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TESIS

CELL DEATH EFFECT OF CD47-AGONIST PEPTIDES ON DIFFERENT TYPES OF LEUKEMIA AND IN A MURINE MODEL

POR

ASHANTI CONCEPCIÓN USCANGA PALOMEQUE

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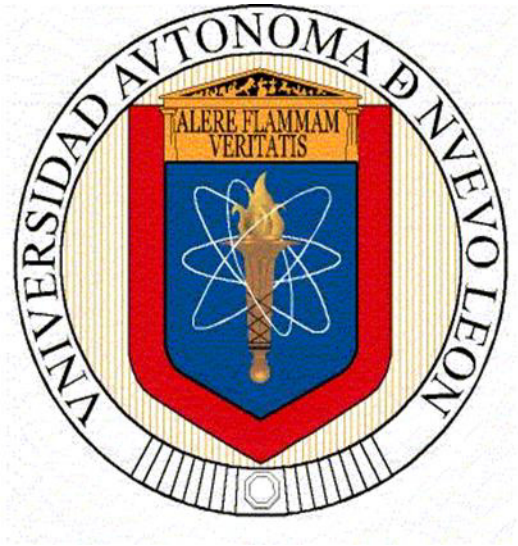

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PRESENTED BY

ASHANTI CONCEPCIÓN USCANGA PALOMEQUE

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ABBREVIATIONS

%	percentage
°C	Celsius
µg	microgram
µg/µL	Microgram per microliter
µL	microliter
µM	micromolar
Ab	antibody
ACD	Accidental cell death
ACDD	Antibody-dependent Cellular Cytotoxicity
ADCD	autophagy-dependent cell death
AIF	apoptosis-inducing factor
ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
Ann-V	Annexin V
ANXA1	annexin A1
APCs	Antigen presenting cells
Ara-C	cytarabine
ATCC	American Type Culture Collection
ATM	Ataxia-Telangiesctasia mutated
ATP	Adenosine Triphosphate
BAP31	B-cell receptor-associated protein 31
Bax	Bcl-2-associated X protein
Bcl- 2	B-cell CLL/lymphoma 2
Bcl- XL	B-cell lymphoma-extra large
BCR-ABL	breakpoint cluster region protein-Abelson murine leukemia viral oncogene homolog 1
BMSC	bone marrow stromal cells
Ca2+	Calcium ion
CBC	Completed Blood Count
CC100	cytotoxic concentration 100
CC50	cytotoxic concentration 50
CD	Cluster of differentiation
CDC	Complementary Dependent Cytotoxicity
c-KIT	tyrosine-protein kinase Kit
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CO2	Carbon Oxigen
CRT	Calreticulin
CTX	cyclophosphamide
CXCL10	CXC-chemokine ligand 10

DAMPs	damage-associated molecular patterns
DC	Dendritic cells
DD1α	death domain 1α
DMSO	Dimethyl Sulfoxide
DSC3	Desmocollin 3
EBV	Epstein-Barr virus
eIF2a	Eukaryotic translation initiation factor 2 alpha
ER	endoplasmic reticulum
FBS	fetal bovine serum
Fc	crystallizable Fraction of antibodies
FEEL-1	fasciclin EGF-like, laminin-type EGF-like, link domain-containing scavenger receptor-1
Fig.	figure
FLT3	FMS tyrosine kinase 3
HLA	The human leukocyte antigen
HMGB1	high-mobility group box 1
HSCs	hematopoietic stem cells
HSP70	heat shock protein 70
HSP90	heat shock protein 90
HTLV-1	human T cell leukemia virus 1
IAP	integrin-associated protein
ICD	immunogenic cell death
IGHV	immunoglobulin heavy chain
IHC	immunochemistry
IL-1β	interleukin-1β
INEGI	Instituto Nacional de Estadística y Geografía
INF	interferon
ITIM	immunoreceptor inhibitory tyrosine
kDa	KiloDaltons
LAMP1	lysosomal-associated membrane protein 1
LDCD	lysosome-dependent cell death
LPR1	Low density lipoprotein receptor-related protein 1
mAb	monoclonal antibody
mAb	monoclonal antibody
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCL1	Myeloid cell leukemia sequence 1
MCV	Mean corpuscular volume
MFI	Mean fluorescence intensity
mg	milligram
min	minutes
mL	milliliter

mm3	cubic milimeter
MMS	multiple domain that crosses the membrane
MPT	mitochondrial permeability transition
MTT	3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide
MW	Molecular weight
MYD88	Myeloid differentiation primary response 88
NCCD	Cell Death Nomenclature Committee
NK	Natural killer
NLR	The nucleotide-binding domain, leucine rich containing protein
NLRP3	NLR family pyrin domain containing 3
nm	nanometers
Omi / Htra2	/high temperature requirement protein A2
PANX1	Pannexin 1
PBMC	Peripheral Blood Mononuclear cells
PBS	Phosphate Buffer Saline
PCD	programmed cell death
PCR	polymerase chain reaction
PDLIM1	PDZ and LIM domain protein 1
PDT	Photodynamic therapy
PE	Phycoeritryn
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PI	Propidium Iodide
PI3K	phosphatidylinositol-3-kinases
PLCγ1	Phospholipase C gamma-1
PS	phosphatidylserine
QVD	Q-VD-OPh
RAF	Rapidly Accelerated Fibrosarcoma
RAGE	Receptor for advanced glycation endproducts
RCD	regulated cell death
RGD	arginine-glycine-aspartic
RIP1	the serine/threonine kinase receptor-interacting protein
ROCK1	Rho-associated, coiled-coil containing protein kinase 1
ROS	reactive oxygen species
RPL	L ribosomal protein
rpm	revolution per minutes
RUNX1	Runt-related transcription factor 1
SDF-1	stromal cells derived Factor 1
SHIP	inositol phosphatase
SHP-1	tyrosine phosphatase domain homologue of Src 2
SIRP-a	alpha signal regulatory protein
Smac / DIABLO	Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI

SNAP25	synaptosomal-associated protein 25
SREC-1	Scavenger Receptor expressed by Endothelial cells
STAT3	Signal transducer and activator of transcription 3
TET2	Tet methylcytosine dioxygenase 2
TIM3	T cell immunoglobulin and mucin domain 3
TKI	tyrosine kinase inhibitor
TLR	Toll-like receptor
TMSB10	Thymosin beta-10
TP53	Tumor preotein p53
TSP-1	Thrombospondin-1
VAMP1	vesicle-associated membrane protein 1
VEGFR	Vascular Endothelial Growth Factor Receptors
ZAP-70	Zeta-chain-associated protein kinase 70

I. ABSTRACT

Recently, it has been demonstrated that peptides derived from the C-terminal domain of the TSP1 (4N1K and PKHB1) can activate CD47 and cause caspase-independent and calcium-dependent regulated cell death (RCD) selectively in cells of patients with chronic lymphocytic leukemia (CLL). Therefore, the aim of this project was to study the potential of CD47-agonist peptides to induce cell death in different types of leukemia *in vitro* and *in vivo,* in an immunocompetent murine model. To achieve this, cell death was evaluated, analyzing phosphatidylserine exposure (Ann-V), cell membrane permeabilization (PI), response to caspase inhibitor (Q-VD-OPh) and calcium chelator (BAPTA) in the different leukemic cell lines, in a murine T cell tumor lymphoblast cell line (L5178Y-R) and in PBMC and murine lymphoid cells. Moreover, the immunogenicity of cell death was evaluated by calreticulin (CTR) exposure, ATP and DAMPs release (HSP70, HSP90, HMGB1, calreticulin). In and *in vivo* model, tumor volume, survival, cell blood count and tumor histology and immunochemistry (IHC) were assessed, at the same time, administration of a prophylactic antitumor vaccination, and determination of immunologic memory were performed. According to the results PKHB1 is more effective to induce cell death than 4N1K, and induced cell death in each one of the leukemia cell lines tested (MEC-1, Jurkat, CEM, MOLT-4 K562, HL-60 and L5178Y-R). PKHB1 also induced a caspase-independent and calcium-dependent cell death in leukemic cells. Cell death induced by PKHB1 was selective of neoplastic cells and neither affect PBMCs nor cell derived from murine lymphoid organs. Furthermore, ATP and DAMPs release indicate that PKHB1 is able to induce immunogenic cell death. The *in vivo*, weekly treatment with PKHB1 induces complete regression of L5178Y-R tumors in immunocompetent BALB/c mice, prolonging overall survival. PKHB1 cytotoxicity was also selective *in vivo*, since none of tissues exposed to this peptide were damaged, and cell blood counts were significantly improved compared to untreated mice. Moreover, the complete regression seems to be related to activation of immune system since the pathological analysis showed PMN and lymphocytes infiltration in the biopsies of treated mice. The IHC revealed the presence of CD4+ and CD8+ lymphocytes. Vaccinated mice showed no tumor growth when re-challenged with L5178Y-R cells. The present study highlights the potential of TSP-mimetic CD47 agonist peptides as therapeutic tools to treat leukemia.

II. RESUMEN

Recientemente, se demostró que los péptidos (4N1K y PKHB1) derivados del dominio C-terminal del TSP1 pueden activar CD47 y causar la muerte celular regulada (RCD) independiente de caspasas y dependiente de calcio, selectivamente en células de pacientes con leucemia linfocítica crónica. (CLL). Por lo tanto, el objetivo de este proyecto fue estudiar el efecto de los péptidos agonistas de CD47 para inducir la muerte celular en diferentes tipos de leucemia *in vitro* e *in vivo*, en un modelo murino inmunocompetente. Para lograrlo, se evaluó la muerte celular, analizando la exposición a fosfatidilserina (Ann-V), permeabilización de la membrana celular (PI), la respuesta al inhibidor de caspasas (Q-VD-OPh) y al agente quelante de calcio (BAPTA) en las diferentes líneas celulares leucémicas, en una línea celular de linfoblastos tumorales de células T murinas (L5178Y-R), y en PBMC y células linfoides murinas. Además, la inmunogenicidad de la muerte celular se evaluó por exposición a calreticulina (CTR), liberación de ATP y DAMPs (HSP70, HSP90, HMGB1, calreticulina). En el modelo *in vivo* se evaluaron el volumen tumoral, la supervivencia, el recuento sanguíneo celular y la histología e inmunohistoquímica (IHC) tumoral, al mismo tiempo, se administró una vacuna antitumoral profiláctica y se determinó la memoria inmunológica. Los resultados muestran que PKHB1 es más eficaz para inducir la muerte celular que 4N1K, y generó la muerte celular en cada línea celular de leucemia probada (MEC-1, Jurkat, CEM, MOLT-4, K562, HL-60 y L5178Y-R). Además, PKHB1 induce una muerte celular independiente de caspasas, pero dependiente de calcio. La muerte celular inducida por PKHB1 fue selectiva de células neoplásicas y no afectó a las PBMC ni a las células derivadas de los órganos linfoides murinos. Además, la liberación de ATP y DAMPs indica que PKHB1 es capaz de inducir la muerte inmunogénica. *In vivo*, el tratamiento semanal de PKHB1 indujo la regresión completa de los tumores L5178Y-R en ratones Balb/c inmunocompetentes, prolongando su vida. PKHB1 no ocasionó daño en los órganos ni tejidos expuestos a él, y los recuentos sanguíneos de células mejoraron significativamente en comparación con los ratones no tratados. Además, la regresión completa parece estar relacionada con la activación del sistema inmune ya que el análisis de patología indicó PMN y linfocitos en las biopsias de los ratones tratados. El IHC revela la presencia de linfocitos CD4 + y CD8 +. Los ratones vacunados y re-estimulados con células L5178Y-R no mostraron crecimiento tumoral. El presente estudio destaca el potencial de los péptidos agonistas de CD47 miméticos de TSP como una herramienta terapéutica para tratar la leucemia.

III. INTRODUCTION

Leukemia is a group of different types of cancer that start in blood-forming tissues including bone morrow and affect cells of the immune system. Leukemia are classified according to their progression in acute or chronic and by the affected cell in lymphocytic or myelocytic. Resulting in four main types: acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), and chronic myeloid leukemia (CML). Together, they represent one of the principal types of cancer worldwide and could affect anyone, being ALL the neoplasia with the greatest mortality rates in young individuals (American Cancer Society, 2018).

Current treatments for leukemia are chemotherapy and specific kinase inhibitors used alone or in combination with immunotherapies. These treatments aim to induce apoptosis, a genetically directed process of cell self-destruction. However, mutations in the apoptotic machinery are common in various types of cancer. Besides, leukemic cells bear and develop in a microenvironment that protects them from dying, making it necessary to search for treatments that can induce the death of leukemic cells regardless of the protective signals of their microenvironment or intrinsic defects that give them resistance to apoptosis.

This work proposes CD47 as a therapeutic target to treat leukemia. CD47 is a transmembrane protein expressed ubiquitously, belonging to the superfamily of immunoglobulins that has different roles in the immune system. CD47 possesses two major ligands, the alpha signal regulatory protein (SIRP-α) and the extracellular matrix protein, thrombospondin-1 (TSP1). SIRPα is present in professional phagocytes such as macrophages and DCs, which control a ¨don't eat me¨ signal that regulates programed cell removal. This signal is used by cancer cells to evade the immune system. Currently, there are some scientific groups working with monoclonal antibodies (mAb) that block the CD47-SIRP-α binding, to promote phagocytosis of tumor cells, helping to fight against this disease.

The binding of TSP1 and CD47 mediates cell adhesion, cell migration, cell proliferation and cell death. Recently, CD47 agonist peptides derived from the C- terminal domain of TSP1

(4N1K, PKHB1) induce a type of regulated cell death (RCD), caspase-independent cell death in different cancer cells. Actually PKHB1, the first serum-stable soluble decapeptide induces the massive mobilization of calcium ions (Ca^{2+}) to the cytoplasm, via phospholipase C gamma-1 (PLCγ1) activation, provoking the dissipation of the mitochondrial membrane potential and finally refractory CLL-cell death. In addition, an interesting feature observed in the cell death induces by PKHB1 is the calreticulin (CRT) exposure to the cell surface, at least in CLL cells (Martinez-Torres et al.,2015). The CRT is an ER chaperone protein that is exposed to the cell membrane as a "don't eat me" signal, after ER stress, and which is deeply involved with the immunogenicity of cell death (Obeid et al.,2007). Also, PKHB1 was shown to reduce tumor volume without damaging liver and kidney in a xenotransplanted NSG mouse model. In an immunodeficient model such as this, it is impossible to observe whether the immune system affects the outcome, and because CD47 plays a key role in the regulation of the immune system, study of its activation through PKHB1 in an immunocompetent model is fundamental.

For all the above, the present thesis work focused on evaluating whether the activation of CD47 with PKHB1 induced similar RCD signaling in other types of leukemic cells, also, on determining whether this peptide induce immunogenic cell death and its effect *in vivo* in an immunocompetent murine model. With this work, it is pretended to a new vision about the use of CD47 agonist peptides and supporting the use of this peptides as an alternative treatment for leukemia.

IV. BACKGROUND

4.1. Cell Death

4.1.1 History, Definition and Classification

The definition of life and death is more problematic than one might suppose. Over the years, extensive knowledge has been acquired about the processes that the morphological and functional unit of live, the cell, carries out, and within which its death is found. The term of cell death has been evolving with the acquisition of new and better data, generated thanks to the technologies that are currently possessed in the areas of microscopy, cytology, immunology, molecular biology, biochemistry, among other branches of the natural sciences.

From a conceptual point of view, cell death can be defined as the permanent degeneration of vital cellular functions. However, the precise limit between a reversible alteration in homeostasis and an irreversible loss of cellular activities seems to be virtually impossible to identify. To this end, the Cell Death Nomenclature Committee (NCCD) proposed two criteria for the identification of dead cells *in vitro*:

1. The permanent loss of the plasma membrane barrier function.
2. The breakdown of cells into discrete fragments (apoptotic bodies) (Galluzzi et al.,2015).

Moreover, the NCCD classifies cell death into two broad categories (Figure 1):

• **Accidental cell death (ACD):** It is the instantaneous and catastrophic demise of cells caused by physical (high temperatures or high pressures), chemical (potent detergents or pH variations) and mechanical (shear forces) agents. This death is insensitive to any type of pharmacological or genetic interventions. Although it can occur *in vivo*, it cannot be prevented or modulated. Nonetheless cells exposed to the mentioned damage could release damage-associated molecular patterns (DAMP´s) that is, endogenous molecules with immunomodulatory functions. Intercepting DAMPs or blocking DAMP-ignited signaling pathways may mediate beneficial effects in a wide array of diseases involving accidental as well as regulated, instances of cell death.

• **Regulated cell death (RCD):** Involves a genetically encoded molecular machinery to destruct itself. This type of death can be modulated by the cell, not only by inhibiting the transduction of lethal signals, but also by the capacity of the cell to mount an anti-stress response and to restore homeostasis (Galluzzi et al.,2015).

Physiological cases of RCD, that occurs not only as a consequence of microenvironmental disturbances but also in the context of embryonic development, tissue homeostasis, and immune response (Fuchs & Steller, 2011) are generally referred to as programmed cell death (PCD) (Galluzzi et al.,2018).

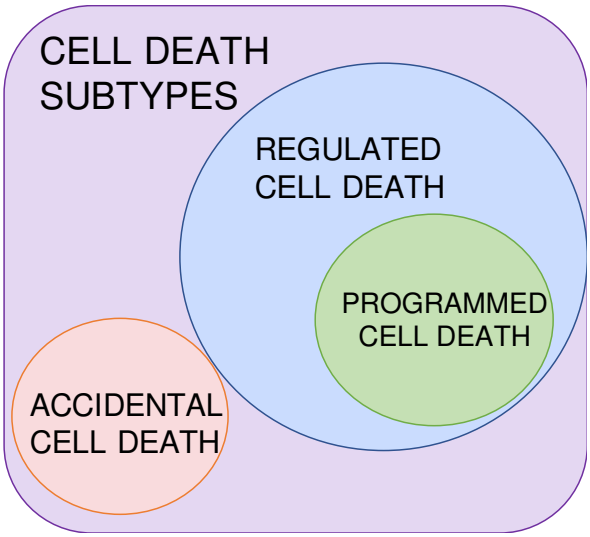

Figure 1. Types of cell death. Cells exposed physical, chemical and mechanical extreme stimuli, succumb in a completely uncontrollable, immediate loss of structural integrity. This type of death is known as: accidental cell death (ACD). On the other hand, cell death can be initiated by a genetically 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 (Extracted from Galluzzi, et al. 2015).

The field of cell death are in continuous progress and novel signaling pathways that orchestrate RCD are still being characterized, to the better understanding of the subtypes of cell death, figure 2 and table 1 summarized an updated classification of the major cell death modalities centered on molecular and essential aspects of the process. Also, for practical purposes, the main types of regulated cell death that have been described are mentioned below.

4.1.2. Types of Regulated cell death (RCD)

Cell death can be classified according to its morphological appearance (which may be apoptotic, necrotic, autophagic, or associated with mitosis), enzymatic criteria (with and without the participation of nucleases or different classes of proteases, such as caspases,

cathepsins, calpains and transglutaminases), functional aspects (programmed or accidental, physiological or pathological) or with immunological characteristics (immunogenic or non-immunogenic) (Melino, 2001). Figure 2 summarizes the known modes of regulated cell death.

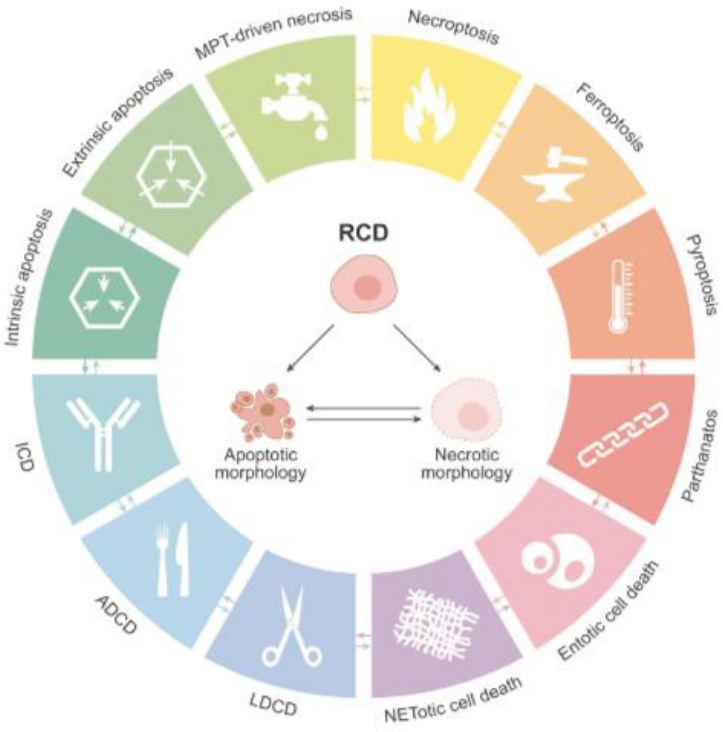

Figure 2. Main cell death modalities. Each of regulated cell death (RCD) modes is initiated and propagated by molecular mechanisms that exhibit a considerable degree of interconnectivity. Furthermore, each type of RCD can exhibit a completed spectrum of morphological features that go from entire necrotic to entire apoptotic, and an immunomodulatory profile ranging from anti-inflammatory and tolerogenic to pro-inflammatory and immunogenic. ADCD: autophagy-dependent cell death, ICD: immunogenic cell death, LDCD: lysosome-dependent cell death, MPT: mitochondrial permeability transition. Imagen adapted from Galluzi et al.,2018.

4.1.3. RCD dependent of caspases

4.1.3.1. Apoptosis

The representative subtype of caspase-dependent PCD is apoptosis. This manifests itself with the cellular disappearance characterized by: cytoplasmic contraction, the initial condensation of chromatin in the nuclear membrane (marginalization) and then the participation of the whole nucleus (pyrosis), nuclear fragmentation (karyorrhexis), minimal alterations of other organelles and a peculiar process, "blebbing", that culminates in the formation of discrete corpses that initially retain the integrity of the plasma membrane (apoptotic bodies) (Kerr, Wyllie and Alastair, 1972, Kroemer et al.,2009) that are efficiently taken up by neigh-boring cells with phagocytic activity and degraded within lysosomes (Galluzi et al.,2018). The biochemical activation of apoptosis occurs through two general routes: the intrinsic pathway, characterized by the release of cytochrome C and the activation of caspase 9, and the extrinsic

pathway, characterized by the activation of the death receptors of the cell surface and the activation of caspase 8 and / or 10. Both pathways converge in the activation of effector caspases, 3, 6 and 7 (Castro-Obregón et al.,2004; Cooper, 2012). In Figure 3 are described the Classical pathways of apoptosis.

