Kenny Misael Calvillo-Rodríguez, Luis Gómez-Morales, Eva Lardé, Thomas Denèfle, Diana Caballero-Hernández, Hélène Merle-Béral, et al. 2018a. "CD 47 Agonist Peptide PKHB 1 Induces Immunogenic Cell Death in T-cell Acute Lymphoblastic Leukemia Cells." *Cancer Science* 110 (1): cas.13885. https://doi.org/10.1111/cas.13885.

Van, Vu Quang, Nobuyasu Baba, Manuel Rubio, Keiko Wakahara, Benoit Panzini, Carole Richard, Genevieve Soucy, et al. 2012. "CD47Low Status on CD4 Effectors Is Necessary for the Contraction/Resolution of the Immune Response in Humans and Mice." *PLoS ONE*. https://doi.org/10.1371/journal.pone.0041972.

Viallard, Jean Francois, J. L. Pellegrin, V. Ranchin, T. Schaeverbeke, J. Dehais, M. Longy-Boursier, J. M. Ragnaud, B. Leng, and J. F. Moreau. 1999. "Th1 (IL-2, Interferon-Gamma (IFN-γ)) and Th2 (IL-10, IL-4) Cytokine Production by Peripheral Blood Mononuclear Cells (PBMC) from Patients with Systemic Lupus Erythematosus (SLE)." *Clinical and Experimental Immunology*.

https://doi.org/10.1046/j.1365-2249.1999.00766.x.
Zhivotovsky, B., and S. Orrenius. 2010. "Cell Cycle and Cell Death in Disease: Past, Present and Future." In *Journal of Internal Medicine*. https://doi.org/10.1111/j.1365-2796.2010.02282.x.

19.BIOGRAPHICAL ABSTRACT

Kenny Misael Calvillo Rodríguez

Candidate for the degree of: Mater of Science with orientation in Immunology

Thesis: Study of the immunogenicity of the cell death induced by the activation of CD47 in tumor cells.

Research field: Health Science

Personal data: Born in Monclova, Coahuila México on March the 7th. 1994. Son of Juan Antonio Calvillo Ramos and Flor Estela Rodríguez Lerma.

Education: Clinical Laboratory Technician by Universidad Autónoma de Nuevo León.

Bsc Chemistry Bacteriology Parasitology by Universidad Autónoma de Nuevo León.
Title of research work "Analysis of the cytotoxic and antitumoral effect induced by the activation of CD47 in the cell line L5178Y-R".

Awards:

- Recognition as first place in the "V National Symposium of Pharmaceutical Sciences and Biomedicine and III National Symposium of Applied Microbiology". With the poster exhibition of the work: PKHB1 induces tumor regresion through regulated cell death and stimulation of the immune system. Monterrey, México, 2018.
- Special distinction for the quality and originality of the thesis work, November 2017.
- Founding member of the Student Society of Chemist Bacteriologist Parasitologist, logistics responsible, 2016-2017.
- 2nd Place in the State Contest "II Creative Young and Inventors". Institute of Innovation and Technology Transfer of Nuevo León and the National Council of Science and Technology Northeast Delegation, 2014.

Professional experience:

- Clinical Laboratory Technician: Biomedical and Biotechnological Developments of México (DEBBIOM, April 2012-July 2017). Activities: taking blood samples and vital signs, processing samples (protection of plasma, antidoping and pregnancy tests), application of good clinical practices / documentation.
- Clinical Laboratory Technician: U.A.N.L. School of Biological Sciences, central teaching laboratory of the Bsc. L.B.G. (August 2013-January 2016). Activities: administration of the laboratory, support in the handling of equipment for teaching practices, preparation of maintenance programs and operating manuals.

- Clinical Laboratory Technician: Medical and Surgical Center "San Bernabé" (September 2012-February 2013). Activities: responsible for laboratory: taking, processing and reporting of blood and bacteriological samples; preparation of culture media and equipment maintenance.

Publications:

Scientific articles on Indexed Journals JCR

- Uscanga-Palomeque AC, Calvillo-Rodríguez KM, Gómez-Morales L, Lardé E, Denèfle T, Caballero-Hernández D, Merle-Béral H, Susin SA, Karoyan P, Martínez-Torres AC*, Rodríguez-Padilla C*. CD47 agonist peptide PKHB1 induces immunogenic cell death in T-cell acute lymphoblastic leukemia cells. Cancer Sci. 2019;110(1):256-268.
- Martínez-Torres AC*, Calvillo-Rodríguez KM*, Uscanga-Palomeque AC, Gómez Morales L, Mendoza-Reveles R, Caballero-Hernández D, Karoyan P, Rodríguez-Padilla C. PKHB1-tumor cell lysate induces antitumor immune system stimulation and tumor regression in syngeneic mice with tumoral T lymphoblasts. Journal of Oncology. https://doi.org/10.1155/2019/9852361.
- Calvillo-Rodríguez KM*, Martínez-Torres AC*, Uscanga-Palomeque AC, Gómez-Morales L, Karoyan P, Rodríguez-Padilla C. PKHB1 induces immunogenic cell death in breast cancer cells. In preparation.