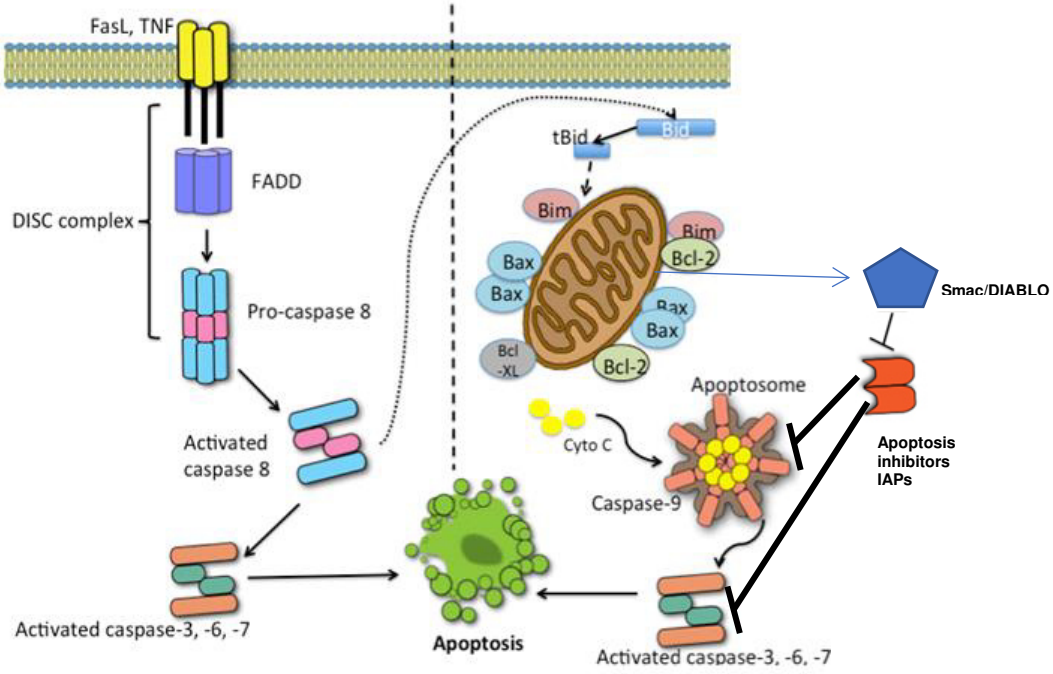

Figure 3. Classical pathways of apoptosis, caspase-dependent death: *Extrinsic pathway* is mediated by death receptors, the main death receptors are FasL and TNF. In summary, the receptors in the plasma membrane receive the extracellular signal, and their ligands are joined forming the DISC complex together with FADD and pro-caspase 8, which is cleavage by this complex and once activated can induce apoptosis through direct cleavage of caspase -3, -7 or through the cleavage of BID and, changing it to tBID (truncated BID) which promotes the release of cytochrome C. This leads to the formation of the apoptosome that caspase 9 activates 3, 6 or 7 effector caspases, culminating in the apoptosis. *Intrinsic pathway:* it is the pathway mediated by the mitochondria. Under conditions of intracellular stress, when the lethal signals are constant, the mitochondria suffer damage, external membrane permeabilization occurs, controlled by the balance of pro and anti-apoptotic proteins of the Bcl-2 family (antiapoptotic Bcl-2, Bcl-XL, Pro-apoptotic Bim, Bax, Bak). The release of cytochrome C, together with APAF-1 and procaspase 9, forms the apoptosome, which activates caspase 9. This process culminates in the activation of effector caspases 3,6 and 7. Image adapted from Cooper, 2012.

4.1.4. RCD independent of caspases

They are types of cell death that do not require the activation of caspases, for that reason, they are also called "non-apoptotic" cell death. The identification of these types of death has increased in recent years, and it has been described that they depend on a variety of stimuli,

cellular tissues and experimental conditions (Martínez-Torres, 2013). The main types of RCD independent of caspases are summarized in Table 1 they are briefly described below.

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.

Table adapted from Martínez-Torres, 2013 and Galluzzi et al.,2018.

4.1.4.1.Autophagy

Autophagy derives from the Greek (fagia) to eat, (self) oneself, that is, *self-digestion*. This is a highly conserved process in evolution, which occurs in all eukaryotic cells, from yeasts to mammals and it has homeostatic and biosynthetic functions (Klionsky, 2005). Autophagy is

activated for the cells to balance sources of energy, response to starvation and removing misfolded proteins or damaged organelles. For that reason, autophagy is generally thought of as a survival mechanism. However, its deregulation is involved in an RCD (Glick, Barth & Macleod, 2010). Autophagy is characterized by sequestration of portions of cytoplasm into a double membrane-bound vesicle called the autophagosome. The autophagosome subsequently fuses with a lysosome, leading to the degradation of the sequestered cytosolic proteins and organelles (Kaur & Debnath, 2015).

Controversy exists about whether autophagy is a real cause of cell death or not, but recently, a group of scientific achieve induce a death that is blocked by pharmacological or genetic inhibition of autophagy. This death, termed "autosis," has unique morphological features include nuclear convolution, increased autophagosomes, nuclear shrinkage, and focal perinuclear swelling. Autosis depends on the cellular Na+, K+ -ATPase; and occurs during treatment with autophagy-inducing peptides, starvation, and cerebral hypoxia–ischemia. It cannot be blocked by caspases or regulated necrosis inhibitors but autosis is inhibited by cardiac glycosides, which are Na+,K+-ATPase antagonists used in clinical medicine (Liu et al.,2013; Fitzwalter & Thorburn, 2015; Klionsky et al., 2016).

In the area of cancer, there is a lot to elucidate on treatments inducing autophagy, since this process can either favor or affect malignant cells (Hanahan and Weinberg, 2011). On the one hand, they have observed that the autophagic machinery can limit DNA damage and chromosomal instability in immortalized epithelial cells deficient in ATG genes (genes related to autophagy) (Mathew et al.,2007). Mice mutated in the Beclin-1 gene or other components of the autophagy machinery are more susceptible to developing cancer, which suggests that the induction of autophagy may serve as a barrier to tumorigenesis and may operate alone or in conjunction with apoptosis (White & DiPaola, 2009). On the other hand, the lack of nutrients, radiotherapy and certain cytotoxic drugs can induce high levels of autophagy that apparently protect cancer cells, which accentuates the resistance of the malignant cells to the treatments. In addition, stressed cancer cells can reduce their size by autophagy and enter a state of reversible latency in the presence of survival stimuli, causing

a relapse of cancer. Therefore, autophagy seems to have contradictory effects on tumor cells and, therefore, tumor progression (Choi, 2012).

4.1.4.2.Regulated necrosis

For a long time, necrosis was considered as an accidental type of cell death and was defined as a type III, meaning that it does not present the morphological features of apoptosis (Type I) or autophagy (Type II) (Kroemer et al., 2009). Now due to the work of several laboratories (Cho et al., 2009, He et al.,2009, Zhang et al.,2009), it is clear that the necrosis can occur in a regulated manner, and that necrotic cell death has a prominent role in multiple physiological and pathological adjustments (Vandenabeele et al., 2010).

Regulated necrosis is defined as a controlled cell death process regulated genetically that eventually results in cellular leakage, and it is morphologically characterized by cytoplasmic granulation, as well as organelle and/or cellular swelling (‘oncosis’) (Vanden Berghe et al.,2014). These morphological hallmarks are shared by multiple RCD modalities, such as necroptosis, parthanatos, oxytosis, ferroptosis, ETosis, NETosis, pyronecrosis and pyroptosis; all of these processes are characterized by a particular aspect of the cell death process. Those form of death are interconnected at the molecular level (Vanden Berghe et al.,2014).

Some factors that trigger regulated necrosis are: DNA damage by alkylating agents, excitotoxins, and the binding of death receptors, among others (Galluzzi et al.,2012). Molecules like, RIP1 (protein of the receptor of interaction) and its homolog RIP3, that belong to the family of the kinases, and are regulators of the survival or death of the cell, participate in physical and functional interactions that in last instance activate regulated necrosis (Vandenabeele et al.,2010).

4.1.5. Immunogenic cell death (ICD)

As mentioned before, the classification of regulated cell death could be by immunological characteristics, and in the past few years the concept of immunogenic cell death (ICD) has

emerged. ICD is a functionally peculiar form of cell death that is associated with the emission of damage-associated molecular patterns (DAMPs), which stimulate an immune response against dead-cell antigens when they derive from cancer cells (Green et al., 2009; Kroemer et al., 2013). Exposure or release of DAMPs could render tumor cell death to be immunogenic, meaning capable of mounting an effective antitumor immunity, thus, converting non-immunogenic forms of cell demise into ICD instances (Ma, 2016).

ICD includes changes in the composition of the cell surface and the release of soluble mediators, in a defined temporal sequence. Such signals operate on a series of receptors expressed by dendritic cells to stimulate the presentation of tumor antigens to T cells (Kroemer et al., 2013). The gold-standard approach to determine whether cell death may be immunogenic relies on vaccination experiments in which murine dying cells are injected into immunocompetent syngeneic mice (Galluzi et al., 2016). However, some distinctive biochemical properties of ICD can be evaluated *in vitro*, these are the exposure of calreticulin (CRT) (Obeid et al., 2007) and other endoplasmic reticulum (ER) proteins like heat shock protein 70 and 90 (HSP70 and 90) at the cell surface (Garg et al., 2015), the secretion of ATP (Garg et al., 2012), and the cell death–associated release of the non-histone chromatin protein, high-mobility group box 1 (HMGB1) (Inoue & Tani, 2014). The figure 4 illustrate the different requirements for the immunogenicity of the cell death. As DAMPs release is the main characteristic of ICD, in the following paragraphs, the most significant alarmins are described.

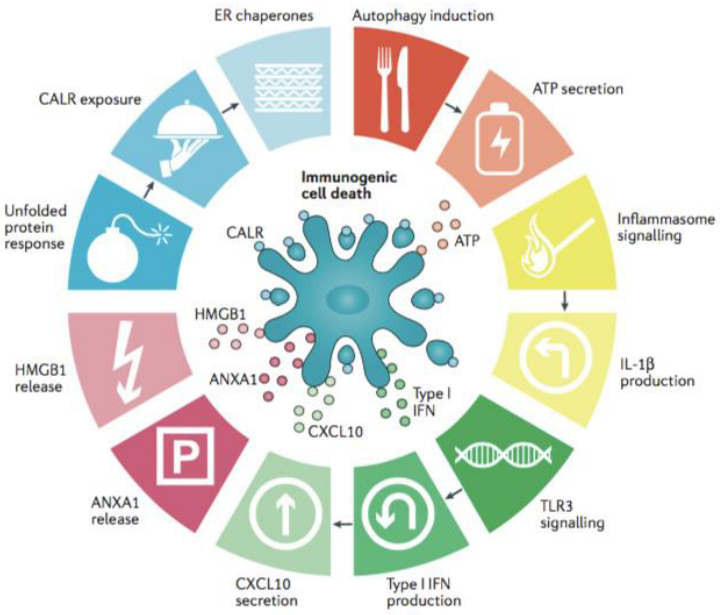

Figure 4. Requirements for the immunogenicity of the cell death. Several processes have been linked to the immunogenicity of cell death, including the unfolded protein response and consequent exposure of calreticulin (CRT) and other endoplasmic reticulum (ER) chaperones on the cell surface; the activation of autophagy and consequent secretion of ATP; the release of interleukin-1β (IL-1β) upon inflammasome signalling; the activation of Toll-like receptor 3 (TLR3), resulting in a type I interferon (IFN) response that stimulates the production of CXC-chemokine ligand 10 (CXCL10); as well as the release of high-mobility group box 1 (HMGB1) and annexin A1 (ANXA1). Imagen taken from Galluzi et al.,2015.

4.1.6. Damage Associate Molecular Patters (DAMPs)

DAMP´s are also named alarmins or danger associated molecular patterns (Bianchi, 2007). They are endogenous biomolecules, hidden from recognition by the immune system under normal conditions (Land, 2015), but exposed or released upon stress, injury or cell death, thereby becoming able to bind cognate receptors on immune cells (Li, Tang & Lotze, 2013). It is necessary to mention that not all DAMPs act as immunogenic danger signals and therefore, do not necessarily participate in ICD. Several DAMPs are crucial for the maintenance of tissue homeostasis and help to avoid auto-immune responses, as they exert immunosuppressive effects, including phosphatidylserine (PS), death domain 1α (DD1α), B-cell CLL/lymphoma 2 (BCL2) (Garg et al.,2015b). Indeed, currently only six DAMPs have been linked to the perception of RCD as immunogenic: (1) calreticulin (CALR), (2) ATP, (3) high-mobility group box 1 (HMGB1), (4) type I IFN, (5) cancer cell- derived nucleic acids, and (6) annexin A1 (ANXA1) (Galluzi et al.,2018). Table 2. summarizes some of the most prominent DAMPs characterized to date, their function and mode of emission, the cell death pathway they are associated with, and their known cognate receptors.

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

DAMPs	Receptors	Localitation 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 and Galluzzi et al.,2016

4.1.6.1.Calreticulin (CRT)

CRT represents the most abundant protein of the endoplasmic reticulum (ER) lumen, (Kroemer et al., 2013). The functions of CRT comprise chaperoning proteins, calcium release and storage, as well as regulation of cell adhesiveness through integrins. Also, it has important immune functions, such as antigen processing and presentation since, CRT is a "eat me" signal for phagocytes like macrophages, neutrophils, and DCs, enhancing the uptake of antigen and maturation of DCs (Showalter et al., 2017) and as a trigger for T_H17 cell priming (Pawaria & Binder, 2011).

CRT translocate from the ER lumen to the surface of stressed and dying cells, this phenomenon occurs as an early ICD- associated event, even before PS exposure (Panaretakis

et al., 2009). This CRT translocation is carried out by elements in a sequential signal transduction that can be enlisted in three modules: 1) ER stress module; where the early activation of the ER-sessile kinase PERK leads to phosphorylation of the translation initiation factor eIF2a, 2) apoptotic module; which involves partial activation of caspase-8, that mediated cleavage of the ER protein of B-cell receptor-associated protein 31 (BAP31) and conformational activation of Bax and Bak; and 3) exocytosis module, which implicated, the CRT transport via the Golgi apparatus, depending on vesicle-associated membrane protein 1 (VAMP1) and synaptosomal-associated protein 25 (SNAP25) (Panaretakis et al., 2009; Galluzi et al., 2018). Defects at any level of this cascade compromise the immunogenicity of RCD *in vivo* (Panaretakis et al., 2009).

4.1.6.2.High mobility group box-1 protein (HMGB1)

HMGB1 is a nuclear protein that binds to nucleosomes and promotes DNA bending. When cells die in a non-programmed way, HMGB1 is released in the extracellular medium (Bianchi, 2007). HMGB1 is perhaps the most studied alarmin, with a variety of functions ranging from its central role as a chromatin-associated protein to an autophagy promoter and to a cytokine or a DAMP (Li, Tang & Lotze, 2013). HMGB1 is constitutively expressed in nuclei of almost all cell types and presents primarily inside nuclei in normal resting cells. When released in the periphery, HMGB-1 interacts with TLR2, TLR4, and the receptor for advanced glycation end products (RAGE) in which it acts as both a chemotactic and proinflammatory mediator (van Zoelen et al.,2009; Yang et al.,2012). The molecular mechanisms that underlie the release of HMGB1 from cells suffer ICD is still unknown. However, danger signalling through the HMGB1–TLR4–MYD88 axis appears to be required for adaptive immune responses against mouse cancer cells succumbing to ICD (Galluzi et al.,2016).

4.1.6.3.Adenosine Triphosphate (ATP)

In response to treatment with chemotherapeutics *in vitro*, cancer cells release adenosine triphosphate (ATP) into the culture supernatant (Kepp et al., 2018). ATP is a fundamental nucleoside to obtain cell energy and is the most abundant intracellular metabolite. It is considered as a “find me” signal recognized by P2Y2 receptors on phagocyte being an

important chemoattraction by dying cells (Kroemer et al., 2013). Also, extracellular ATP promotes formation of inflammasome through activation of NLR family pyrin domain containing 3 (NLRP3) and with it the release of proinflammatory cytokines such as IL-1β and IL-18 (Latz, Xiao & Stutz et al., 2013).

The ICD-associated release of ATP is carried out through a complex mechanism that involves (1) The change of vesicular ATP stores from lysosomes to autolysosomes; (2) the exposure on the plasma membrane of lysosomal-associated membrane protein 1 (LAMP1); (3) Rho-associated, coiled-coil containing protein kinase 1 (ROCK1)-mediated and myosin II-dependent cellular blebbing; and (4) the opening of pannexin 1 (PANX1) channels. Those are caspase-dependent mechanisms (Martins et al., 2014). Moreover, the secretion of ATP by cells exposed to ICD inducers requires an undamaged autophagic machinery, in the majority of the models (Keep et al., 2014). Autophagy-deficient tumor cells exhibit reduced ATP release *in vivo*, correlating with an impaired recruitment of APC´s, absent antitumor immune responses and therapeutic failure (Michaud et al., 2011).

4.1.6.4.Heat Shock Proteins (HSPs)

A family of proteins that play an important role as chaperones are the Heat shock proteins; they help to make a correct folding of nascent and misfolded proteins. HSP70 and 90 are associated to the ICD because these proteins when are secreted actively, perform functions such as inducing the secretion of proinflammatory cytokines since they are able to interact with several receptors (including TLRs). In addition, HSPs can cooperated with APCs allowing the cross-presentation of peptides associated with them (Bianchi,2007).

Collectively, the exposure and/or release of the proteins described above is crucial for the immunogenicity of dead cancer cells.

4.1.7. ICD inductors: Treatments with Immunogenic ability

ICD can be initiated by a set of stimuli, including viral infection, chemotherapeutics, specific forms of radiation therapy, and hypericin-based photodynamic therapy (Galluzzi et al., 2018). Recently the search for chemotherapeutics which can stimulate immune responses has

emerged. ICD inducers do not have a general structure-function relationship making it discovery almost impossible. However, a characteristic of most ICD inducers is their ability to induce reactive oxygen species (ROS) associated to the ER stress (Garg et al., 2015).

Inductors of ICD are classified based on their ability to induce ER stress, a) type I: treatment that indirectly initiate an ER stress response, such as the anthracyclines, mitoxantrone, oxaliplatin and cyclophosphamide that primarily target cytosolic proteins, plasma membranes, or nucleic proteins (Inoue & Tani 2014). b) type II: treatments that are directly linked to ER stress such as photodynamic therapy (PDT) or oncolytic viruses that have as a target the ER to cause cell death (Inoue & Tani 2014; Showalter et al.,2017). Table 3 show the main treatment for cancer with the ICD-induce capacity.

It is important to mention that despite the capacity of certain treatments to induce immunogenic death, defects in the immune system of cancer patients, result in non-functional treatments (Kepp et al.,2009). For that reason, *in vivo* vaccination (figure 5) and the *ex vivo* immunological analysis of the antitumor immune response that involves DC and T cells are necessary to confirm the ability of certain drug to act as ICD inducer (Kepp et al.,2014).

Table 3. Treatment with ICD-induce capacity.

Anticancer agent	DAMPs	Major targets by ICDinducers	Preclinical observations for inciting antitumorimmunity
Classification of type I ICD inducers			
Anthracyclines (doxorubicinand idarubicin), Mitoxantrone,Oxaliplatin, UVC irradiation,g-irradiation	Ecto-CRT, ERp57,HMGB1 , and ATPrelease	Nucleus (DNA or DNArelatedproteins for cellmitosis)	In vivo antitumor effect is mitigated by depletion ofCD8+ T cells. Immunogenicity requires ecto-CRT in prophylactic tumor vaccination mouse models
Anti-EGFR antibody—7A7	Ecto-CRT, ERp57, ecto-Hsp70, ecto-Hsp90	Cell surface receptor(EGFR)	Contribution of CD4+ T and CD8+ T to 7A7-triggered suppression of metastasis in mice model
Cyclophosphamide(CTX)	Ecto-CRT, HMGB1release	Nucleus (DNA)	Metronomic doses of CTX deplete Treg from bedand tumors, CTX modulates DCs to produce IL-12
Bortezomib	Ecto-HSP90, Ecto-CRT, HSP70	Cytosol (26S proteasome)	Cytotoxicity of NK cells against bortezomib treated cells increased
Cardiac glycosides	Ecto-CRT HMGB1 and ATP release	Cell surface (Na + /K + -ATPase, enzyme)	Prophylactic antitumor immunity is partially dependent on CD8+ T cells accompanied with Th17 cells
Classification of type II ICD inducers			
Coxsackievirus B3 (CVB3),an oncolytic virus	Ecto-CRT, HMGB1 translocation, ATPrelease	ER (ROSgeneration)	Intratumoral CVB3 administration markedly recruited NK cells and granulocytes, both of which contribute to the antitumor effects
Hypericin-based PDT	Ecto-CRT, ecto-Hsp70, ecto-Hsp90, HMGB1, ATPrelease	ER (ROSgeneration)	PDT -hypericin therapy provokes antitumorimmunity in both prophylactic and therapeutic murine tumor models. Same therapy-treated tumor cells result in phenotypic maturation of DCs and robust CD4+ T and CD8+ T cell expansion

Adapted from Inoue & Tani, 2014

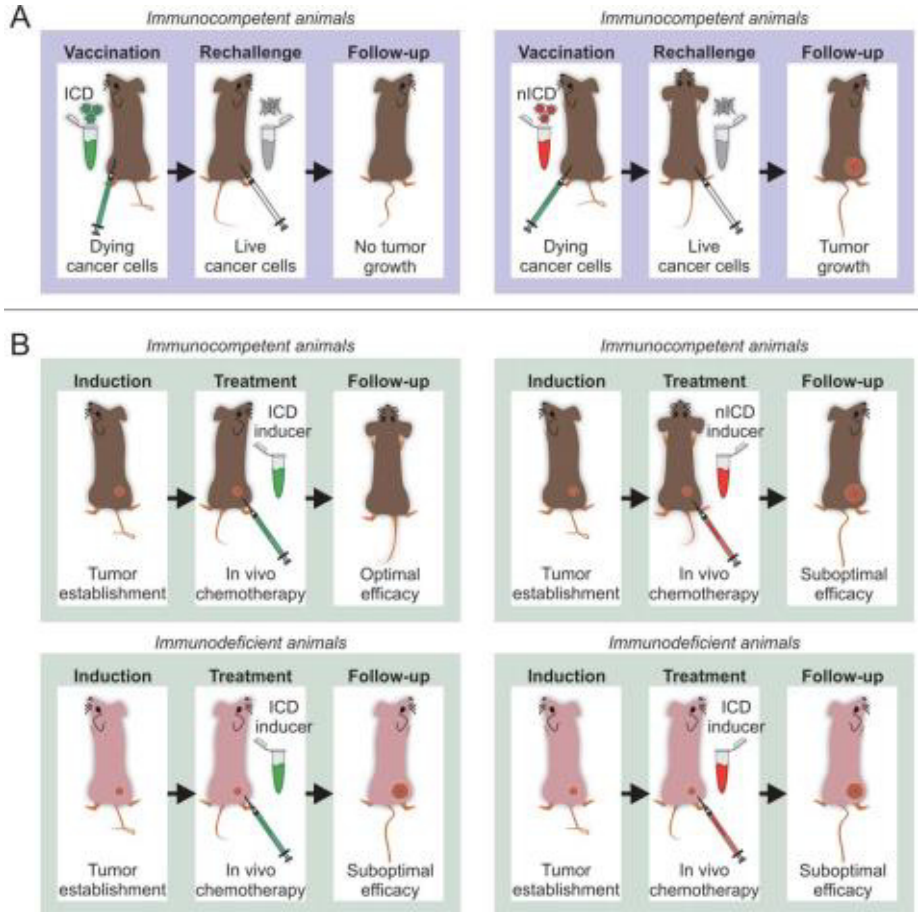

Figure 5. The gold-standard to detect ICD inducers. A. Vaccination assays. Murine cancer cells are treated *in vitro* with a putative inducer of immunogenic cell death (ICD) and eventually injected s.c. into one flank (vaccination site) of immunocompetent syngeneic mice. Seven days later, mice are challenged with living cancer cells of the same type into the contralateral flank (challenge site). The development of neoplastic lesions at the challenge site indicates that the stimulus under investigation is unable to cause immunogenic cell death. Conversely, the absence of tumor, indicates an ICD inducer. **B**. Therapeutic assays. Immunocompetent and immunodeficient syngeneic mice bearing grafted, genetically-driven or chemically-induced subcutaneous or orthotopic tumors are treated with a putative ICD inducer at therapeutic doses, followed by the monitoring of tumor size over a 1–3 weeks period. In this setting, bona fide ICD inducers mediate optimal antineoplastic effects in immunocompetent, but not in immunodeficient, mice. Adapted from Kepp et al.,2014.

Through the different ways of death exposed previously, we can notice that there are still big questions, about how they regulate each other, how one type of death can affect or empower another, to achieve the homeostasis of the organism. Clarification about the regulation of cell death can help us understand the causes of diseases such as cancer, as well as the causes of chemoresistance. That is why the knowledge of the RCD is an indispensable tool for the generation of more effective therapies directed against diverse neoplasia. Among them are

leukemias, types of hematopoietic cancers that despite having treatments available to combat them, have resistance to these treatments.

4.2. Leukemia

4.2.1. Generalities

Leukemia, also known as hematologic or blood cancer, is a type of cancer that starts in blood-forming tissue, such as the bone marrow, and causes large numbers of abnormal blood cells to be produced and enter the blood (U.S. National Library of Medicine, 2018). This type of cancer was studied in this work.

Leukemia is classified according to their evolution as acute or chronic, and according to their affected cell line they will be lymphoid or myeloid (Ortega-Sánchez et al.,2007). It is prudent to mention that the process by which blood cells are generated has its origin in the primitive cells that produce blood from the bone marrow (hematopoietic stem cells, HSCs) and is known as hematopoiesis. In hematopoiesis, the differentiation of hematopoietic stem cells occurs to give rise to the progenitor cells of myeloid and lymphoid origin, which in turn give rise to all the other specific types of blood cells, erythrocytes, leukocytes and platelets, Figure 6.

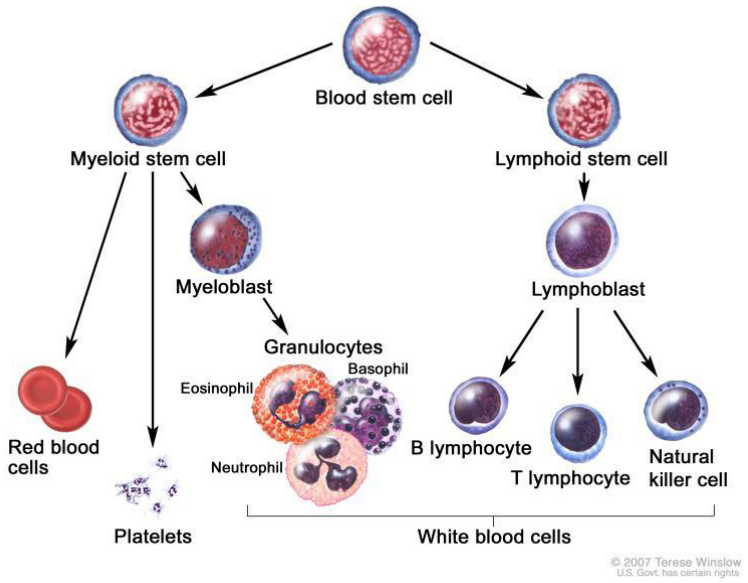

Figure 6. Hematopoiesis. This image shows that all blood cells are produced by the blood stem cells. The myeloid pathway leads to the differentiation of red blood cells, platelets and some white blood cells (Granulocytes), and the lymphoid pathway leads to the differentiation of white blood cells (lymphocytes). Taken from the NCI, 2018.

Any of the blood-forming cells of the bone marrow can become a leukemic cell. Like other tumor cells, leukemic cells have characteristics that distinguish them from non-neoplastic cells. These characteristics are: a signal of constant proliferation, evasion of growth suppressors, capacity for invasion and metastasis, immortality, induction of angiogenesis and resistance to cell death (Hanahan and Weinberg, 2011). Therefore, the leukemic cells, no longer mature normally, begin to reproduce rapidly (in acute leukemia and late phases of chronic leukemia) and become immortal, usually accumulating in the bone marrow, displacing healthy cells. In most cases, leukemic cells enter the bloodstream rapidly and can spread to other organs, such as the lymph nodes, the spleen, the liver, the central nervous system (the brain and spinal cord), among others. When this happens, they can prevent other cells in the body from performing their functions, due to the decrease in healthy blood cells, which makes it difficult to obtain oxygen for the tissues, as well as being able to control bleeding, or fight infections (National Cancer Institute, 2015).

4.2.2. Epidemiology of leukemia in the world

Leukemia represents a serious health problem, because it affects individuals of all ages. It is estimated that around 352,000 people in the world were diagnosed with leukemia in 2012, differentially according to location (Cancer Research UK, 2012). The majority (91%) of leukemia cases are diagnosed in adults over 20 years of age. Among adults, the most common types are CLL (36%) and AML (32%). While ALL is more common in people under 20 years of age, representing 56.1% of cases. In 2017, the United States, reported 62,130 new cases of leukemia, while deaths are estimated at 14,300 (9.1%) for men and 10,200 (5.1%) for women (National Cancer Institute, 2018).

4.2.3. Epidemiology of leukemia in Mexico

In Mexico, the Instituto Nacional de Estadística y Geografía (INEGI) reported that in 2013, the main cause of hospital morbidity among the population under 20 years of age is cancer of hematopoietic organs (58.7% of men and 62% of women) and for 2013, of the total deaths due to malignant tumors in the population under 20 years old, 57.1% are men and 42.9% are women (INEGI, 2015).

Leukemia is one of the most aggressive conditions that can affect childhood, and it shows a growing trend (although to a lesser extent than the other cases mentioned) in the last ten years. Between 2004 and 2012, 38,598 people died from this type of cancer in Mexico, with a growth of almost 14% between the number of cases registered in 2004 compared to those recorded in 2013 (INEGI, 2015). Table 4 shows the mortality rate due to the main types of cancer in our country, in the population under 20 years of age, where it can be seen that the neoplasms of hematopoietic organs occupy the first place. Table 5, shows the mortality rate in adults over 20 years old, where it is appreciated that hematopoietic cancer occupies the 6th (INEGI, 2015).

Table 4. Mortality rate of the main malignant tumors in the population under 20 years of age, according to sex. 2013.

For every 100 thousand inhabitants for each sex			
Main malignant tumors	Total	Men	Women
Hematopoietic organs	2.57	2.87	2.26
Encephalon and other parts of the central nervous system	0.66	0.75	0.57
Bones and joint cartilages	0.35	0.35	0.36
Lymphatic tissue and related	0.31	0.40	0.22
Tumors of ovaries / testicles	0.17	0.23	0.11
Digestive system	0.17	0.22	0.12

Note: The International Statistical Classification of Diseases and Related Health Problems (ICD-10) was used, codes: C15-C26, C40 C41, C56, C62, C70-C72, C81-C96.
Source: INEGI (2015). Mortality statistics. Dynamic cubes; and CONAPO (2015). Projections of the Population 2010-2050. Processed INEGI.

Table 5. Mortality rate of the main malignant tumors in the population aged 20 years and over, according to sex. 2013.

For every 100 thousand inhabitants for each sex			
Main malignant tumors	Total	Men	Women
Digestive organs	32.52	33.98	31.20
Respiratory and intrathoracic organs	10.58	14.71	6.83
Male reproductive system	8.44	17.75	NA
Breast cancer[1]	7.59	0.14	14.36
Female genital organs	6.78	NA	12.93
Hematopoietic organs[2]	5.68	6.28	5.14
Urinary tract[2]	4.12	5.51	2.85
Germ cells (testicle and ovary)[1]	3.48	1.30	5.46

Note: The International Statistical Classification of Diseases and Related Health Problems (ICD-10) was used, codes: C15-C39, C50-C61, C63-C68, C88-C95.
1.For men, breast cancer and testicular tumor are not one of the main causes of death but the data is included for comparison purposes.
2.For women, cancer in the hematopoietic and urinary tract organs are not the main causes of death, but the data is included for comparison purposes
NA: Not applicable
Source: INEGI (2015). Mortality statistics. Dynamic cubes; and CONAPO (2015). Projections of the Population 2010-2050. Processed INEGI.

As mentioned above, there are different types of leukemia and the main characteristics, their subtypes, as well as their epidemiology, risk factors, diagnosis, treatment and prognosis are mentioned below.

4.2.4. Acute Lymphocytic Leukemia (ALL)

4.2.4.1.Characteristics and Subtypes

Acute lymphocytic leukemia (ALL), also called acute lymphoblastic leukemia, is a malignant disorder of lymphoid progenitor cells, characterized by the accumulation of early forms of lymphocytes (blasts) in the bone marrow. ALL can be divided into different subtypes that depend on age (ALL in adults or pediatric ALL), according to the type of lymphocyte of origin (ALL of B cells or T cells), immunological findings (ALL of pro-B cells, pre-B, mature B, immature or mature Timic) and genetic findings (eg BCR-ABL positive or negative)

(Gokbuget and Hoelzer, 2009). Combining all subtypes of ALL, their 5-year survival rate is 35% (Bassan and Hoelzer, 2011; Lang et al.,2015).

4.2.4.2.Cytogenetics of ALL

Translocations are the most common type of DNA change that can lead to leukemia. A translocation means that the DNA of a chromosome breaks off and binds to a different chromosome. The most common translocation in ALL in adults (15% to 30% of patients) gives rise to the Philadelphia chromosome (Ph) and involves the exchange of DNA between chromosomes 9 and 22, t (9; 22) (q34; q11) with the fusion of the BCR-ABL1 gene (Thomas, 2007; Ravandi, 2011). Other less common translocations are those that occur between chromosomes 4 and 11, t (4; 11), or between chromosomes 8 and 14, t (8; 14). Currently, the presence of t (4; 11) translocations, as well as an additional chromosome 8 or an absent chromosome 7, represent a poor prognosis for the remission of the disease.

4.2.4.3.Epidemiology, risk factors, diagnosis, prognosis and treatment.

ALL is the most common neoplasm in children and represents 75-80% of acute leukemia in this age group. The incidence of ALL in childhood is 3-4 cases per 100,000 in children under 15 years. Although the disease affects children of all ages, the incidence rates show two peaks, one in children between 2 and 5 years old and other in adults of 60 years, having a slight predominance in men (Bathia and Robinson, 2003). Although most cases of ALL occur in children, the majority of deaths due to this type of leukemia occur in adults (McNeer & Bleyer, 2018). In 2018, approximately 5,960 new ALL cases will be diagnosed and around 1,470 people will die from this cause. The statistics mentioned are in the United States (American Cancer Society, 2018).

The diagnosis of this disease, as well as that of the other types of leukemia, is usually by chance in routine check-ups, specifically in blood count, where high leukocyte counts are observed. Subsequently, the bone marrow examination is performed to confirm the diagnosis of this type of leukemia. If more than 25% of bone marrow cells are cancerous lymphocytes,

we continue to perform immunophenotyping, which indicates whether the leukemia is of myeloid or lymphoid origin (American Cancer Society, 2018).

There are some risk factors for the development of acute lymphocytic leukemia, which include:

1. Exposure to high levels of radiation: This risk factor is shared with Acute Myeloid Leukemia (AML).
2. Exposure to certain chemical substances: such as benzene.
3. Viral infections: for example, human T cell leukemia virus 1 (HTLV-1) and Epstein-Barr virus (EBV) may be related to rare forms of leukemia.
4. Hereditary syndromes: It is not a hereditary disease, but there are genetic changes that generate certain syndromes that seem to increase the risk of ALL, such as Down syndrome, Klinefelter, Bloom, Fanconi anemia, Neurofibromatosis and Ataxia-telangiectasia (Hoelzer et al.,2002).

The general treatments for ALL are chemotherapies, combining two or more medications. The most common chemotherapeutic agents to treat ALL are vincristine, Doxorubicin, Cytarabine, L-asparaginase, Etoposide, Methotrexate, among others (American Cancer Society, 2018). Continuing with the treatments we have the directed therapy, in this case the white molecule is the protein of the BCR-ABL gene. The drugs that attack this protein are tyrosine kinase inhibitors (TKIs) and include imatinib, dasatinib, nilotinib, bosutinib and ponatinib. These drugs were developed to treat CML, but they were found to be useful for patients with the Philadelphia chromosome (Apperley, 2015, American Cancer Society, 2018). Another option to treat ALL is the use of monoclonal antibodies such as Blinatumomab, which can bind to CD19 and CD3 at the same time, CD19 is present in B cells, including those of leukemia and lymphoma and CD3 is present on the surface of T lymphocytes. By binding to these two proteins, said antibody binds both cancer cells and cells of the immune system, thereby awakening the latter to attack leukemic cells (Molhoj et al.,2007). The use of this antibody is limited to acute B-cell lymphocytic leukemias. Stem cell transplantation is an option, mainly when you want to increase the doses of chemotherapy. The blood producing stem cells that are used for transplantation, can come

from peripheral blood, bone marrow or umbilical cord, the drawback of this practice is that it is necessary that the HLA of the patient and donor are compatible. Surgery and radiotherapy are not recommended in ALL (American Cancer Society, 2018).

The 5-year survival rate in adults is about 30-50% compared to the 90% in children (McNeer & Bleyer, 2018). However, the cause of the remaining 70-10% that does not achieve remission of the disease is not fully understood. About 10-20% of patients die at the beginning of induction treatments and the other 10% refractory to remission (Bassan et al.,2004). Unfortunately, about half of the patients with complete remission suffer a relapse of the disease. Chemotherapy regimens are unable to induce long-lasting remissions in most post-relapse patients, so overall survival at 5 years after relapse is 7% (Dinner et al.,2014; Kantarjian et al.,2010). While in some places like the United States estimate a general cure rate of between 20 to 40% (Sive et al.,2012; O'Brien et al.,2008; Quian and Shen, 2014).

The reason why patients present refractoriness in ALL are not clear, however it is likely that they are related to multiple factors such as the subtype of acute lymphocytic leukemia, age (it has been seen that the highest rates of patients cured are those of young patients), leukocyte count, immunophenotypes, genetic changes, among others (Rowe et al.,2005, Robak, 2004, Chiaretti et al.,2006, Qian and Shen, 2014). Holleman and his team of researchers took leukemia cells from 173 children with B-cell ALL and performed sensitivity tests *in vitro*, challenging these cells to 4 drugs (prednisolone, vincristine, asparaginase, and daunorubicin). They found that about 124 genes are associated with resistance in ALL of B cells (Holleman et al.,2004). Among the genes found to confer resistance to prednisolone in ALL is overexpression of the antiapoptotic gene MCL1. Asparaginase-resistant ALLs overexpress several genes encoding ribosomal proteins such as RPL3-RPL6 and RPL11, and in altered vincristine-resistant ALL, altered expression of cytoskeletal and extracellular matrix genes, example of such genes are TMSB10, PDLIM1 and DSC3 (Holleman et al.,2004). Imatinib, a TKI that targets BCR-ABL, has presented chemoresistance due to the acquisition of point mutations in the kinase domain of BCR-ABL (especially in the contact sites T315I, E255K and Y253H) (Hofmann et al.,2004, Thomas, 2007, Ravandi, 2011, Soverini et al.,2014).

4.2.5. Chronic Lymphocytic Leukemia (CLL)

4.2.5.1.Characteristics and subtypes

Chronic lymphocytic leukemia (Chronic Lymphocytic Leukemia, CLL) is a type of cancer characterized by excess B lymphocytes. In this neoplasm, leukemic cells often accumulate slowly, and many people suffering from this disease show no symptoms during the first few years. years (American Cancer Society, 2015).

The CLL can be divided into two groups:

• CLL of a very slow development.

• CLL of a rapid development.

Generally, leukemia cells that overexpress CD38 and ZAP-70, and that do not show mutations of the variable region gene of the immunoglobulin heavy chain (IGHV), are the most rapidly developing (Rassenti et al.,2004; Schroers et al.,2005).

4.2.5.2.Cytogenetics of CLL

More than 80% of CLL cases have genetic aberrations. In most cases of CLL, a change can be found in at least one of these chromosomes. Most often this change is a deletion. Loss of one part of chromosome 13 is the most common deletion, other chromosomes such as 11 and 17 may also undergo deletions. Sometimes an additional chromosome 12 (trisomy 12) is present (American Cancer Society, 2015).

4.2.5.3.Epidemiology, risk factors, diagnosis, prognosis and treatment.

Chronic lymphocytic leukemia (CLL) represents approximately a quarter of the new cases of leukemia in the US, while in Mexico there are no precise statistics. The risk that a person has during their lifetime of developing CLL is approximately 1 in 200 people, and the risk is slightly higher in men than in women. Each year, there are approximately 15,000 new diagnoses and 5,000 deaths from CLL in the United States (Siegel et al.,2014, American Cancer Society, 2015).

The risk factors for the development of this disease are:

• Exposure to certain chemicals, such as the orange agent (herbicide).

• Family history, blood relatives have twice the risk of developing this cancer

• Incidence according to sex, the reason why it is more frequent in men than in women is unknown.

• Race / Ethnic group, CLL is more common in North America and Europe.

Most patients are asymptomatic at the time the disease begins, and the diagnosis is often made by chance. For the diagnosis of CLL, an absolute monoclonal lymphocyte count that equals or exceeds 5000 cells / mm3 is required, plus a phenotype that combines the presence of CD19, CD20, CD5 and CD23 (Hallek et al.,2008). This immunophenotype is essential to differentiate CLL from other lymphoproliferative disorders (Nabhan and Rosen, 2014). Flow cytometry is used to diagnose the disease.

Standard treatments for CLL include combinations of purine analogs, alkylating agents, and monoclonal antibodies directed to CD20 such as Rituximab, Obinutuzumab, and Ofatumumab and another mAb called alemtuzumab, directed against CD52 that is found on the surface of CLL cells. and of many T lymphocytes. In addition, the efficacy of various tyrosine kinase inhibitors has recently been demonstrated, as inhibitors of PI3Kδ (Idelalisib, formerly called GS-1101 and CAL-101), which is in phase I test and can be used in conjunction with Rituximab with a good prognosis. These treatments increase the progression-free survival and response rate and overall survival of patients with CLL who were unable to undergo standard chemotherapy, due to the toxicity of the treatment (Furman et al.,2014).

The response rate to combined treatments that include monoclonal antibodies such as rituximab and drugs such as cyclophosphamide and fludarabine is 73%, however complete remission is only in 25% of the cases, that is, more than half of the cases relapse into the disease (Dighiero and Hamblin, 2008). The combination of the monoclonal antibody alemtuzumab with prednisolone, administered to patients with advanced refractory CLL, achieved a remission of 60% in a few patients with some defect in TP53 (Pettitt et al.,2006).

The cause of drug resistance by CLL is mainly due to mutations in TP53. However, the deletion or mutation of TP53 only explain between 25% to 50% of the refractory cases, so

other components in the p53 pathway, such as ATM and miR-34a, may be contributing to chemoresistance (Martínez- Torres, 2013).

4.2.6. Acute myeloid leukemia (AML)

4.2.6.1.Characteristics and subtypes

Acute Myeloid Leukemia (AML), also called acute myelocytic leukemia or acute non-lymphocytic leukemia, accounts for the majority of the remaining cases. AML starts from the myeloid cells that make up white blood cells (which are not lymphocytes), red blood cells or platelets.

The subtypes of AML are coarse, the World Health Organization, classifies by groups, which depend on several factors, roughly we have:

- AML with certain genetic abnormalities
- AML with changes related to myelodysplasia
- AML related to previous administration of chemotherapy or radiation
- AML not specific
- Myeloid sarcoma
- Myeloid proliferations related to Down syndrome
- Neoplasm Blastocyst Plasmacytoid Dendritic Cells
- Undifferentiated acute and biphenotypic leukemia (Dohner et al.,2010)

4.2.6.2.Cytogenetics of AML

The cytogenetics of AML is complex, because multiple genes participate in the development of this disease (see the review of the state of the art: Estey and Dohner, 2006). Changes in certain genes, such as FLT3, c-KIT and RAS, are common in AML cells. Similarly, when translocations occur, they can activate the oncogenes or deactivate genes, such as RUNX1 and RARa, which would normally help the blood cells to mature. Other unfavorable anomalies in AML, are the elimination of a part of chromosomes 5 or 7, the translocation or inversion of chromosome 3, the translocation of chromosomes 6 and 9, 9 and 22, among others, table 4, indicates the characteristic most unfavorable karyotype for AML.

Table. 6. Adverse karyotypic characteristic of patients with AML

Adverse karyotypic features of AML	
Confirmed	**Complex Karyotipe; inv (3)/t(3;3);7**
Probable	**t(6;9); t(6;11);t(11;19)(q23;p13.3);-5;del(5q)**

(Information taken from Estey and Dohner, 2006.)

4.2.6.3.Epidemiology, risk factors, diagnosis, prognosis and treatment.

Acute myeloid leukemia is the most common myeloid leukemia, in the United States an estimated 20,830 new cases of AML (1.3% of all new cases of cancer) and estimated deaths for 2015 are 10,460 (1.8% of all deaths from neoplasms). It is a little more common in men than in women and is rare in people under 45 years of age (National Cancer Institute, 2015). Tobacco use is a proven risk factor for AML. Like ALL, exposure to radiation and chemicals such as benzene increases the risk of developing this type of leukemia. The use of some chemotherapeutics such as alkylating agents, those containing platinum and topoisomerase II inhibitors are associated with the risk of AML. Examples of alkylating agents are cyclophosphamide, mechlorethamine, procarbazine, chlorambucil, melphalan, busulfan, and carmustine. Platinum medications include cisplatin and carboplatin, while topoisomerase II inhibitor drugs include etoposide, teniposide, mitoxantrone, epirubicin, and doxorubicin. Similarly, certain genetic and blood disorders such as polycythemia vera, essential thrombocythemia, and idiopathic myelofibrosis may increase the risk of developing AML (American Cancer Society, 2018).