Scientific Articles in Arbitrated Journals

- Kenny M. Calvillo-Rodriguez, Ashanti C. Uscanga-Palomeque, Luis Gomez-Morales, Diana E. Caballero-Hernandez, Philippe Karoyan, Cristina Rodriguez-Padilla, Ana C. Martinez-Torres. PKHB1 induces tumor regresion through regulated cell death and stimulation of the immune system. RCFB, Ed. Esp. 2, pg. 68. 2018.
- Ashanti C. Uscanga-Palomeque, Kenny M. Calvillo-Rodriguez, Luis Gomez-Morales, Diana E. Caballero-Hernandez, Philippe Karoyan, Cristina Rodriguez-Padilla, Ana C. Martinez-Torres. The CD47 agonist peptide, PKHB1 induces immunogenic cell death in acute T-cell lymphocytic leukemia cells. RCFB, Ed. Esp. 2, pg. 39. 2018.
- Uscanga-Palomeque, A., Martínez-Torres, AC, Calvillo-Rodríguez, KM, Gómez-Morales, L., Caballero-Hernández, DE & Rodríguez-Padilla, C. PKHB1 induces tumor regression through regulated cell death and stimulation of the immune system. XXIX National Congress of Research in Medicine. Monterrey, México, October 4-7.

Presentations:

Posters

- Activation of CD47 by the peptide PKHB1: a promising treatment against leukemia in the "21 State Meeting of Health Research", Monterrey, México, 2018.
- CD47 agonist peptide, PKHB1, induces selective cell death in leukemia cell lines and induces tumor regression in vivo, in the congress "Young Researchers In Life Sciences" Paris, France, 2018.

- PKHB1 induces tumor regression through regulated cell death and stimulation of the immune system. "V National Symposium of Pharmaceutical Sciences and Biomedicine and III National Symposium of Applied Microbiology", Monterrey, México, 2018.
- The tumor lysate obtained by PKHB1 induces the immune system activation and tumor regression. "VI National Symposium of Pharmaceutical Sciences and Biomedicine and IV National Symposium of Applied Microbiology", Monterrey, México, 2019.
- TSP1-mimetic peptide PKHB1 induces immunogenic cell death in T cell leukemia. "Young Researchers In Life Sciences" Paris, France, 2019.
- The activation of CD47 in breast cancer stimulates the antitumor immune response by inducing immunological memory. "6th CTR Scientific-Expo", Monterrey, México, 2019.

Oral presentations

- Errors in the preanalytical phase of the clinical laboratory. Introductory course of sampling (1CITM) by the Student Society of Chemicals Bacteriologists Parasitologists of the School of Biological Sciences, 2016.
- Analysis of the cytotoxic and antitumor effect induced by the activation of CD47 in the cell line L5178Y-R. Meeting of Young Researchers, Center for Internationalization, UANL. Monterrey, México, 2017.
- CD47 agonist peptide, PKHB1, induces selective cell death in leukemia cell lines and induces tumor regression *in vivo*. "European Workshops on Cell Death", Fuggi, Italy 2018.
- The CD47 agonist peptide, PKHB1 induces immunogenic cell death in acute T-cell lymphocytic leukemia cells, in the "V National Symposium of Pharmaceutical Sciences and Biomedicine and III National Symposium of Applied Microbiology", Monterrey, México, 2018.
- The activation of CD47 in breast cancer stimulates the antitumor immune response by inducing immunological memory. "VI National Symposium of Pharmaceutical Sciences and Biomedicine and IV National Symposium of Applied Microbiology", Monterrey, México, 2019.

Additional courses:

- Animal models for research laboratories. UANL, School of Biological Sciences, 2017.
- 8th International Symposium on Immunopathogenesis in Cancer and Infectious Diseases. UANL, School of Biological Sciences, November 2015.
- Cardio-pulmonary resuscitation. Biomedical and biotechnological developments Monterrey, 2014.
- Management of Hazardous Biological-Infectious Waste, module 1 basic security. School of Biological Sciences, COBBIO, 2014.
- Prevention of infections in health personnel. Faculty of Medicine, UANL, 2012.
- 6th International Quality Control Cycle. IFCC. BIO-RAD. (Videoconferences). Doctors Hospital, Monterrey, Nuevo León, 2012.