Like the other types of leukemia, diagnosis is made thanks to routine examinations, clinical history and physical examination. For confirmation of this disease, bone marrow aspiration and biopsy are required, and specialized tests such as flow cytometry, FISH, cytogenetics, among others, are necessary for the typing of leukemia (Dohner et al.,2010). The main markers selected to diagnose AML using flow cytometry are CD3, MPO, TdT, CD34, CD117 cytoplasmic (Dohner et al.,2010). Fluorescent in situ hybridization (FISH) is an option to detect gene rearrangements, such as RUNX1-RUNX1T1, CBFB-MYH11, and the fusion of MLL and EVI1 genes, or loss of chromosome 5q and 7q (Frohling et al.,2002 Lughthart et

al.,2008). Frequently the FISH technique is necessary to identify MLL fusion partners in 11q23 translocations (Dohner et al.,2010).

For more than 30 years, chemotherapy drugs that are most commonly used to treat AML are cytarabine (cytosine arabinoside or ara-C) and drugs of the anthracycline class (such as daunorubicin [daunomycin], idarubicin and mitoxantrone) (Estey and Dohner, 2006: Ferrara and Schiffer, 2013). Other chemotherapy drugs that may be used to treat AML include: Cladribine (Leustatin, 2-CdA), Fludarabine (Fludara), Etoposide (VP-16), among others. Likewise, stem cell transplantation is used and the use of monoclonal antibodies such as gemtuzumab ozogamicin, a monoclonal antibody against CD33, in combination with calicheamicin, is being studied, giving important clinical improvement in AML patients (Ferrara and Schiffer, et al. 2013). Currently, TKIs are also used, mentioned to treat ALL, these TKIs inhibit different kinases, including RAF kinases, VEGFR, c-KIT and FLT3, examples of these inhibitors used to treat AML are Tandutinib, Sorafenib, Sunitinib, Midostaurin, KW-2449, Lestaurtinib, Quizartinib, among others (Grunwald and Levis, 2013).

Chemotherapy is preferably given to patients under 60 years and can cure from 20% to 75% of treated patients, this wide range is due to the cytogenetic variability of leukemia cells. However, the same chemotherapy treatments in patients older than 60 years, cure less than 10% due to the toxicity of the treatment and the association of old age with cytogenetic anomalies involving chromosomes 5 and 7 (mentioned in the cytogenetic section of AML). A greater understanding of the pathogenesis of AML is necessary for the development of specific therapies, especially in elderly people (Estey and Dohner 2006). About 50% of patients with adverse karyotypes (Table 4) of AML do not respond to standard treatments, due to these genetic anomalies (Estey and Dohner, 2006).

The activation of mutations in the FMS tyrosine kinase 3 (FLT3) gene results in the survival and proliferation of leukemic blasts and is associated with poor prognosis. This type of mutations is present in more than 30% of AML cases. Internal duplications in tandem FLT3, are associated with adverse prognosis and drug resistance, this mutation is present in 23% of

cases of AML (Marcucci et al.,2007, Grunwald and Levis, 2013). Another cause of resistance to drugs, is the use of TKIs, which do not manage to stay in the patients' plasma for a long time and since there is not enough of them they do not inhibit FLT3, but they are not effective (Grunwald and Levis , 2013). Other recurrent mutations in genes such as TET2, RUNX1 and TP53 have been described in AML and are associated with chemoresistance (Kadia et al.,2015).

4.2.7. Chronic myeloid leukemia (CML)

4.2.7.1.Characteristics and subtypes

Chronic myeloid leukemia (CML), also known as chronic granulocytic leukemia, is a clonal myeloproliferative disorder, in which there is increased proliferation of the granulocyte cell line without losing the ability to differentiate. This disease is characterized by a chromosomal translocation that generates the oncogene Bcr-Abl (homolog of the viral oncogene of murine leukemia of Abelson, ABL-1 and gene of the region of the conglomerate of rupture points (breakpoint cluster BCR region), that encodes a constitutive kinase activity (Zhou and Xu, 2015) The translocation occurs between chromosomes 9 and 22, giving a shorter chromosome 22, known as the Philadelphia chromosome, which is present in most patients with CML (Rowley, 1973; Jabbour and Kantarjian, 2014).

Bcr-Abl (oncogenic tyrosine kinase) is responsible for initiating and maintaining the CML cell leukemia phenotype. This oncoprotein is also responsible for the phosphorylation, activation and deregulation of intracellular signaling proteins that regulate the survival and growth of progenitor cells in the bone marrow (Tauchi et al.,1994; Kabarowski and Witte, 2000; and Xu, 2015).

CML can be classified into three phases of the disease:

• Chronic Phase (CP): less than 10% blasts in blood or bone marrow samples

• Accelerated Phase (AP), presents with severe anemia, splenomegaly and infiltration to organs, the blast count is between 10% - 20.

• Blast phase (BP), presents as an acute leukemia and has symptoms such as hemorrhages, fevers and infections, the blast count is greater than 20% and these cells are spread from the bone marrow to other organs (Jabbour and Kantarjian, 2014).

4.2.7.2.Cytogenetics of CML

The Philadelphia chromosome (t (9; 22) (q34; q11)) was first described in this type of leukemia. It is present in all cases of CML and results in two critical events in the disease: 1) The gene provides a unique biomarker for diagnosis and monitoring of the response to treatment and 2) The fusion tyrosine kinase is used as a target therapeutic.

4.2.7.3.Epidemiology, risk factors, diagnosis, prognosis and treatment.

The American Society Against Cancer of the United States estimates that by 2015, there will be approximately 6,660 new cases of CML and 1,140 people will die from this cause. About 10% of all leukemia cases are of this class, this disease affects mainly adults and rarely occurs in children (American Cancer Society, 2015). The main risk factors for developing this disease are exposure to radiation, age and incidence according to sex, as it affects slightly more men than women.

Many people with CML have no symptoms when diagnosed with the disease, and like other leukemias, their diagnosis is casual. The blood count results with a large number of white blood cells and when seeing the peripheral blood smear, most of the leukocytes are observed in their immature forms. The disease must be confirmed through the analysis of the bone marrow. Other tests that help confirm are, the karyotype determination, to find the presence of the Philadelphia chromosome, t (9; 22) (q34; q11), fluorescent in situ hybridization (FISH) to locate the BCR-ABL gene, as well as the PCR to see the amplification of the same gene (Jabbour and Kantarjian, 2014).

Currently, small molecules, inhibitors of tyrosine kinase (TKIs), are used for the treatment of CML. There are some commercially available, these include imatinib, dasatinib, nilotinib, bosutinib and ponatinib. The TKIs are viable options for the initial CML-CP management (Jabbour and Kantarjian, 2014). Other treatments are the use of chemotherapeutic agents such as hydroxyurea, cytarabine (Ara-C), busulfan, cyclophosphamide and vincristine, which can be used in combination with TKIs (American Cancer Society, 2015).

The survival rate of CML is not known with precision, but in a study conducted on patients with this disease and treated with imatinib, it was determined that about 90% of them lived 5 years after the start of treatment (Druker et al.,2006). In subsequent studies, the use of imatinib ceased to be effective, since around 60% of patients relapsed in the disease and had chemoresistance to this drug, fortunately, these patients responded to other TKIs. Unfortunately, around 10% to 15% of patients with CML continue to be resistant to treatment with TKIs (Apperley, 2015).

Several mechanisms of primary and acquired resistance have been identified for CML, these mechanisms include the amplification of the BCR-ABL1 gene, the overexpression of the glycoprotein, resistant to multiple drugs (MDR1) and the low activity of the drug transporter, OCT1 (Le Coutre et al.,2000; Mahon et al.,2000; White et al.,2012). However, the most frequent mechanism for chemotherapeutic resistance against CML appears to be the development of point mutations in the ABL1 kinase domain, which results in decreased binding of TKI drugs (Apperley, 2015).

4.2.8. Side effects of current treatments in leukemia

Although current treatments generate a good survival prognosis in some types of leukemia, most of them are toxic to patients, generating side effects. The side effects of using this mAb are neurological problems, such as seizures, fainting, difficulty speaking, confusion and loss of balance (American Cancer Society, 2015). On the other hand, we have the case of tyrosine kinase inhibitors, which can generate vascular obstruction, pulmonary hypertension, pancreatitis and hepatitis, cardiovascular, cerebrovascular, cytopenia among others (Apperley, 2015). In the general case of chemotherapy, the adverse effects are muscle pain, bone pain, fever, headaches, tiredness, nausea and vomiting, bone marrow damage, neuropathies, lung damage and many others (American Cancer Society, 2015). On the other hand, neoplasms generate resistance to these treatments, making them less and less effective, and bone marrow, the origin of leukemia, generates a microenvironment that contributes to this resistance, as well as the survival of all kinds of neoplasms (Meads et al.,2008).

4.2.9. Tumor Microenvironment in Bone Marrow.

As mentioned above, resistance to chemotherapeutic agents is a cause that hinders the cure of cancer. Some of the mechanisms by which cancer cells become resistant to drugs are inherent to the intrinsic characteristics of the tumor cell, such as its genetic instability and high mutation rate, as well as the expression of MRD genes (resistant to multiple drugs), inhibition of the expression of the target molecule, to which the drug is directed, among others. While on the other hand the microenvironment where both the tumor and the drug are found, can make it difficult for the drug to perform its function (Alonso, 2009). Regarding the tumor microenvironment, Meads and colleagues point out that the bone marrow promotes a protective environment for neoplastic cells, where they maintain interactions with other cell types such as stromal cells, osteoclasts and osteoblasts, which provide them with protection and provide them with antiapoptotic and proliferative signals (Meads et al.,2008). This interaction with its microenvironment is the main cause of resistance to treatments for all types of leukemia (Meads, et al.,2008).

It has been shown that this resistance is given by soluble factors, such as cytokines and stromal cells derived Factor 1 (SDF-1), and by components of the extracellular matrix such as fibronectin, present in the bone marrow (Meads et al.,2008). It is known that Interleukin-6 (IL-6) induces the activation of the signal transducer and activator of transcription 3 (STAT3), conferring protection against Fas-mediated apoptosis, through the positive regulation of the anti-apoptotic protein Bcl- XL (Catlett et al.,1999; Cheu and Van Ness, 2002). Another example of how the microenvironment of the bone marrow favors the protection of leukemic cells is the "homing" that the neoplastic cells have, thanks to the cell surface receptor coupled to protein G (CXCR4) which is overexpressed in cells malignant and its ligand, SDF-1, is constitutively expressed by bone marrow stromal cells (BMSC) (Burger and Kipps, 2006; Jung et al.,2006). On the other hand, integrins type αvβ3 enhance survival, proliferation and migration, and are over-expressed in tumor cells (Gou and Giancotti, 2004). These integrins induce the adhesion of tumor cells to the BMSC, involving the Notch1 receptor with its Jagged ligand and leading to the increase in p27Kip1, which causes cell arrest in the G1 phase (Hazlehurst et al.,2000). This leads to inhibition of growth and protection against drugs that cause apoptosis (Jundt et al.,2004). That is why the search

for treatments that can induce the death of leukemic cells, regardless of the protective signals of their microenvironment or intrinsic defects that give them resistance to apoptosis, is necessary to combat resistance to treatments.

With the previous information about the importance of leukemias and how the microenvironment where they develop contributes to the ineffectiveness of the treatments used for their remission. The search for new and better therapeutic agents that are effective despite the microenvironment and in patients in whom classical therapies have no effect is necessary. On the other hand, the knowledge of resistance to a specific type of death, such as apoptosis, leaves us open to explore the alternate pathways of regulated death. The white molecule chosen for this project, CD47, is described below, which is involved in various biological processes and which we believe may induce regulated cell death in the different types of leukemia, previously discussed.

4.3. CD47

4.3.2. Generalities

The Cluster of Differentiation 47, CD47, is a highly glycosylated transmembrane protein. The molecular weight calculated for CD47 in humans is 31-35 kDa depending on the length of the C-terminal cytoplasmic tail, but due to the glycosylation in its Ig domain, its migration in SDS-PAGE gels is 45-55 kDa (Brown and Frazier, 2001). It was originally reported by several groups of researchers who named it in various ways, including Rh-related antigen, since it is not found in the erythrocytes of patients with Rh null hemolytic anemia (Miller et al.,1987), integrin-associated protein (IAP), due to its physical association with different types of integrins (Brown *et al.,*1990), another way of calling it was OA-3 antigen (Campbell et al.,1992) since it was reported that said molecule was identical to the antigen of cancer, OA3, overexpressed in ovarian carcinoma cells and finally, CD47 (Mawby et al.,1994). CD47 is an integrin-associated protein (IAP) belonging to the immunoglobulin superfamily. It is a receptor for Thrombospondin (TSP-1), a ligand for the alpha and gamma signal regulatory protein (SIRPα, SIRPγ), and has also been shown to be associated with integrins from the same cell (Brown and Frazier, 2001). This molecule is expressed ubiquitously and plays multiple roles in the homeostasis of the organism. It has an active role in neuronal

development and in the regulation of the immune and cardiovascular systems, among others. It also plays a central role in proliferation, invasion, angiogenesis, apoptosis, and evasion of the immune system (Sick et al.,2012; Soto-Pandoja et al.,2013; Martínez-Torres, 2013).

In cancer, CD47 can play a double role, on the one hand, cancer cells can use this molecule to evade surveillance of the immune system, since the binding of CD47 to SIRPα, prevents phagocytosis by promoting tumor progression (Sarfati et al.,2008). However, on the other hand, the activation of CD47 in the cells of the immune system by the binding with TSP-1 controls the inflammatory response, including the activation and migration of cells of the innate and adaptive immune system (Linderberg et al.,1996; Li et al.,2002; Chin et al.,2009). It also inhibits angiogenesis (Soto-Pantoja et al.,2013) and induces cell death in several types of cancer cells (Mateo et al.,1999, Barbier et al.,2009, Roue et al.,2003, Marle-Béral et al.,2009; Bras et al.,2007). Therefore, CD47 is a versatile protein with multiple, clinically important functions (Martínez-Torres, 2013).

4.3.2. Structure of CD47

CD47 belongs to the immunoglobulin (Ig) superfamily. It has a single IgV type domain at its N-terminal end, followed by a highly hydrophobic presenilin domain with five transmembrane segments [the multiple domain that crosses the membrane (MMS)] and ends in a C-terminal cytoplasmic sequence whose length oscillates between 3-36 amino acids. In this intracellular sequence is where alternative splicing occurs, giving rise to four isoforms of CD47 (Fig. 4) (Brown and Frazier, 2001, Soto-Pantoja et al.,2015).

The IgV domain is N-glycosylated and modified with an O-glycosaminoglycan. The five N-glycosylation sites (Fig. 3) are not required for binding to SIRPα (Subramanian et al.,2007), but glycosaminoglycan modification is required for thrombospondin-1 (TSP-1) signaling through of CD47 (Kaur et al.,2013). As regards the modification of glycosaminoglycan, it occurs in Ser64 and Ser79 of the IgV domain, resulting in an isoform of more than 250kDa (Kaur et al.,2013). On the other hand, this extracellular region can undergo a proteolytic cut,

regulated by glucose levels. The decrease in glucose leads to the proteolytic cleavage of the IgV domain, which results in a fragment less than 30kDa (Maile et al.,2008).

Additionally, CD47 has two different disulfide bridges. The first is conserved within its IgV type region and the second disulfide bond binds Cysteine33 (Cys33) present in the IgV domain to Cys263 which is found in the last extracellular loop of the transmembrane domain. These disulfide bridges (Fig. 4) can regulate protein conformation and access to other proteins and are essential for the interaction of CD47 with SIRP-α and the response of T cells to Ca2 + (Rebres et al.,2001; Subramanian et al.,2007).

Alternative splicing occurs in the terminal Carboxyl region. Each of the four cytoplasmic tails generated (Fig. 4) have been found in vivo (Reinhold et al.,1995); being the isoform 2 the predominant one. The second most abundant isoform is 4, which has the longest cytoplasmic extension and is found mainly in neurons, the peripheral nervous system, the intestine and the testes (Brown and Frazier 2001). While isoform 1 is mainly expressed in keratinocytes (Reinhold et al.,1995). None of the cytoplasmic extensions of CD47 has any known reason for enzymatic activity or protein interaction (Brown and Frazier, 2001).

All of the above indicates that both post-translational modifications imply a high degree of regulation, not necessarily related to the expression of the protein. This regulation can be modified by the interaction of CD47 with other intracellular and / or membrane-associated proteins. On the other hand, the availability or functionality of CD47 may be different depending on the type of cell. Therefore, CD47 interactions may be of great importance for signal transduction in both physiological and pathophysiological conditions (Soto-Pantoja et al.,2013, Martínez-Torres, 2013).

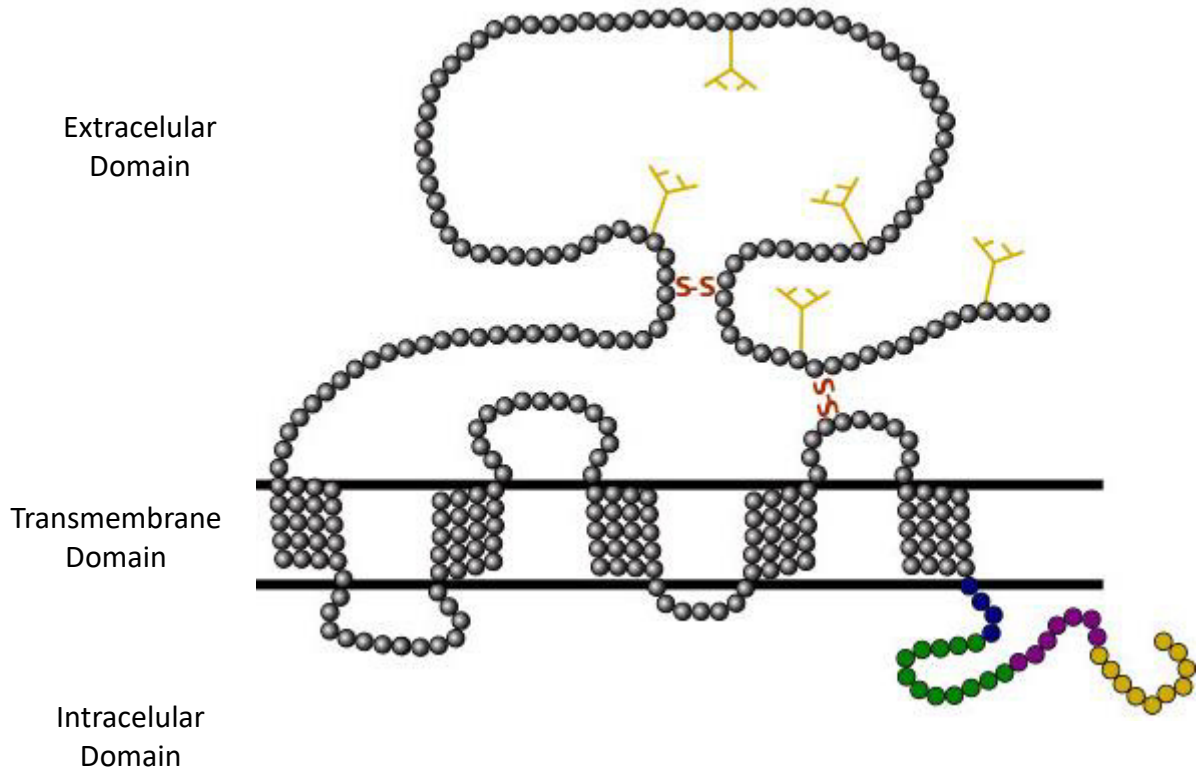

Figure 7. Structure of CD47. It shows the extracellular IgV domain, with its 5 glycosylation sites (in yellow), followed by the 5 transmembrane segments (MMS) (in gray), the disulfide bridges (ss in red) and finally the Carboxyl domain. cytoplasmic terminal with its 4 possible alternative splicings, where isoform 4 is the complete molecule.

4.3.3. Interactions and ligands of CD47

Thanks to the IgV domain, CD47 is able to interact with three classes of extracellular ligands, the main ligand secreted for CD47 is TSP-1, a homotrimeric glycoprotein whose C-terminal domain binds to CD47 (Isenberg et al.,2009). The second ligand is the family of signal regulatory proteins that consists of 3 members, of which only SIRPα and SIRPγ are considered CD47 receptors. Finally, it has been shown that CD47 can interact with various integrins. Table 6 summarizes the main ligands of CD47 and the biological effects of these associations, the interaction and the effects of this, depending on the type of cell and the specific situation.

Among the integrins that interact with CD47 is the widely expressed RGD (arginine-glycine-aspartic) receptor αvβ3, the platelet fibrinogen receptor αIIbβ3 and the collagen receptor α2β1. Only these integrins have been coprecipitated with CD47 in leukocytes (αvβ3) (Lindberg et al.,1993), platelets (αvβ3 and αIIbβ3) (Chung et al.,1997), in melanoma cells (Gao et al.,1996) and in ovarian carcinoma (αvβ3) (Frazier et al.,1999) and smooth muscle cells (Wang and Frazier, 1998) and platelets (α2β1) (Chung et al.,1999). The structural requirements for coprecipitation and functional cooperation have been carefully examined only for association CD47 with αvβ3. In this case, the Ig domain of CD47 is required for both functional and physical interaction (Brown and Frazier, 2001). The interaction is explained in more detail below, with its two main ligands, SIRPα and TSP-1. Table 5 summarizes the main ligands of CD47 and the biological effects of these associations, the interaction and the effects of this, depending on the type of cell and the specific situation. Next, the interaction is explained in depth, with its two main ligands SIRPα and TSP-1.

4.3.3.1.Signal Regulatory Protein Alpha (SIRPα)

The SIRPs are transmembrane glycoproteins of the immunoglobulin superfamily and consist of 3 domains: one variable type Ig (IgV), farther from the membrane (D1) and two constant Ig type, close to the membrane (D2 and D3) (Fig. 8).

The members of this family are SIRPα (CD172A), SIRPβ (CD172b) and SIRPγ (CD172g). SIRPα is highly expressed by neurons and myeloid cells, SIRPβ is expressed by neutrophils and macrophages, whereas SIRPγ is mainly expressed in lymphocytes and NK cells (Barclay and Brown, 2006).

The main cellular ligand for SIRPα is CD47, which can also bind to SIRPγ but with an affinity approximately 10 times lower than to SIRPα (Hatherley et al.,2008). In contrast to the clearly defined signaling functions of SIRPα, those of SIRPγ are poorly understood. SIRPγ is a transmembrane protein that lacks a cytoplasmic domain, therefore, it is thought that the signal occurs indirectly by the lateral association with other membrane proteins (Brooke et al.,2004, Matozaki et al.,2009).

Table. 7. Main Ligands of CD47 and their consequences in cells.

CD47 partners		Principal biological / cellular consequences
Extracellular partners		
SIRPα		Inhibition of phagocytosis, stimulation of cell-cell fusion, T-cell activation, neutrophil and monocyte transmigration, …
SIRPγ		Leukocyte transendotelial migration, T-cell proliferation
Thrombospondin-1 (C-terminal domain)		Programmed cell death (PCD), inhibition of angiogenesis, inhibition of nitric oxide signaling, cell adhesion, cell proliferation, cell survival, …
Lateral / membrane partners		
Direct partnerts	**Multiple Integrins**	**αvβ3:** PCD, monocyte-cytokine synthesis, melanoma cells spreading **α2β1:** Migration and proliferation of smooth muscle cells **α2:** COX-2 expression and intestinal epithelial cell migration **αIIbβ3:** Platelet activation **α4β1:** adhesion of sickle reticulocytes, B-cell migration αs, α6β1, αMβ2…
	VEGFR2	Inhibition of VEGFR signaling
Indirect partnerts	**CD36**	Inhibition of NO signaling
	FAS	Stimulation of Fas-mediated apoptosis
Intracellular partners		
Direct partnerts	**Gi proteins**	Syk: LYN & FAK phosphorylation – platelet activation PKA: PCD, decrease in cAMP-platelet activation, migration ERK: T cell adhesion, inhibition of smooth muscle cell migration PI3K: migration, spreading, proliferation
	PLIC-1	Cytoskeletal regulation for cell spreading and motility, regulation of heterotrimeric G-protein function
	BNIP3	Leukemic and activated T-cell PCD (4N1K)
Indirect partnerts	**Drp-1**	CLL PCD (immobilized CD47mAb)
	Rac	Neuronal development
	Cdc42	B-cell migration, neuronal development
	Src Kinases	Epithelial cell spreading and migration, neuronal development
	Protein 4.2	Rh complex integrity on red blood cells
	HIF-1α	Leukemic T PCD (S-S diabody from scFv of MABL)

Adapted from Martínez-Torres, 2013.

SIRPα in its extracellular portion is structurally related to the T cell and B cell antigen receptors, and through the D1 region it interacts with CD47. The cytoplasmic domain of SIRPα contains various tyrosines that when phosphorylated can create an immunoreceptor inhibitory tyrosine (ITIM). These motifs are binding sites for the phosphatases SHP-1, SHP-2 (with tyrosine phosphatase domain homologue of Src 2) and SHIP (inositol phosphatase) (Matozaki et al.,2009).

The interaction of CD47 with SIRPα mediates several functions in leukocytes, including the transmigration of neutrophils and monocytes (Parkos et al.,1996, De Vries et al.,2002). Furthermore, it was shown that this interaction negatively regulates the signaling of some Toll-like receptors (TLR), as well as the secretion of inflammatory cytokines and the triggering of the leukocyte oxidative catastrophe (Martínez-Torres, 2013, Ide et al.,2007 Smith et al.,2003; Dong et al.,2008).

Finally, the CD47-SIRPα interaction regulates phagocytosis mediated by monocytes, macrophages and dendritic cells (Ishikawa-Sekigami et al.,2006; Oldenborg et al.,2001; McCracken et al.,2015), since CD47 serves as a signal "do not eat me". This generated signal involves the activation of the SIRPα ITIM and the recruitment of the SHP-1 and -2 phosphatases, which results in the inhibition of phagocytosis. Consequently, CD47 prevents phagocytosis of the macrophage itself. The signal does not eat me was discovered for the first time in erythrocytes, and later it was extended to leukocytes. In addition, after these observations it was demonstrated that the CD47-SIRPα binding participates in the regulation that allows macrophages to discern between a viable and an apoptotic cell (Martínez-Torres, 2013).

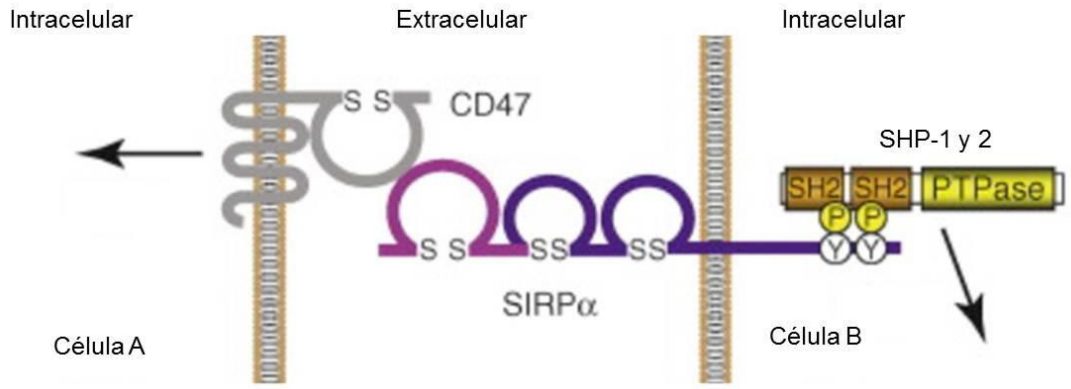

Figure 8. Structure and interaction of CD47 / SIRPα. SIRPα is constituted by 3 Ig-like domains, in the extracellular region and in the intracellular region it has phosphorylation sites for tyrosine, these sites bind to tyrosine phosphatases proteins, SHP-1 and 2, and activate them. CD47 is a ligand of SIRPα, the N-terminal end of Ig type of SIRPα binds to the Ig domain of CD47. The trans interaction of these two proteins mediates intracellular signals in both directions. (Taken from Martínez-Torres, 2013)

4.3.3.2.Thrombospondin-1 (TSP-1)

Thrombospondins comprise a family of multidomain calcium-binding glycoproteins composed of five members, called TSP-1 to TSP-5, said proteins are encoded by five genes. Platelet thrombospondin or TSP-1, as it is now known, is the prototype member of this family (Fig. 5). TSP-1 and -2 are homotrimeric molecules and have identical structural domains, while TSP-3, -4 and -5 are homopentimers and missing the repeating type 1 and procollagen regions found in TSP-1 and 2. TSP 1 and 2 have a complex pattern of generalized expression during embryogenesis, while TSP 3, 4 and 5 are more localized expression at specific stages of development (Lopez-Dee et al.,2011).

TSP-1 has several binding domains to membrane or extracellular matrix receptors, mediated cell-cell and cell-extracellular matrix interactions. The structure of the TSP-1 is shown in fig. 5. Briefly, this protein is formed by an N-terminal globular domain, three diffusible chains: Type I (properdin type repeats), type II (epidermal growth factor type) and type III (calcium binding repeats) and a globular domain. C-terminal, which joins CD47 (Gao and Frazier, 1994, Gao et al.,1996). In fact, TSP-1 is the main soluble ligand of CD47 (Martínez-Torres, 2013). The main cell binding site that was identified is located at the C-terminal end of TSP-1 (Kosfeld and Frazier, 1992). With the use of synthetic peptides, the cell binding activity was localized into two sequences, which contain the VVM motif, this discovery brought the development of small peptides with the VVM motif corresponding to the sequences of TSP-1. Said peptides include, 7N3 (1102-FIRVVMYEGKK-1112) and 4N1 (1016-RFYVVMWK-1024), the latter being highly conserved in all TSP species and isoforms (Kosfeld and Frazier, 1993). In addition, it should be mentioned that the group of Gao and Frazier also identified CD47 as a receptor for TSP-1 in many cell types (Gao and Frazier, 1994). Later, the same group developed the peptide, 4N1K (K-RFYVVMWK-K) which corresponds to 4N1 flanked by two Lys residues, this modification was made to improve the solubility of 4N1 without affecting the binding to CD47 (Gao et al.,1996).

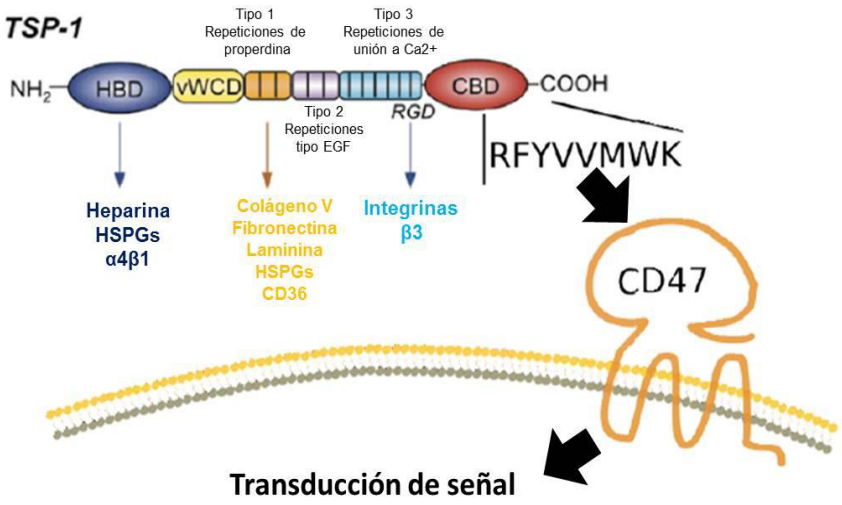

Figure 9. Structure of TSP-1. Said molecule comprises different domains, including the heparin binding domain (HBD). The von Willebrand domain C. Three repeats of properdin (type 1), which bind to CD36, to collagen V, fibronectin and proteoglycan heparan sulfate. Three EGF type repeats (type 2), and seven calcium-binding repeats (type 3) that bind to β3 integrins through the RGD motif and the terminal carboxyl domain (CBD), several TSP-1 domains interact with different components of Extracellular matrix or membrane receptors, as already mentioned, also shows the key amino acid sequence, responsible for the ligation of TSP-1 with integrins (RGD) and RFYVVMWK for interaction with CD47. Image taken from Martínez-Torres 2013.

4.3.4. Interactions of CD47 / TSP-1

Floquet and collaborators proposed a molecular model of the interaction of CD47 with the C-terminal domain of TSP-1, they showed that the 4N1 sequence is hidden within a hydrophobic compartment of TSP-1, avoiding any interaction. However, when it is close to CD47 and the phospholipid bilayer, the hydrophobic cleavage opens allowing 4N1 recognition (Floquet et al.,2008). The biological consequences of CD47 / TSP-1 ligation is broad and depend on the cell type, its association with other molecules, its conformation, its distribution on the cell surface, the anchoring mode, and the particular situation in which these are produced. processes (Martínez-Torres, 2013). These mechanisms play a key role in the regulation of the organism's homeostasis.

The interaction of CD47 / TSP-1 is involved in various biological processes. There is a history that this relationship mediates the inhibition signal of nitric oxide (NO), having

repercussions in the cardiovascular system. This is because NO is a radical gas that regulates blood flow and pressure, decreasing the amount of adhesion molecules in platelets and altering calcium levels in vascular smooth muscle cells of the arteries, among other mechanisms. When TSP-1 inhibits the enzyme that censes and activates NO production, soluble guanylyl cyclase (sCG) in platelets and endothelial cells, vasodilation and chemotaxis are controlled (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 FGF-2 in vitro and in vivo (Kanda et al., 1999). In addition, CD47 inhibits vascularity functions, as observed in a study conducted by Isenberg et al. In 2008, in which null mice for CD47 and TSP-1 increased the number of blood vessels in a skin graft (Isenberg et al., 2008, Martínez-Torres, 2013). On the other hand, the interaction CD47 / TSP-1 participates in the regulation of several aspects of the immune system, both innate and adaptive, because it is involved in different processes such as inflammation, migration, elimination, adhesion and expansion. In the innate immune response, the binding of CD47 / TSP-1 plays an important anti-inflammatory role, besides suppressing the production of IL-2 in human monocytes and predicts its differentiation to dendritic cell (CD) (Demeure et al., 2000; Armant et al., 1999). The loss of CD47 prevents the maturation of DCs and the production of inflammatory cytokines (Demeure et al., 2000). In the adaptive response CD47 plays an important role in the activation of T cells, because they express integrins αvβ1, CD47 and TSP-1, facilitating their interaction, which triggers both inhibitory and stimulatory functions (Martínez-Torres, 2013). It is important to mention that there are differences in the distribution and expression of these two proteins in the cells, which influences the interactions between both proteins. TSP-1 is produced by professional antigen-presenting cells, and T cells express it on its surface (Li et al.,2002), whereas CD47 expression depends on the cell type of the immune system, for example T cells, express high levels of CD47, compared with monocytes and DC dendritic cells (Lindberg et al.,1993).

With the above, it is easy to realize that the interaction between these two molecules, have a broad spectrum of functions and the one that interests in this work is the ability to induce death to tumor cells.

4.3.5. CD47 as a therapeutic target in cancer

In recent years, the search for therapeutic targets against neoplasms has been the subject of several investigations. The various biological functions in which CD47 is immersed made the eyes of different groups of researchers rested on it. Mainly due to its characteristic of the *Janus* type molecule, which on the one hand exerts the "do not eat me" signal that can be exploited by neoplastic cells and contribute to their survival and progression. This is because it has been observed that cells overexpressing CD47 become resistant to phagocytosis mediated by macrophages and dendritic cells, by the binding of CD47 to SIRPα, as is the case of renal carcinomas (Nishiyama et al.,1997), prostatic (Vallbo and Damber, 2005), and of squamous cells (Suhr et al.,2007), T-cell water lymphocytic leukemia (Raetz et al.,2006), multiple myeloma (Rendtlew et al.,2007), cells mother of acute myeloid leukemia (Jaiswal et al.,2009) and in other types of leukemia (Chao et al.,2010). On the other hand, it can inhibit angiogenesis by interacting with its TSP-1 ligand and induce cell death in chronic lymphocytic leukemia cells (Mateo et al.,1999) and mother's cancer (Manna and Frazier, 2004). The understanding of CD47, its ligands and signaling pathways, is fundamental for the development of therapies directed towards CD47, which are capable of treating devastating diseases such as cancer.

4.3.6. CD47 in cell death regulated in cancer

In this context, several studies with monoclonal antibodies and peptides from the C-terminal region of TSP-1 against CD47 have been shown to be able to induce regulated cell death in different cell types. The first study described, on regulated cell death mediated by CD47, was carried out by Mateo and his collaborators in 1999, where they used, attached to a plate, the monoclonal antibody (mAb) anti-CD47 B6H12, TSP-1, and the peptide 4N1K to induce death in CLL cells (Mateo, et al.,1999). Almost at the same time another group led by Pettersen, found that using only the anti-CD47 soluble antibody (Ad22 and 1F7, but not B6H12 or 2D3), induced cell death to the cell line derived from acute T-cell leukemia (Jurkat) (Pettersen et al.,1999). Both groups found that the soluble antibody B6H12 was not able to generate RCD, these findings showed the importance of the complex regulation between the CD47 binding model and the induction of death (Martínez-Torres, 2013).

Subsequently, different research groups continued to evaluate the induction of RCD in different cell types, using mainly anti-CD47 soluble mAbs, such as Ad22, 1F7, B6H12, 2D3, mAb-MABL, CC2C6 among others. Some examples of cancer studied were: in the cell line CCRF-CEM, in ALL, JOK-1, in CLL (Uno et al.,2007; Leclair et al.,2018), mammary carcinoma (MCF-7) (Manna and Frazier, 2004), cells of multiple myeloma (KPMM2) (Kikuchi et al.,2005), among others. Likewise, TSP-1 and the 4N1 and 4N1K peptides adhered to the plate were used to test the RCD induction in leukemia and breast cancer cells (Manna and Frazier, 2004).

Furthermore, in a study on the recombinant fusion protein SIRPα-Fc, it was observed that this protein did not induce CD47-dependent death (Lamy et al.,2003). While, Brooke and his team reported that the binding of SIRPγ to CD47, if it could induce programmed cell death in the cell line of acute T-cell leukemia (Jurkat) and in the line of human histiocytic lymphoma (U937) (Brooke et al. al., 2004). On the other hand, it was shown that CD47 increases Fas-mediated apoptosis (Manna et al.,2005).

The main characteristics of the RCD mediated by CD47, observed by different researchers, in cancer cells are:

a) A fast process.

b) Independent of caspases.

c) Depolarization of the mitochondrial membrane without release of proapoptotic proteins (Cytochrome C, AIF, Smac / DIABLO, Omi / Htra2).

d) Production of reactive oxygen species (ROS).

e) Exposure of phosphatidylserine.

f) Permeabilization of the plasma membrane.

g) Absence of DNA fragmentation or condensation of chromatin (Martínez-Torres, 2013).

Most of the research done in recent years on the induction of RCD by CD47, has been made through the use of mAb, which are designed to block, imitate or stimulate the effects of its interaction with one of its main ligands. The mechanisms by which anti-CD47 antibodies can

facilitate the elimination of tumor cells are shown in Figure 6. In fact, Weissman and his team described a process called "Programmed Cell Elimination", which consists of blocking the signal "Do not eat me" induced by the interaction of CD47-SIRPα, to induce cell recognition and elimination, by using mAbs to block said interaction (CD47-SIRPα). It is currently the most advanced strategy in clinical trials (Chao et al 2012; Edris et al.,2012). In the same way, these antibodies are being used to enhance the cytotoxicity mediated by CD8 + T cells in conjunction with the application of radiotherapy (Soto-Pantoja, et al.,2014).

But as mentioned throughout the work, the importance of CD47 in different biological processes, suggest that the application of antibodies that block its function, can bring side effects, such as anemia, thrombosis and hypertension, because it is found on the surface of erythrocytes, as well as other vascular endothelial smooth muscle cells and platelets, in which CD47 inhibits NO signaling. Some of these effects have already been confirmed in vivo, thanks to the study by Bauer et al., where mice injected intravenously with miap301, a murine anti-CD47 antibody, suffered acute increases in mean and diastolic blood pressure (Bauer, et al.,2012).

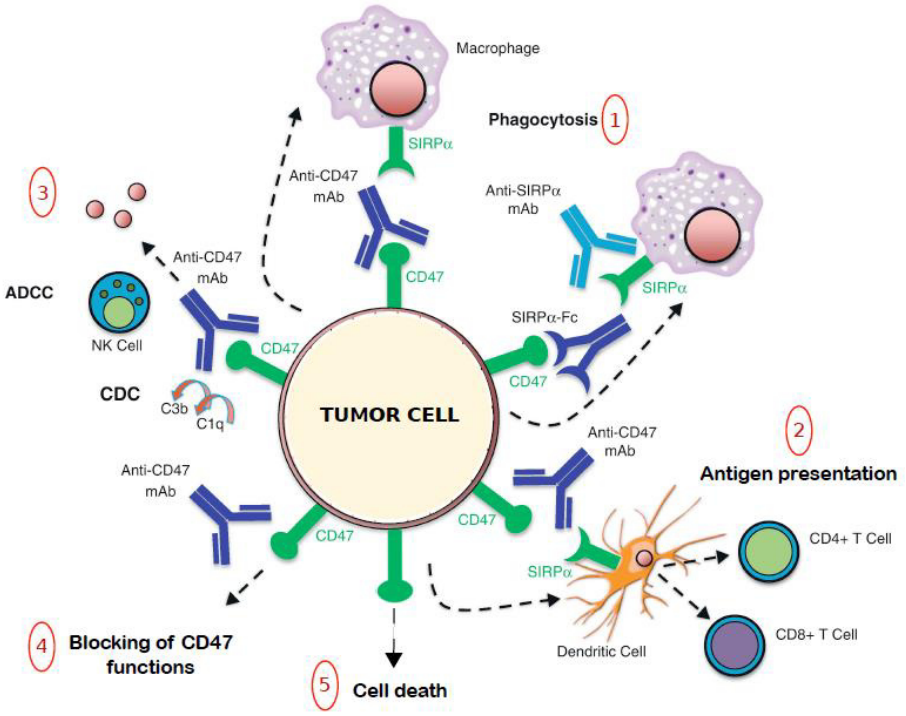

Figure 10. Use of CD47 as a target to eliminate cancer cells. The use of monoclonal antibody (mAb) against CD47 can induce the elimination of neoplastic cells through multiple mechanisms. 1) Through the phagocytic absorption of tumor cells by macrophages: by inhibiting the CD47-SIRPα interaction with an anti-CD4 mAb, with a mAb against SIRPα or by using the recombinant protein of SIRPα (which is shown as a bivalent Fc fusion protein). 2) Anti-CD47 antibodies can stimulate an adaptive antitumor immune response that leads to the phagocytic absorption of tumor cells carried out by dendritic cells and the subsequent presentation of antigens to CD4 and CD8 T cells. 3) Through the induction of Antibody-dependent Cellular Cytotoxicity (ADCC) and NK-mediated Complementary Dependent Cytotoxicity (CDC): Anti-CD47 Antibodies can eliminate tumors through mechanisms dependent on the crystallizable Fraction of antibodies (Fc). 4) Blocking the functions of CD47 can also promote tumor reduction by blocking several of its actions on tumor cells. 5) Finally, stimulation of CD47 can induce direct cell death. Taken from Martínez -Torres 2013.

On the other hand, the study of peptides derived from TSP-1 were scarce, mainly due to the proteolytic degradation suffered by the peptides when administered in a soluble form and due to their capacity to form aggregates, thus limiting their therapeutic use. However, in this year 2015, an article was published where they demonstrated that the activation of CD47, through the use of synthetic peptides derived from TSP-1, selectively induces RCD, both in vitro and in vivo. In this study, they administered soluble peptides 4N1K and PKHB1 (which is more stable in serum, compared to 4N1K) in the CLL cell line (MEC-1), as well as in primary cells of patients with CLL, obtaining RCD. In addition, a coculture of cells from patients with

chemoresistance and stromal cells was used to simulate the tumor microenvironment, which is known to generate a protective environment for leukemic cells, and even so the peptides had an effect, causing RCD. On the other hand, in an CLL-xenograft model carried out in NOD / scid gamma (NSG) mice, they demonstrated that the injection of CD47 agonist peptides reduces the size of the tumor, without inducing anemia or toxicity in blood, liver or kidney. (Martínez-Torres, et al.,2015). Such activation, caused by the mentioned peptides, causes RCD by means of the activation of phospholipase C gamma1 (PLCγ1), a protein that is overexpressed in CLL cells, as demonstrated in that same publication. In short, it was demonstrated that the over-activation of PLCγ1 leads to the massive increase of calcium ion (Ca2 +) in the cell cytoplasm, inducing the activation of serine proteases (serpases), the depolymerization and degradation of actin, the loss of potential of the mitochondrial membranes, and finally to the death of the leukemic cell (Martínez-Torres, et al.,2015).

The above represents a watershed in the studies of the induction of RCD mediated by CD47, since it gives us bases to look for other ways in which CD47 can be used as a therapeutic target, not only in leukemias, but in other types of neoplasms and conditions that involve the immune and cardiovascular system. It also generates more evidence about the complexity of CD47, which, depending on its ligands, is the task that this protein will play. It can be said that CD47 is still considered a protein not fully understood, due to its inherent capacity to interact with other molecules that also have multiple functions depending on the cells that express them. For this reason, the study of CD47 with its ligands and possible co-stimulatory molecules is of utmost importance, in order to develop strategies that favor the regulation of various cellular biological processes, including the death of tumor cells. The present project will precisely focus on determining if the type of regulated cell death is the same for the different types of leukemia *in vitro*, determine If PKHB1 is able to induce tumor regression in an immunocompetent mouse model *in vivo*, as well as finding the implication of the immune system when CD47 is binding with its agonist peptides. This in order to give bases for the use of these peptides as possible treatments to patients with chemoresistant leukemias.

V. JUSTIFICATION

Leukemia is considered the leading cause of death from neoplasms in patients under 20 years of age in Mexico and nearly 4,830 deaths in adults occur annually in our country due to this disease, turning it into a serious public health problem, regardless of age.

Despite the efforts made and treatments currently available, leukemia acquire chemoresistance, mainly because of the acquisition of intrinsic mutations of the leukemic cells and microenvironment in which they develop (the bone marrow), which provides them with favorable signals to continue with the pathological progress of the disease. This leads us to the search for new and better therapies that can combat these hematopoietic neoplasms, independently of the main causes of resistance to current treatments and looking for treatments that can induce an effective anticancer immune response.

Recently, immunogenic cell death (ICD) has emerged as an innovating concept in anticancer therapies development, due to this form of RCD is able to induce the "wake up" of immune system, allowing the recognition and eradication of malignant cells. However, a restricted number of chemotherapeutics can trigger ICD of neoplastic cells, making necessary the seek for treatment able to induce ICD.

In this context, peptides derived from TSP-1, a ligand of CD47, induce a caspase-independent, but calcium-dependent cell death in chronic lymphocytic leukemia cells, independently of protective factors of the microenvironment and intrinsic mutations. Interestingly, PKHB1 can induce calreticulin (CRT) exposure in CLL cells, which gives us a hint of its potential ability to be an ICD-inducer. Therefore, we verified if PKHB1 could induce a similar RCD described for CLL in different types of leukemia and in immunocompetent murine model *in vivo*. Also, we determined whether PKHB1 was able to induce ICD. Elucidating the mechanism by which these peptides act, could provide new alternatives for the treatment of chemoresistant leukemia.

VI. HYPOTHESIS

CD47 agonist peptides selectively induce a fast, caspase-independent and calcium-dependent cell death on leukemic cells *in vitro*, being these peptides able to induce immunogenic cell death and exhibit antineoplastic activity *in vivo* in an immunocompetent environment.

VII. OBJECTIVES

7.1.General Objective

To study the mechanism of cell death induced by CD47 agonist peptides on a panel of leukemic cells and their *in vivo* effects in an immunocompetent environment.

7.2.Specific Objectives

7.2.1. *In vitro*:

- Analyze the cell death induced by CD47-agonist peptides on different types of leukemia cell lines and to choose the best one to further analysis.
- Determine whether induced cell death on a panel of leukemic cells is caspase and calcium dependent.
- Evaluate whether induced cell death is immunogenic.

7.2.2. *In vivo*:

- Evaluate the antitumor activity of PKHB1, in a murine model with an intact immune system.
- Analyze the role of the immune system in the elimination of the tumor through the use of PKHB1.

VIII. MATERIAL AND METHODS

This project involved an international collaboration between the Laboratory of Immunology and Virology, where the main part of this project was carried out, and the Biomolecules Laboratory of the Oncodesing research center in Villebon Sur Yvette, France, where Pr. Philippe Karoyan, provided the different CD47 agonist peptides.

8.1.CD47 agonist peptides

The peptides used were 4N1K and PKHB1, which were developed, synthesized and their affinity tests were realized at the Laboratory of Biomolecules of the Oncodesign research center by Pr. Philippe Karoyan. The amino acid sequences and characteristics of the peptides that were used are shown below:

PKHB1: kRFYVVMWKk. MW : 1,385.3.

4N1K: KRFYVVMWKK. MW: 1,385.0.

8.2.Cell-Treatment with Peptides

The purified peptide powder was aliquoted and freshly diluted in ultra-pure water before treatment. Peptide concentration was measured at 280 nm (Thermo Scientific NanoDrop^{TM} 2000).

8.3.Cell culture

Leukemia cell lines used in this work were MEC-1 (CLL), Jurkat, CEM and MOLT-4 (T-ALL), HL-60 (AML) y K562 (CML). Peripheral blood mononuclear cells (PBMCs) (human and murine), CD4+ and CD8+ human T cells and primary cultures cells from mice lymphoid organs were obtained from healthy individuals and used as control healthy cells. Cells were maintained in complete medium (RPMI 1640 supplemented with 10% of fetal bovine serum [GIBCO by Life technologies, Grand Island, NY, USA], 2 mM L-glutamine, 100 U/mL of penicillin-streptomycin [GIBCO by Life technologies, Grand Island, NY, USA]), and incubated at 37 ºC in a controlled humidified atmosphere with 5% ± 0.5% CO_2. Washes were performed with phosphate buffer saline (PBS, pH 7.2) 1X (GIBCO by Life technologies,

Grand Island, NY, USA), after removing medium from the container. Cell count was performed using trypan blue (0.4%. Sigma-Aldrich, St Louis, MO, USA), a Neubauer chamber and an optic microscope (Zeiss Primo star, Gottingen, Germany) as proposed by the ATCC's standard protocols, or with and automatic cell counter (Beckman coulter, Vi-CELL XR cell counter, Indianapolis, USA).

8.4.Cell death induction and inhibition, analysis by flow cytometry

Annexin-V-APC (0.1 µg/mL; BD Biosciences, USA) was used for the assessment of phosphatidylserine exposure, propidium iodide (PI, 0.5 µg/mL. Sigma-Aldrich, Eugene MO, USA) for cell viability analysis. Cell death was recorded in a BD Accury C6 (BD Biosciences, USA) in the total population (10,000 cells), and data were analyzed using FlowJo software (Ashland Oregon, USA). To induce cell death, $1x10^6$ cells/mL were treated for 2 h with PKHB1 (200 µM) and as control for caspase-dependent apoptosis, cells were incubated for 24 h with etoposide (200 µM, Enzo life science). For the inhibition assays, calcium chelator, BAPTA (5 mM, CalbioChem, Merck, Billerica MA, USA); or the broad-spectrum caspase inhibitor Q-VD-OPh (QVD, 10 µM, BioVision, Milpitas CA, USA); were added 30 min before inducing cell death.

8.5.Calreticulin exposure

To quantify the percentage of calreticulin located at the cell surface, flow cytometry was used. In a 96 well plates were plated 1 x 10^6 cells / mL, treated with PKHB1 (200 µM) and incubated for 2h. The cells were harvested, washed with PBS1X and stained with Calreticulin-PE (BD) antibody (1:1000) in FACS buffer (PBS 1X + 2% FCS). The staining was incubated for 1h in dark at room temperature. Then, the cells were washed with PBS 1X by centrifugation at 1600 rpm for 5min and resuspended in 100 µL of FACS buffer and calreticulin exposure was recorded in a BD Accury C6 (BD Biosciences, USA) in the total population (10,000 cells), and data were analyzed using FlowJo software (Ashland Oregon, USA). Also, to observe the CRT on cell surface, a confocal microscopy was used. First, poly L- lysine was added to sterile coverslips placed inside the 6-well plates, one day after, the poly – L- lysine was washed with PBS1X and $1x10^6$ cells / mL, were seed it. The treatment

was added (PKHB1 200 µM) and incubated for 2h. Then, the cells were stained with Calreticulin-PE (BD) antibody (1:500) and incubated 1h, and additionally the Hoechst staining was added.

8.6.Western Blot

In a 6-well plate, 1 x 10^6 cells / mL were seeded in a serum-free culture medium and treated with PKHB1 (200 µM) or left alone (Control) for 2 h. Every supernatant was recovered into a sterile tube, also, total protein was isolated from the cellular pellet using 100 µL of lysis buffer (Set 2x buffer; 20mM Tris pH 6.8, 2mM de EDTA, 300mM de NaCl and SDS 2%). Concentration from both was measured with the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA) and 50µg of protein was loaded in linear SDS-PAGE gels (12%). After blotting, nitrocellulose filters were probed with primary antibodies against HMGB1 (HAP46.5: sc-56698), HSP70 (C92F3A-5: sc-66048), HSP90 (F-8: SC-13119) and Calreticulin (F-4: sc373863). Anti-mouse or anti-rabbit coupled to horseradish peroxidase served as secondary antibody (Santa Cruz Biotechnology, California, USA). Visualization was performed with ECL substrate system (Thermo Scientific).

8.7.ATP release assay

Leukemia cells (1 x 10^6 cells / mL) were seeded in serum-free culture medium and treated by PKHB1 (200µM) for 2h. Supernatant was transferred into sterile tubes (Eppendorf Biopur, Westbury, NY) and extracellular ATP was immediately measured by a luciferase assay (ENLITEN kit, Promega) following the instructions by the manufacturer. Bioluminescence was assessed by optical top reading in a microplate reader (Synergy HT, BioTek, Software Gen5, Winooski, VT, USA) at 560 nm using a white polystyrene 96 well (Corning Incorporated, NY, USA).

8.8.HMGB1 release assay

To determine HMGB1 concentration in the supernatant of untreated and treated cells with PKHB1 (CC_{50} and CC_{100} for each cell line), leukemia cells (1 x 10^6 cells / mL) were seeded in serum-free culture medium. Supernatant was transferred into sterile tubes (Eppendorf

Biopur, Westbury, NY) and extracellular HMGB1 was immediately measured using the HMGB1 ELISA kit (BioAssay ELISA kit Human) for CEM and MOLT-4 cell lines and HMGB1 ELISA kit (BioAssay ELISA kit Mouse) for L5178Y-R cell line, following the instructions by the manufacturer. Absorbance was assessed by optical top reading in a microplate reader (Synergy HT, BioTek, Software Gen5, Winooski, VT, USA) at 450 nm.

8.9.Animals

The protocol for the animal study was approved by the Animal Ethical Committee (CEIBA) of Universidad Autonoma de Nuevo León, School of Biological Science, number: 01/2015. Also, all experiments were conducted according with the Mexican law NOM-062-ZOO-1999. Six-to-eight-week-old BALB/c female mice were obtained and housed at the bioterium of Immunology and Virology Department, School of Biological Sciences. The mice were maintained in controlled environmental conditions (25ºC and a 12 h light/dark cycle) and were fed with rodent food (Science diet) and water *ad libitum*. Animals were organized in three groups with thirteen mice each one, were performed:

1. Control: Tumor without treatment, injected *intra-peritoneally (i.p.)* with saline solution.
2. PKHB1: Tumor treatment with peptide (200µg/200µL), injected *i.p.* once a week
3. Healthy: Without tumor and treatment.

8.9.1. Establishment of the tumor in BALB / c mice

The tumor was established by the subcutaneous injection of $1x10^6$ L5178Y-R cells in 100 µL of PBS, into the left hind of the leg. The cell count for the preparation of the injections was carried out by the trypan blue exclusion method.

8.9.2. Scheme of treatment and tumor index

Tumor volume and weight were measured three times per week using a caliper (Digimatic Caliper Mitutoyo Corporation, Japan) and a digital scale (American Weigh Scale-600-BLK, USA), respectively. When the tumor reached 100 mm^3, the first PKHB1 injection (200µg/200µL) was applied (day 0), following the scheme showed in table 8

.

Table 8. Diagram of treatment and tumor measurement *in vivo*

Control (without treatment; 200µL saline solution)	**PKHB1 peptide** (200µg/200µL)	**Healthy** (without tumor and treatment)
13 mice	13 mice	6 mice
Days of treatment ***Once at week (7,14,21,28,35 days)*** **and measuring the volume and weight of tumor three times per week**		
Day 42: Necropsy; Extraction and measurement of tumor		

The tumor volume was estimated according to the following formula: tumor volume (mm^3) = $4\pi / 3 * A * B * C$. Also, final tumor weight was assessed, after mice sacrifice. The data obtained was graphed and subjected to a statistical analysis, obtaining the significant difference between the groups.

8.9.3. Survival assessment

Mice behavior was monitored throughout the experiment, survival was also evaluated while the treatment lasted. Mice were sacrificed when tumors reached a size of $2cm^3$, and stablished humane endpoint in guidelines for ethical treatment of mice and rats in cancer research (Workman et al., 2010). The Survival over time was represented by the Keplan-Meier graph.

8.9.4. Spleen, Thymus, Lymph Nodes, and Bone Marrow cells extraction

Spleen, thymus, lymphatic node, and bone marrow cells were obtained from female Balb/c mice post-sacrifice. Spleen cells were obtained through perfusion using a syringe with 10mL of PBS. Thymocytes and lymph node cells were obtained by maceration, recovering the cells into a 15mL tube with 10mL of PBS. Bone morrow cells were obtained from only one femur and tibia per mouse, cells were flushed with 5mL PBS into a 15mL centrifuge tube. Every cell suspension was centrifuged at 1,600 rpm for 10 min, washed twice with PBS and counted using trypan blue staining.

8.9.5. Blood and PBMCs isolation

Blood was obtained by cardiac puncture of sacrificed mice, and separated in two microcentrifuge tubes, one to perform Complete Blood Count and other for PBMC isolation, which was performed by density gradient centrifugation using Ficoll-Hypaque-1119 (Sigma-Aldrich, St Louis, MO, USA). Also, human PBMCs were obtained from total blood of healthy donors, blood was collected by venipuncture. Briefly heparinized blood was diluted in Phosphate Buffered Saline 1X at equal volume (1:1), then diluted blood was carefully placed over Ficoll-Hypaque (1:2), followed by centrifugation at 1,600rpm for 30 minutes without acceleration and brake. After centrifugation, the white blood cells were collected with a 5mL serological pipette, washed and counted. $4x10^5$ cells were seeded in a 96 well plate with RPMI medium to performed cell death induction or isolation of CD4+/CD8+ through primary antibody (CD4; MT310 sc-19641 y CD8; 32-M4 sc-1177) and cell death induction was measured by Annexin-V-APC. In brief, after PBMCs insolation cells were seeded ($4x10^6$/mL) into 96-well plates, and then treated with PKHB1 during 1.5h. Then 1 µL of antibodies against CD4 or CD8 (1:100) was added and incubated for 30min. After this, the cells were collected, washed and centrifuged at 1600rpm 10min. Finally, the secondary antibody-FICT (1:100) and Ann-V-APC were added in Annexin binding buffer and incubated 30min at 4°C and cell death was recorded in a BD Accuri C6 (BD Biosciences, USA) in the total population (10,000 cells).

8.9.6. Completed Blood Count (CBC)

The heparinized blood collected from mice, was used to perform a CBC. The blood was sent to a specialized veterinary laboratory for analysis, using the Automatic Hematology Analyzer (KontroLab, Rome, Italy). Blood smears was performed placing a drop of blood on one end of a slide and using another slide held at 45° angle as spreader, pushed forward rapidly and smoothly to obtain a thin layer of blood on the slide. Then blood smears were fixed with methanol (Química Meyer, México) and stained with Wright's (Hycel, Mexico, D.F.), and were observed under the microscope, to perform differential blood white cells counts.

8.9.7. Prophylactic vaccinations

L5178Y-R cells (1.5, 3 or 5 x10 ^{6}) were treated with 300µM of PKHB1 (CI $_{100}$) for 2 h Cell death was confirmed using trypan blue staining and flow cytometry. Treated cells were inoculated subcutaneously in 100 µl of PBS into 8-week-old female BALB/c mice into the left hind of the leg, whereas 2 x 10^{6} untreated control cells were inoculated into the right hind 7 days later. Tumor volume was measured as mentioned previously and animals were sacrificed upon appearance of distress, tumor ulcerations or bearing tumors in excess of 20–25% of the body mass.

8.9.8. Long-term immunological memory assays

Mice which tumor were induced as described previously and with complete tumor regression after PKHB1 administration, were re-challenged with 2 x 10 ^{6} cells in 100 µL of PBS, injected into the opposite limb, and tumor volume was measured like described for the *in vivo* model.

8.9.9. Histology and immunohistochemistry

Tissues and organs were obtained from the different groups of mice and fixed in 10% neutral formalin, processed routinely, embedded in paraffin and sectioning (5µm thickness) and stained with H&E (Sigma- Aldrich, St Louis, MO, USA). Histopathological analysis was done by a veterinarian pathologist (Professional certificate 2593012). Also, immunohistochemistry was done using standard procedures. Slides were deparaffinized using a bath water at 60ºC for 10min, and the tissue was hydrated through xylene (10 min), 100%, 96%, 70, 50% Alcohol (5 min each). Then the slides were blocked using 3% H_2O_2/methanol solution (1:9) for 10min to block endogenous peroxidase activity, washed twice with PBS 1X for 7min and 100µL blocking buffer (10% Bovine Serum Albumin in PBS 1X) was added; this was done in a humidified chamber at room temperature for 45min. Then, samples were washed twice with PBS for 7min and slices were incubated with 100µL of the appropriate primary antibody (CD4;MT310 sc-19641 y CD8; 32-M4 sc-1177), overnight at 4ºC. The next day slices were washed with PBS 1X and the biotinylated secondary antibody was added for 4 hours into a humidified chamber at room temperature. Slices were then washed twice with PBS and diaminobenzidine was applied for 3 min and

the reaction was stopped washing with H_20, and the slides were counterstain using hematoxylin for 2 min and wash. Finally, the slides have to be dehydrated through 6 changes of water, 50%, 70%, 96%, 100% alcohol and xylene 5min each, then were coverslipped using resin as mounting solution and observed under the microscope.

8.10. RNA isolation, cDNA synthesis, and PCR amplification

RNA extraction was prepared from murine splenocytes, thymocytes, lymph nodes, bone marrow and L5178Y-R murine lymphoma cell line and leukemic cell lines, using TRIzol Reagent (Invitrogen by life technologies, Carlsbad CA, USA). cDNA was prepared from RNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City CA, USA) for RT-PCR. RT-PCR was performed using a murine CD47 primers; obtained from Lamy et al. (2007); Forward: 5 -GTTCAGCTCAAC TACTGT-3 and Reverse 5-CTCTTATTCGTATGGCTG-3 with an expected PCR-product size of 535pb. The primers used for Human CD47 were forward 5-GATCAGCTCAGCTACTAT-3 and Reverse 5 -ACAATGACAG TGATCACT-3) with an expected PCR-product size of 523pb. Both for 35 cycles (denaturation at 95°C for 40s, annealing at 52°C for 30s, and extension at 72°C for 40s). Actin (430pb) was used as loading control.

8.11. Cell viability assay: MTT

Viability was determined using the tetrazolium, 3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MO, USA) assay. In a 96-well plate, $1x10^6$ cells/mL were seeded and treated with PKHB1 (200µM) for 2h. 20 µL of MTT (5 mg/mL, dissolved in PBS) per sample were added to the 96-well plate, and incubated for 50 min at 37 °C. The plate was centrifuged for 15 minutes at 1600rpm and supernatant was removed by decanting the plate on paper without hitting the plate to prevent salt loss. 100µL Dimethyl Sulfoxide (DMSO, Bio Basic Canada Inc., Ontario, Canada) were added per well, then incubated for 10min at room temperature and the analyzed at 570nm in a microplate reader (Synergy HT, BioTek, Software Gen5, Winooski, VT, USA.) to obtain the absorbance before determining the percentage of viability. Assays were performed in triplicate and plotted including standard deviation.

8.12. Statistical Analysis

Data were analyzed using t-test (parametric) and Mann-Whitney tests (non-parametric) performed in GraphPad Prism statistical software (San Diego CA, USA). Mice were assigned at random to treatment groups for all mouse studies and, where possible, mixed among cages. There were no mice excluded from experiments. Experiments were repeated at least three times. *P* values considered significant as follows: $p < 0.05$; $p < 0.01$ and $p < 0.001$.

IX. RESULTS

9.1.Expression of CD47 mRNA in the cell lines tested.

CD47 is a ubiquitously expressed protein, however, we confirmed its expression in different human leukemic cell lines and PBMCs, as well as in mouse lymphoid organs and in a murine T cell lymphoblastic tumor cell line (L5178Y-R) which was used in the *in vivo* model. Figure 11 shows that CD47 mRNA is consistently expressed in all the cell lines. Using housekeeping genes as controls, it could be observed that all the human cell lines as well as human PBMCs, the murine cell line L5178Y-R and mouse lymphoid organs cells express similar CD47 mRNA levels.

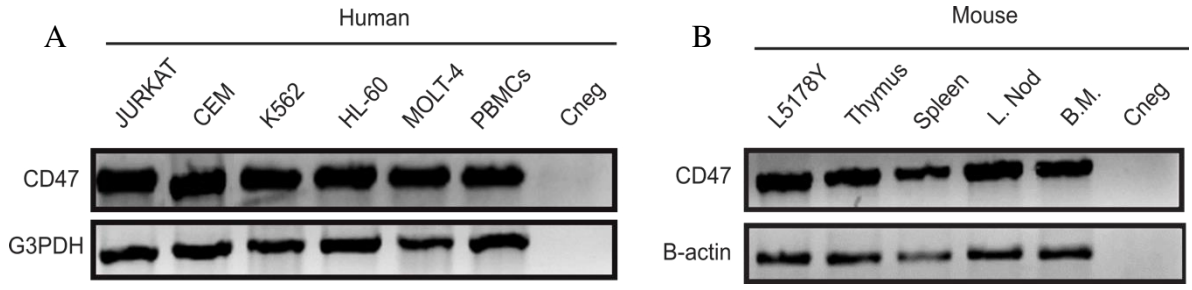

Figure. 11. CD47 mRNA expression in cell lines and mouse organs. RT-PCR was performed in the human leukemic cell lines and human PBMCs **A**. as well as **B.** in mouse lymphoid organs, using mRNA (5µg/µL). G3PDH and β-actin were used as housekeeping genes.

9.2.Cell death induction by PKHB1 and 4N1K in different types of leukemia cell lines.

Once confirmed that the cells that we were going to use express CD47 the next step was to test the cytotoxicity of the CD47 agonist peptides 4N1K and PKHB1 in a panel of leukemic cells. Cell death was measured by tracking the phosphatidylserine (PS) exposure with Ann-V and the plasma membrane permeabilization (PMP) with PI. The cells were incubated for 2 h with crescent concentrations of the peptides (100, 200 and 300 µM). Figure 12 shows that the cell death is increased in a concentration-dependent manner in every cell line tested. Also, the chart displayed that PKHB1 is more effective to kill neoplastic cells than 4N1K. The concentration of PKHB1 necessary to induce cell death in around 50% of the cells (half cytotoxic concentration, CC_{50}) was 200 µM in MEC-1 (59%±3), JURKAT (56%±5), CEM

(46%±6), K562 (53%±3) and HL-60 (50%±5), but 150 µM in L5178Y-R (52%±5), and 300 µM in MOLT-4 (47%±5), while 4N1K is needed in higher concentrations (300 µM) to harvest similar results in MEC-1 (55%±4), JURKAT (47%±4) CEM (44%±12), MOLT-4 (40%±5), K562 (53%±4) and HL-60 (50%±7), while in L5178Y-R, 200 µM 4N1K induces the 54%±2 cell death. The murine cell line resulted more sensitive to both peptides, being CC_{50} 150 µM PKHB1 and 200 µM 4N1K, while MOLT-4 resulted more resistant with a CC_{50} of 300 µM to PKHB1 and 4N1K.

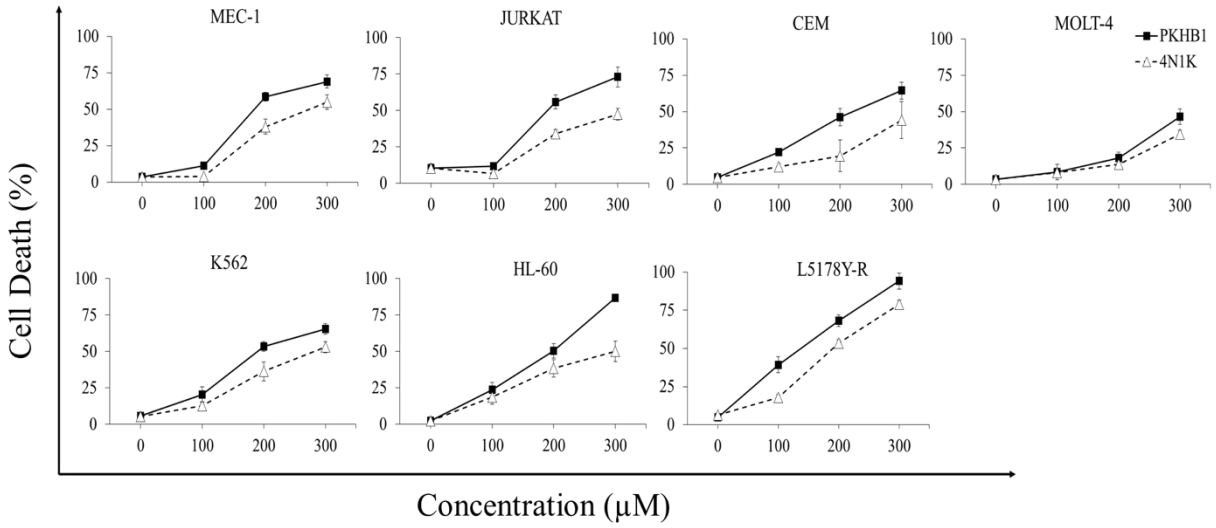

Figure 12. CD47 agonist peptides induce cell death in different types of leukemia. Cell death was measured by the amount of cells with Ann-V^+/PI^+ staining at different concentrations of 4N1K or PKHB1 and represented in line charts as the means (± SD) of triplicates of at least three independent experiments.

Because PKHB1 resulted in a low dose to induce cell death, the further experiments were performed with PKHB1.

9.3.Evaluation of PKHB1 selectivity to induce cell death *in vitro*.

The cytotoxic effect of PKHB1 is evident in neoplastic cells, therefore, the next step was to determine whether PKHB1 affects non-neoplastic cells. Figure 13 A shows representative dot plots of Ann-V/PI-stained MEC-1, JURKAT, CEM, MOLT-4, K562, HL-60, L5178Y-R cells, human and murine PBMCs treated with increasing PKHB1 concentrations. It can be observed that the number of cancer cells with double positive staining increases when

PKHB1 concentration augments, while these characteristics of cell death (PS exposure and PMP) are evidently diminished in both human and murine PBMCs. In fact, these results were observed in triplicates of at least three independent experiments, and are graphed in Figure 13 B.

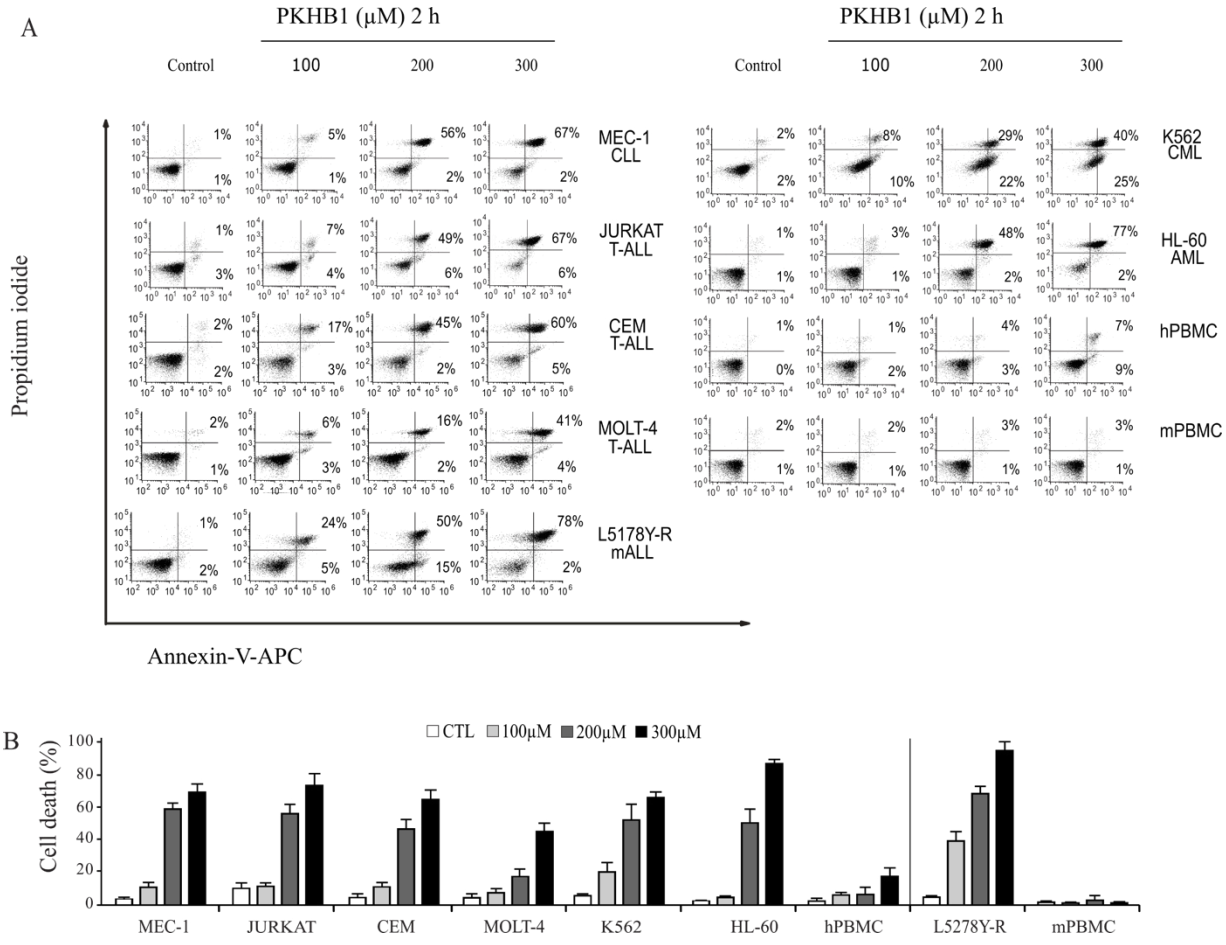

Figure 13. PKHB1 induces cell death on leukemia cell line panel, but not on healthy cells. A. Cell death was measured by Annexin-V-APC (right quadrant) and PI (uper quadrants) staining. A representative dot plot of MEC-1, Jurkat, CEM, MOLT-4, HL-60, K562 human leukemia cells, and L5178Y murine cell line, and human PBMCs and murine PBMC without treatment (Control) and treated with 100, 200 and 300 μM PKHB1 for 2 h. **B.** Cell death is quantified by the amount of Ann-V^+/PI^+ staining at different concentrations of PKHB1 and represented in graph as the means (± SD) of triplicates of at least three independent experiments. The statistic was performed using *t* student test.

Moreover, CD4+ and CD8+ human T cells were obtained from healthy donors, treated with PKHB1 and cell death was measured with Ann-V/PI staining. The percentage of both T cell types in each donor does not change after treatment with PKHB1, as depicted in Figure 14 A. In addition, PKHB1 did not induce cell death in CD4+ and CD8+ T cells, as shown in Figure 14 B. These results denoted the selectivity of PKHB1 to induce cell death just in malignant cells.

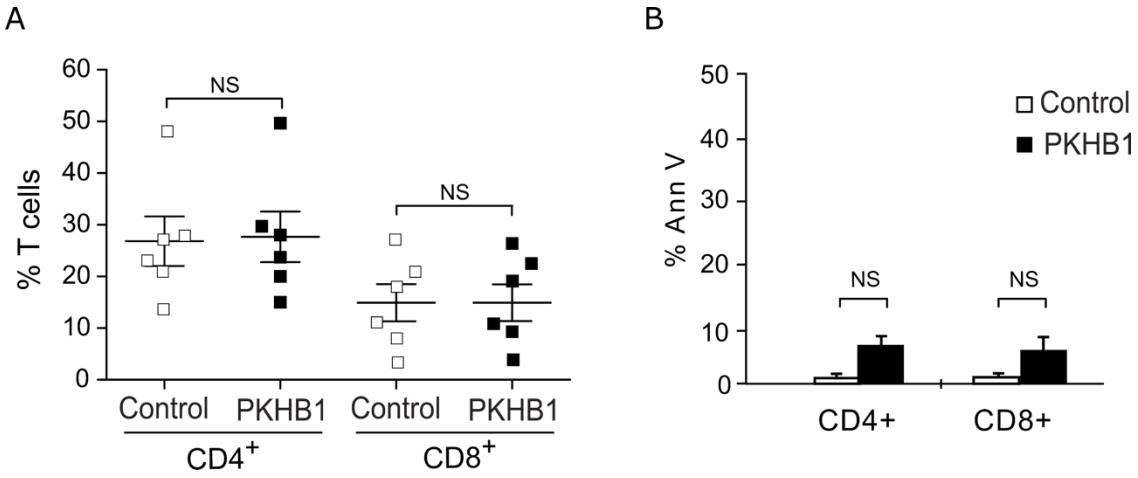

Figure 14. PKHB1 does not induce cell death on CD4+ and CD8+ T cells. A. Percentage of CD4+, CD8+ T cells from each donor, without treatment and treated with PKHB1. **B**. Cell death was measured by Annexin-V-APC and PI staining and represented in graph as the means (± SD) of triplicates of at least three independent experiments. The statistic was performed using *t* student test.

9.4.Determination of caspase and calcium dependence in cell death induced by PKHB1 on different types of leukemia cells

The knowledge and evidence that indicated the ability of PKHB1 to induce a quick and selective cell death with characteristics such as phosphatidylserine exposure and plasma membrane permeabilization not only in CLL (Martínez-Torres, et al. 2015; Denèfle, et al. 2016) but also on different types of leukemia cells led us to think that PKHB1-induced cell death in other types of leukemia cells might also share similar biochemical features with CLL. Since caspase independence is a recurrent feature of CD47-dependent cell death (Oldenborg, 2013) and one of the major pathways regulated by CD47 signaling include calcium homeostasis (Soto-Pantoja, 2015), and PKHB1-induced killing of CLL cells has been shown to depend on a sustained calcium influx (Martínez-Torres, et al. 2015), we tested

the ability of PKHB1 to induce death in cells pre-incubated with a pan-caspase inhibitor (Q-VD-OPH) or an extracellular Ca^{2+} chelator (BAPTA). As seen in Figure 15, caspase inhibition did not prevent PKHB1 to kill MEC-1 (59% ±3 to 55% ±4), JURKAT (51% ±3 to 48% ±5), CEM (51% ±4 to 48% ±5), MOLT-4 (57% ±4 to 51% ±6), K562 (53% ±3 to 52% ±4), HL-60 (47% ±7 to 45% ±3), or L5178Y-R (52% ±5 to 49% ±3) cells; nevertheless, extracellular calcium chelation significantly reduced PKHB1-induced cell death in all cases: MEC-1 (59% ±3 to 18% ±5), JURKAT (51% ±3 to 19% ±4), CEM (51% ±4 to 18% ±11), MOLT-4 (57% ±4 to 38% ±3), K562 (53% ±3 to 24% ±14), HL-60 (47% ±7 to 24% ±8), and L5178Y-R (52% ±5 to 21% ±8).

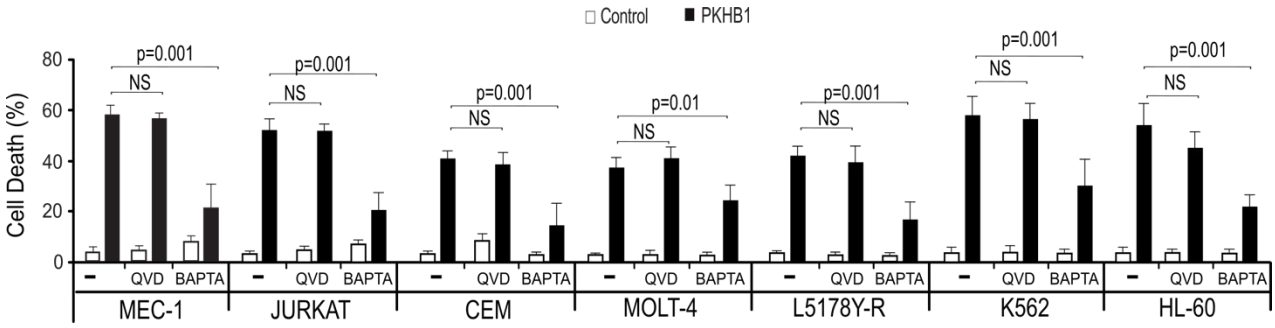

Figure 15. PKHB1 induces caspase-independent but calcium-dependent cell death on leukemia cell lines. Graph represents cell death percentage of T-ALL cells without treatment (Control) or treated with PKHB1 (200µM, 2 h) and left alone (-) or pre-incubated for 30min with QVD or Ca^{2+} chelator BAPTA in the different cell lines tested. The data represent the means (± SD) of triplicates of at least three independent experiments. The statistic was performed using *t* student test.

9.5.*In vivo* effect of PKHB1 in immunocompetent mice model.

Since, it was verified *in vitro* that PKHB1 induces RCD in the different types of leukemic cells, even in the murine T cell lymphoblastic tumor cell line (L5178Y-R), the next objective of this project was to scale up the *in vivo* the effects of PKHB1 in an immunocompetent murine model. Therefore, in order to carry out experimentation with animals, the cytotoxicity of PKHB1 in murine cells was tested.

First, indirect cell viability analysis was performed on primary cultures of cells coming from bone marrow (BM), spleen, thymus and lymph nodes of healthy BALB/c mice. As seen in

Figure 16, PKHB1 treatment did not reduce significantly cell viability of the cells assessed. Moreover, cell viability in bone marrow cells augmented. The average of the percentages for the different primary cultures were: BM (119% ± 0.2), spleen (79% ± 0.1), thymus (98% ± 0.1) and lymph nodes (92% ± 0.07).

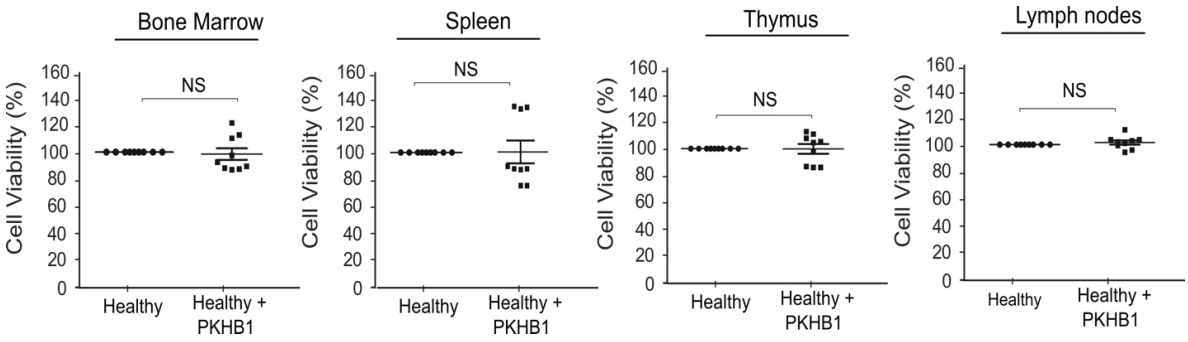

Figure 16. PKHB1 does not affect cell viability of lymphoid organs in immunocompetent mice. Cell viability of bone marrow, spleen, thymus and lymph nodes from healthy mice (without tumor) measured by MTT assay (n=9). The statistic was performed using Mann Whitney Test.

Second, healthy mice were injected with 200µg of PKHB1 dissolved in 200µL injectable water and weight of the whole mice (Figure 17A), vital organs (Figure 17B) and lymphoid organs (Figure 17C) and the number of lymphoid cells (Figure 17D) were compared between healthy mice without treatment (Control) and healthy mice with PKHB1 (PKHB1). The results did not show significant differences between total weight of control and PKHB1-treated mice (Figure 17A). Also, no differences were found in heart, lungs, liver and kidneys weight between control and PKHB1-treated mice (Figure 17B). Similarly, although the graph shows an increase in the spleen weight and a decrease in the thymus weight of PKHB1-treated mice compared with the control, statistically there is no significant difference in the weight of the lymphoid organs (Figure 17C). In the same way, there is no significant difference in the number of cells coming from the different lymphoid organs, although in the graph there is an increase in the cell number from the spleen (Figure 17D). On the other hand, a complete blood count was performed to determine if there are differences in red blood cells (RBC), white blood cells (WBC) and platelets between control and PKHB1-treated mice. Table 9 specified that there are no significant differences in RBC, WBC or platelet between controls and mice treated with PKHB1.

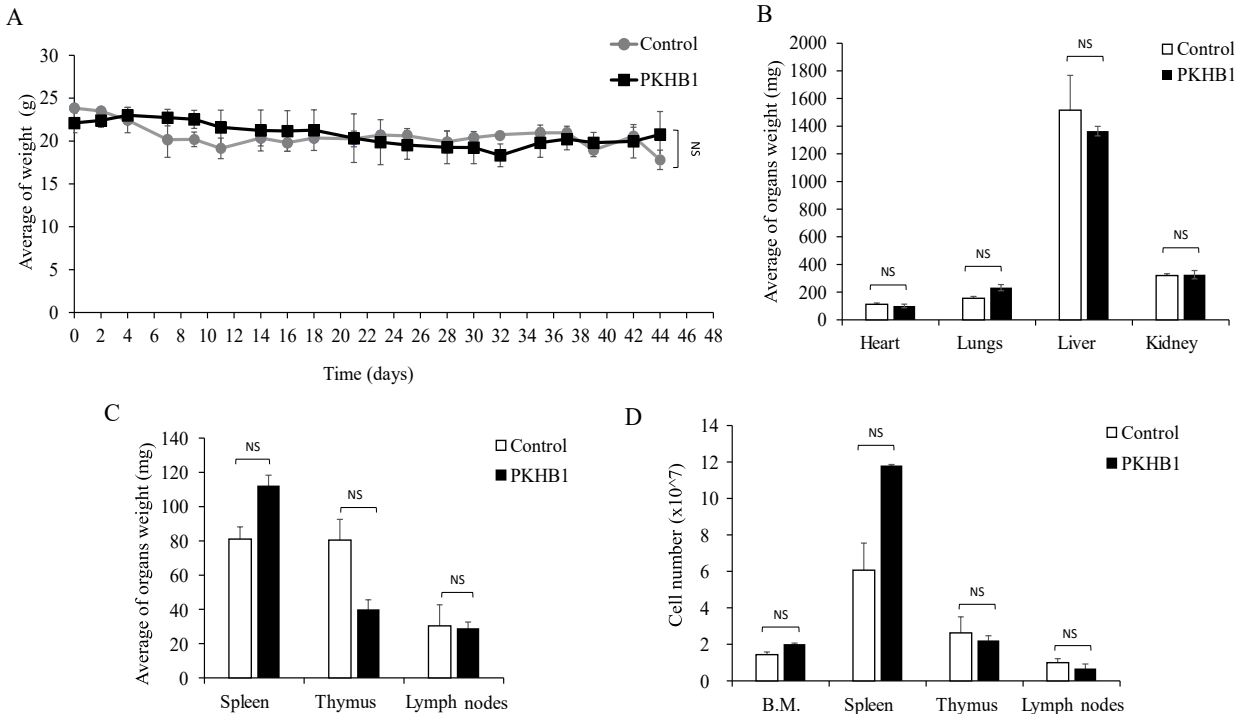

Figure 17. PKHB1 treatment does not affect total weight, organs weight, neither affect cellularity of lymphoid organs in immunocompetent mice. A. the average of the weights of healthy mice, without tumor (Control) and healthy mice injected with PKHB1 (PKHB1) were measured throughout the time (days) and graphed. **B.** vital organs like heart, lung, liver and kidney and **C**. lymphoid organs like spleen, thymus and lymph nodes were obtained from both groups and weights were measured. **D.** Cell number of bone marrow, spleen, thymus and lymph nodes from Control and PKHB1 groups were counted using trypan blue staining and microscopy (Control n=3; PKHB1 n=3). The statistical analysis was performed using Mann Whitney Test.

Table 9. Comparison between Complete blood count (CBC) of Control and PKHB1 groups.

Parameter	Unit	Control	PKHB1
Erytrhocytes	x 10^6/µL	5.3(±0.91)	5.3(±0.45)
Hemoglobin	gm/dL	10.7(±1.31)	10.7(±0.45)
Hematocrit	%L/L	0.3(±0.06)	0.3(±0.02)
MCV	ftL/cell	62.7(±2.31)	61.3(±1.70)
MCH	pg/cell	20.5(±0.95)	20.0(±1.53)
MCHC	gm/dL	32.7(±1.17)	32.7(±1.56)
Platelet	x 10^9/L	387.3(±105)	277.7(±90.8)
Leukocytes	x 10^3/µL	3.9(±0.44)	5.6(±1.25)
Neutrophils	%	14.7(±9.24)	33.0(±12.8)
Segmented neutrophils	%	13.0(±7.81)	30.0(±11.36)
Neutrophil band	%	1.7(±1.53)	3.0(±2.7)
Eosinophils	%	3.0(±1)	0.3(±.58)
Basophils	%	0.0(±0)	0.0(±0)
Monocytes	%	0.3(±0.58)	0.7(±1.15)
Lymphocytes	%	80.0(±5.29)	66.0(±11.14)

MCV=Mean corpuscular volume; MCH= Mean corpuscular hemoglobin; MCHC= Mean corpuscular hemoglobin concentration.

9.6.Assessment of volume tumor, tumor weight and survival of mice treated with PKHB1.

As PKHB1 was innocuous in non-neoplastic cells derived from BALB/c mice, the *in vivo* experimentation on its anti-tumor activity was initiated. The effects of PKHB1 were evaluated in BALB/c mice with syngeneic tumor of L5178Y-R cells. Mice subjected to weekly intraperitoneal (i.p.) administrations of PKHB1 (200µg/200µL) showed an important reduction in volume since the second application, compared with untreated mice (Figure 18A). Moreover, PKHB1 administration resulted in complete regression of the tumor in most of the cases after the 6th dose. This tumor regression was reflected in a significant reduction in tumor weight from 4.54g ±0.92 to 0.39g ±0.88, control and PKHB1-treated, respectively (Figure 18B). Reduction of tumor prompted by PKHB1 increased the survival of mice, as reflected in the Kaplan-Meier survival curve in Figure 15 C, that evinces an 80% of survival at day 44, whereas at day 21 all the untreated mice had perished. The mice that showed complete tumor regression were maintained for further analysis.

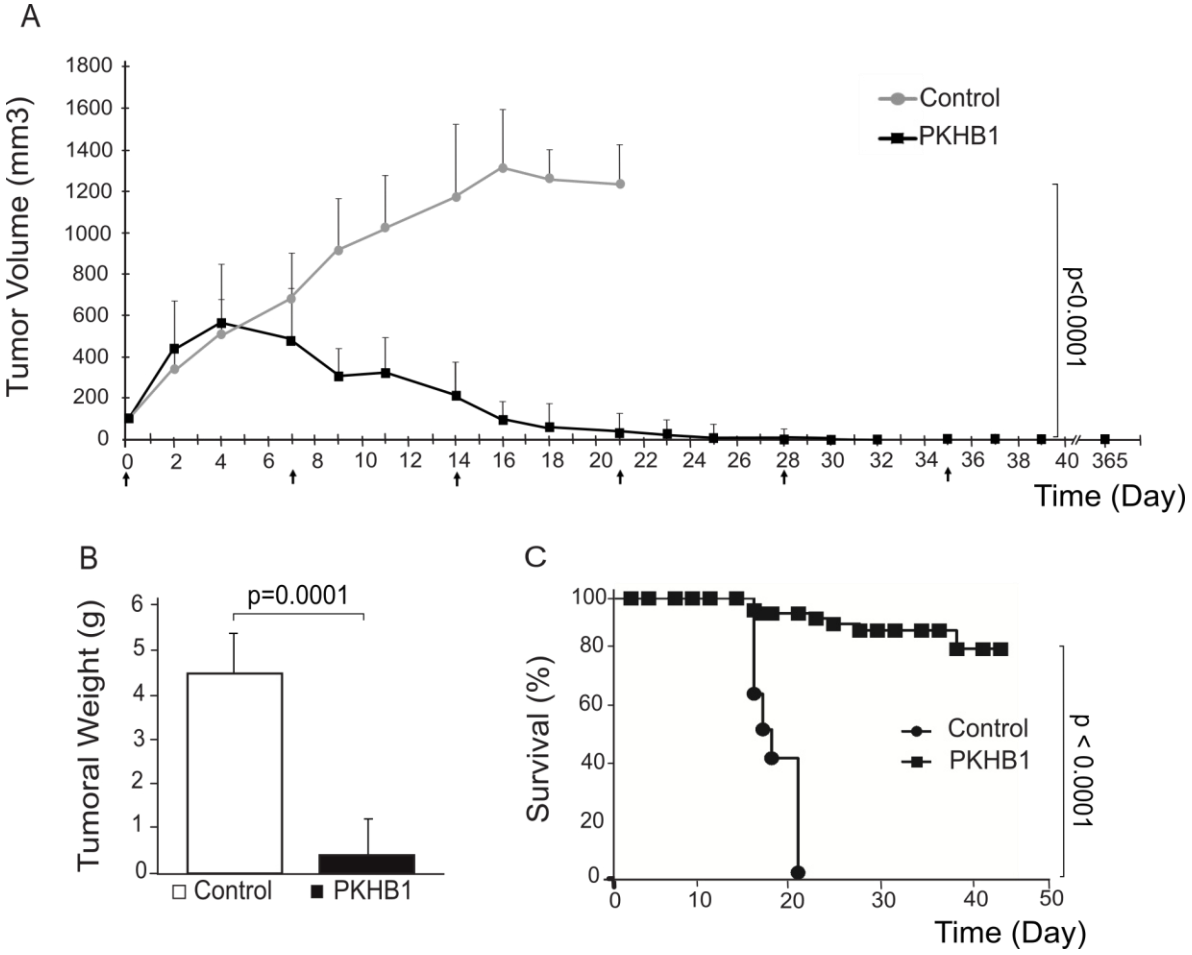

Figure 18. PKHB1 treatment decreases tumor volume and weight, increasing the survival of immunocompetent mice. A. On the graph, the mean (± SD) of the tumor volume (mm^3) through the time (days) of the untreated (Control; n = 13) group and that treated with PKHB1 (200 µg / 200 µL; n = 13). The arrows indicate injections of PKHB1. Tumor volume was calculated as follows V = 4π / 3 * A * B* C where 4π / 3 is a mathematical constant, A= width, B= high, and C= depth. **B.** Mean (± SD) plot of net tumor weight excised from control or PKHB1-treated mice. **C**. Survival over time is represented by the Kaplan-Meier graph.

9.7.Evaluation of PKHB1 effect on blood count, lymphoid organs and vital organs.

After the experimentation period, mice were sacrificed, and blood samples and histological sections from lymphoid and vital organs from both groups (control and PKHB1-treated mice) were obtained to determine PKHB1 toxicity *in vivo.* Histology slides of lung, hearth, kidney, and liver as well as lymphoid organs, did not show any evidence of alteration in PKHB1-treated mice, moreover, metastasis to liver was prevented in them as it is shown in Figure 19A. Besides, cell counts from lymphoid organs that belonged to control mice, PKHB1-treated mice or healthy mice were performed by the trypan blue exclusion technique using

an automatic cell counter (Figure 19B). Cell number in BM, spleen and thymus coming from PKHB1-treated mice was significantly higher than those coming from control mice, and significantly lower in lymph nodes. Conversely, cell number of PKHB1-treated mice was more similar to that of healthy mice, in fact there was no significant difference between cell number of healthy and PKHB1-treated mice in BM and spleen.

Additionally, complete blood count (CBC) tests were performed at day 18 and are depicted in Figures 19C-E. The CBC shows that tumor-bearing mice (both untreated and treated with PKHB1) presented significantly lower number of erythrocytes, compared with healthy mice (Figure 19C) but there was not a significant difference between the number of platelets in any of the three groups (Figure 19D). Nonetheless, the WBC differential analysis shows no significant difference between healthy mice and those treated with PKHB1, whereas untreated tumor mice presented a significant difference from the other two groups in all leukocyte types (Figure 19E).

Altogether, these results reaffirm PKHB1's innocuity in immunocompetent mice and indicate that the immune system is left unaffected by the treatment.

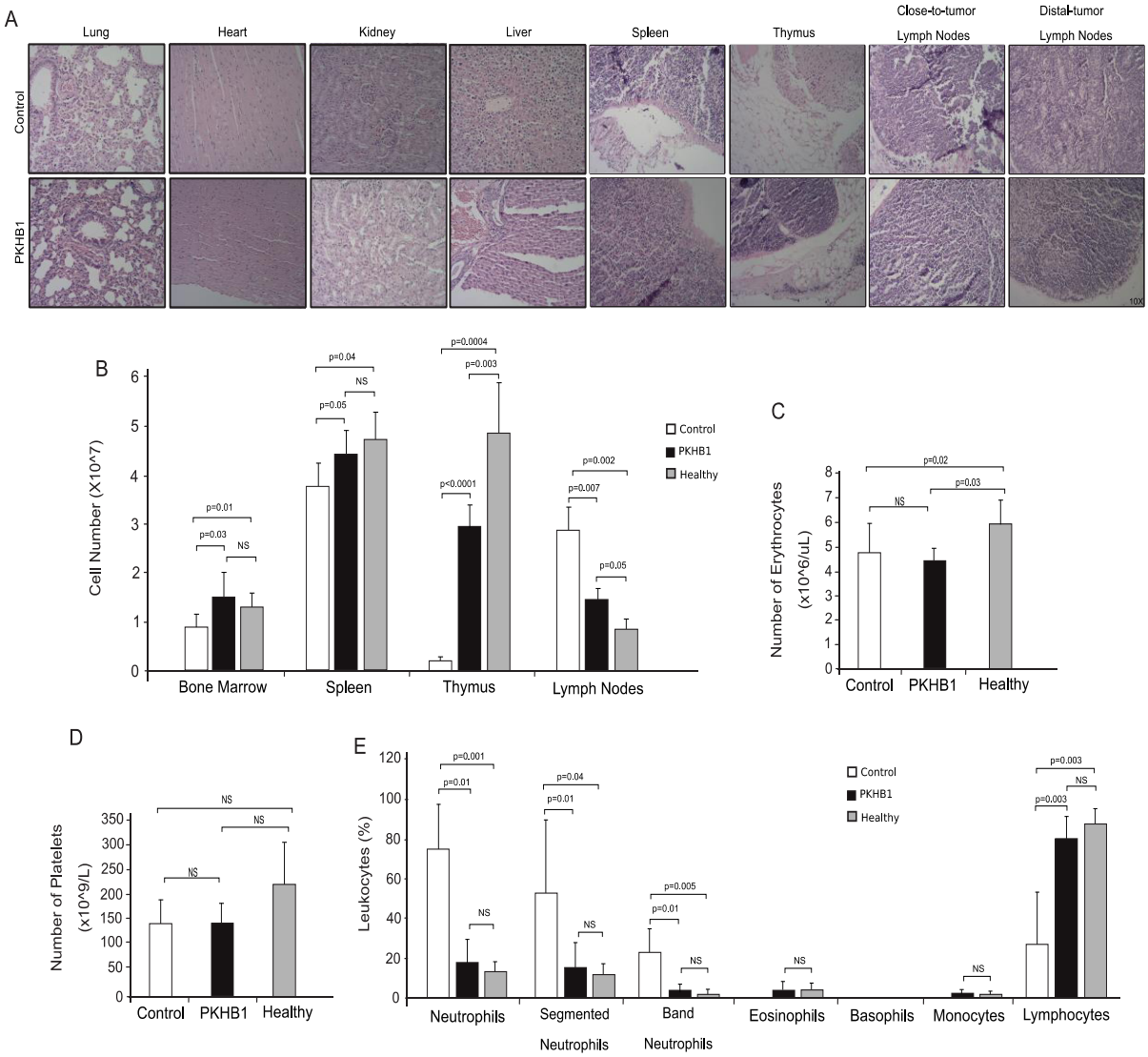

Figure 19. PKHB1 does not affect vital nor lymphoid organs and improves cell blood counts in immunocompetent mice. A. Histology of the different vital organs analyzed, stained with H & E. The control group without treatment presents metastasis to the liver. **B.** Cell count of lymphoid organs (bone marrow, spleen, thymus and lymph node) from mice with tumor without treatment (Control), mice with tumor treated with PKHB1 (200μM, 2h) or mice without tumor nor treatment (healthy) was performed using trypan blue staining (n=6). **C.** Number of erythrocytes. **D.** Number of platelets from mice with tumor (control), PKHB1-treated and healthy. **E.** Different types of leukocytes are displayed in the graph, which represents the mean (± SD) of the percentage of cells obtained using hematic biometry analysis.

9.8.Evaluation of tumor tissue after treatment with PKHB1

Having determined that PKHB1 was able to induce complete tumor regression in an immunocompetent model without damaging lymphoid or vital tissue and improved CBC compared to untreated mice, the histological sections of tumors from control and PKHB1-treated mice were also obtained and analyzed. The morphological and cellular changes on the tumors are depicted in Figure 20, where it is appreciated that untreated group presented undifferentiated lymphoid cells, presumably L5178Y-R cells, some of them performing mitosis, while in PKHB1-treated mice, tumors are a mixture of lymphocytes and polymorphonuclear cells (PMN) both evaluated on day 18 after having started the experiment. Additionally, at day 30 (after every control mouse had perished and when complete tumor regression was reached) the histological slides of tumors from PKHB1-treated mice showed what seems to be an anti-tumor immune response. Figure 20 displays a granuloma of necrotic cells surrounded by giant cells was found where the tumor was inoculated. Since it seemed that PKHB1 treatment induced the recruitment of immune system cells to the tumor site, knowing that tumor-infiltrating leukocytes play a key role in complete tumor regression, it was decided to carry out an immunohistochemistry in the tumor sections to verify whether $CD4^+$ or $CD8^+$ cells had infiltrated to the tumor. The immunohistochemistry manifested the presence of both $CD4^+$ and $CD8^+$ cells in the histological slides of PKHB1-treated mice but not in the untreated control group (Figure 20B).

The above suggests that PKHB1 is able to induce tumor regression despite of the presence of the immune system and that PKHB1 favors the participation of the immune system to account for complete tumor regression.

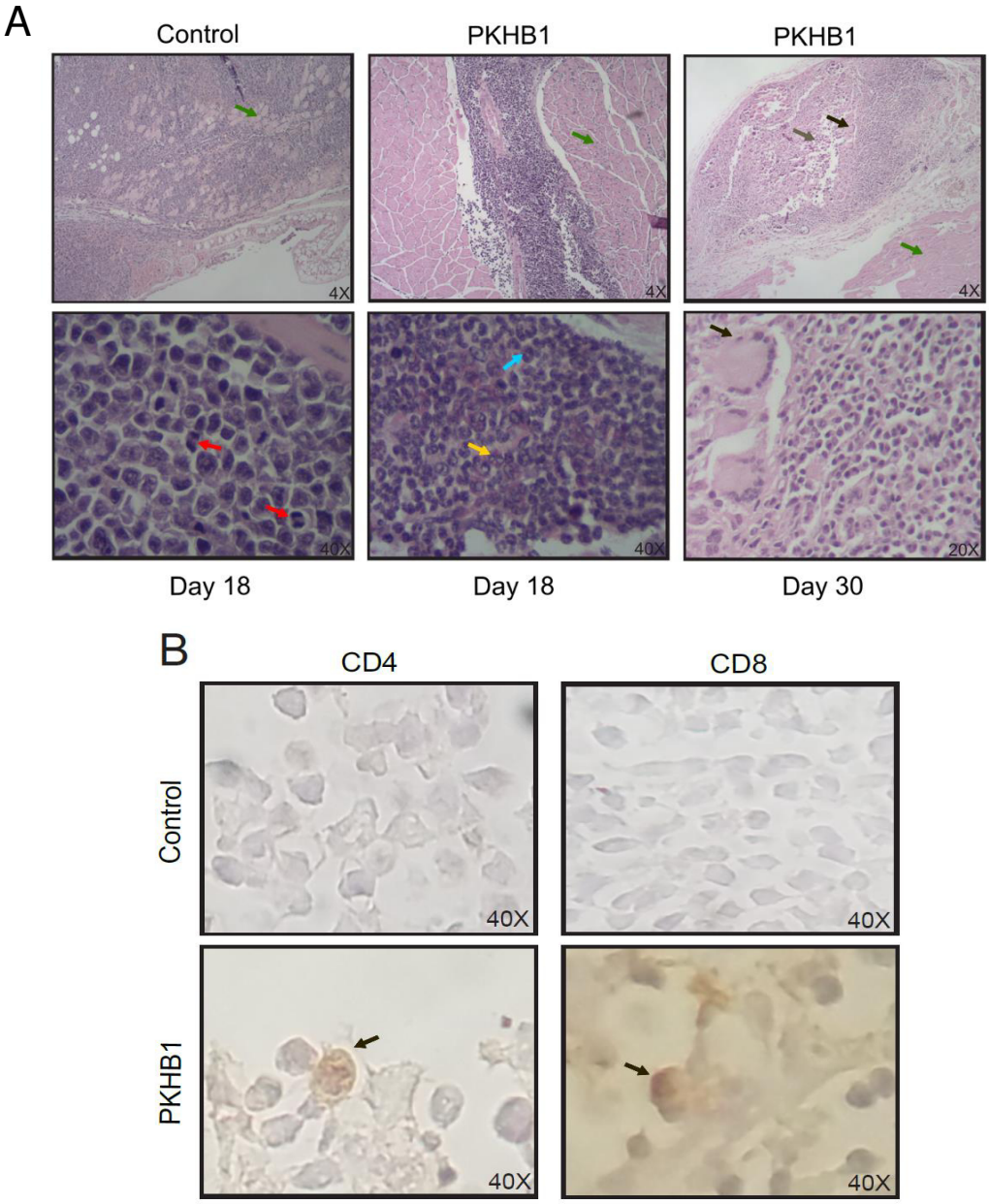

Figure 20. PKHB1 treatment induce recruitment of immune system cells. A. Histology from tumors of control and PKHB1-treated mice (day 18), stained with H&E. The control group without treatment presents lymphoblast cells and mitotic cells (red arrow; left,16 days), while in the treated with the peptide are less undifferentiated lymphoid cells and lymphocytes (blue arrow) and eosinophils (yellow arrow) are observed in response to a lymphomatous focus in muscle tissue (meddle, 18 days) that disappears through time. On day 30, giant cells (black arrow) and necrosis (brown arrow) are observed, normal tissue is signals with green arrow. **B**. Immunohistochemistry, $CD4^+$ and $CD8^+$ cells were labeled in tumor tissue without treatment and treated with PKHB1. Only in tissue treated with PKHB1 there is migration of T lymphocytes. The arrows point to the cells positive for the labeling of the different lymphocytes.

The recruitment of cells from the immune system to the tumor site in PKHB1-treated mice together with previous work reporting that PKHB1 induce calreticulin exposure on the surface of dying CLL cells (Martínez-Torres, et al. 2015) sparked our concern regarding whether PKHB1 was capable to induce immunogenic death. To address these questions, CEM and MOLT-4, T-cell acute lymphocytic leukemia human cell lines (T-ALL) and L5178Y-R as homologues of murine T-ALL were used.

9.9. Induction of calreticulin exposure by PKHB1 treatment in CEM, MOLT-4 and L5178Y-R cell lines.

The next step was to measure CRT exposure, since this protein is highly involved in early phases of ICD, in most cases even before PS exposure (Panaretakis *et al.,*2009). The levels of CRT on the cell surface were first assessed by flow cytometry. Although T-ALL cells showed the presence of ecto-CRT, a significant increase in CRT exposure onto the surface of CEM, MOLT-4 and L5178Y-R cells was observed when these were incubated with PKHB1 (Figure 21A). CRT exposure in PKHB1-treated cells surpassed the one from controls by more than half (Figure 21B). This was observed using confocal microscopy as well (Figure 21C), ratifying that PKHB1 is able to induce CRT exposure in all the cell lines tested.

The fact that PKHB1 was able to provoke CRT exposure in L5178Y-R, hinted us what would have been happening in the *in vivo* model, because such signal was expected to enable phagocyte recognition of the dying lymphoblastic cells.

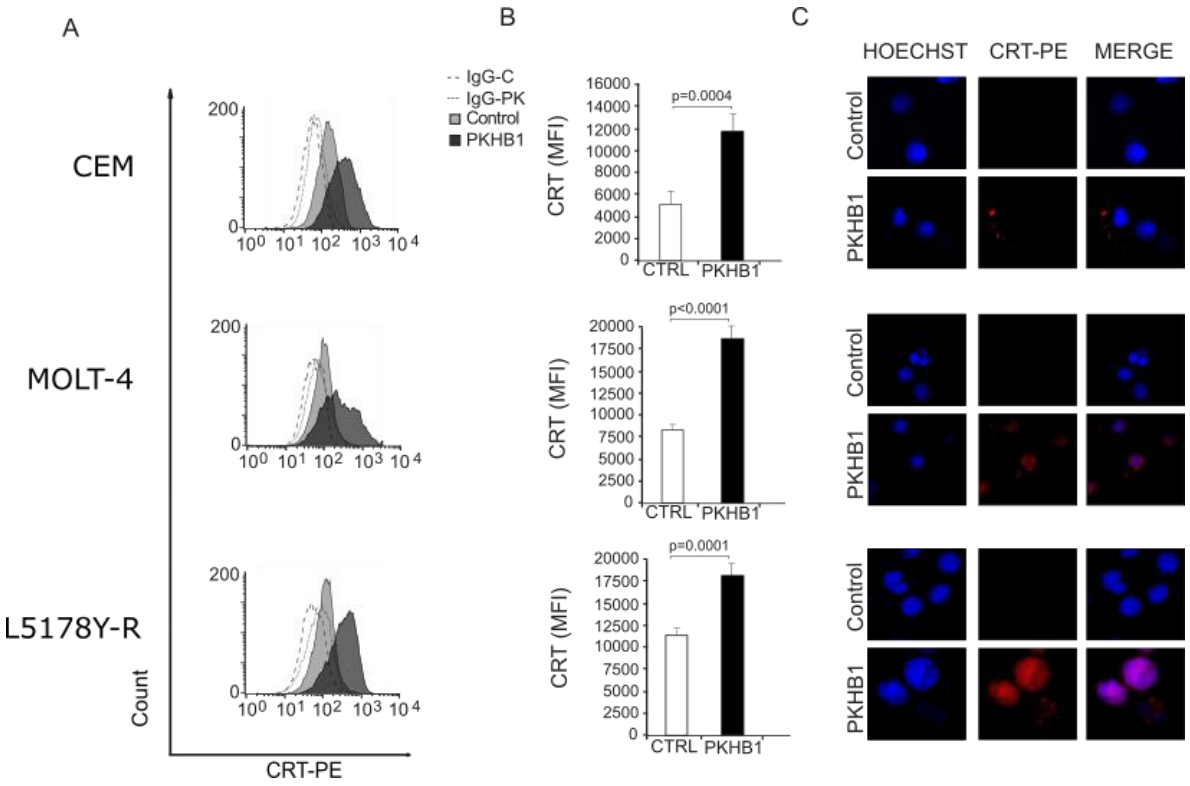

Figure 21. PKHB1 induces calreticulin exposure. A. Charts are a visual representation of the detection of surface CRT in CEM, MOLT-4 and L5178Y-R using FACS. Negative controls, with IgG isotype antibodies, are shown in dotted (IgG-C) and solid (IgG-PK) lines, while Gray is the basal CRT (control) and black represents cells treated (PKHB1). **B.** CRT exposure on T-ALL cells was observed in triplicates of at least three independent experiments and are graphed as the relative MFI for the calreticulin-positive cell for CEM, MOLT-4 and L5178Y-R cell lines. **C.** ECTO-CRT was observed in the cell treated with PKHB1 by CRT-PE staining and nucleus was stained with Hoechst 33342 and visualized by confocal microscopy. (Olympus X70, Mechanic zoom 7).

9.10. Expression and release of heat shock proteins, calreticulin and HMGB1 in cells and supernatant.

Since PKHB1 was able to cause the exposure of calreticulin, the idea that it could induce immunogenic cell death arose. To make sure that PKHB1 caused ICD it was decided to measure the expression and release of the main DAMPs: HSP90, HSP70, CRT and HMGB1. The presence of these DAMPs was determined both in cellular lysates and in supernatant of untreated cells and cells treated with PKHB1 at CC_{50} and CC_{100} for each cell line tested using the *Western blot* assay and the expression of each DAMP in each treatment and cell line were quantified using densitometry. Figure 22A displays the presence of HSP90, HSP70, calreticulin and HMGB1 in the cellular lysates of controls in CEM, MOLT-4 and L5178Y-R cell lines. Conversely, a decrease in the expression of the same DAMPs is observed in the

PKHB1-treated lysates, especially at CC_{100} in HSP70 and CRT. On the other hand, the expression of the mentioned DAMPs increased in the supernatants of PKHB1-treated cells compared with their control (Figure 22B). HSP90 release was more evident at CC_{50} in the three cell lines analyzed, while in HSP70 and CRT major release was observed at CC_{100}. In addition, the amount of HMGB1 protein was too low to be optimally quantified by *Western blot*. Indeed, only in L5178Y-R cell lysates the expression of HMGB1 was appreciable, but in the supernatant of the same cell line, it was not detected (Figure 23, bottom). However, in general, these results indicate that PKHB1 prompts heat shock proteins, calreticulin and HMGB1 release to the extracellular medium.

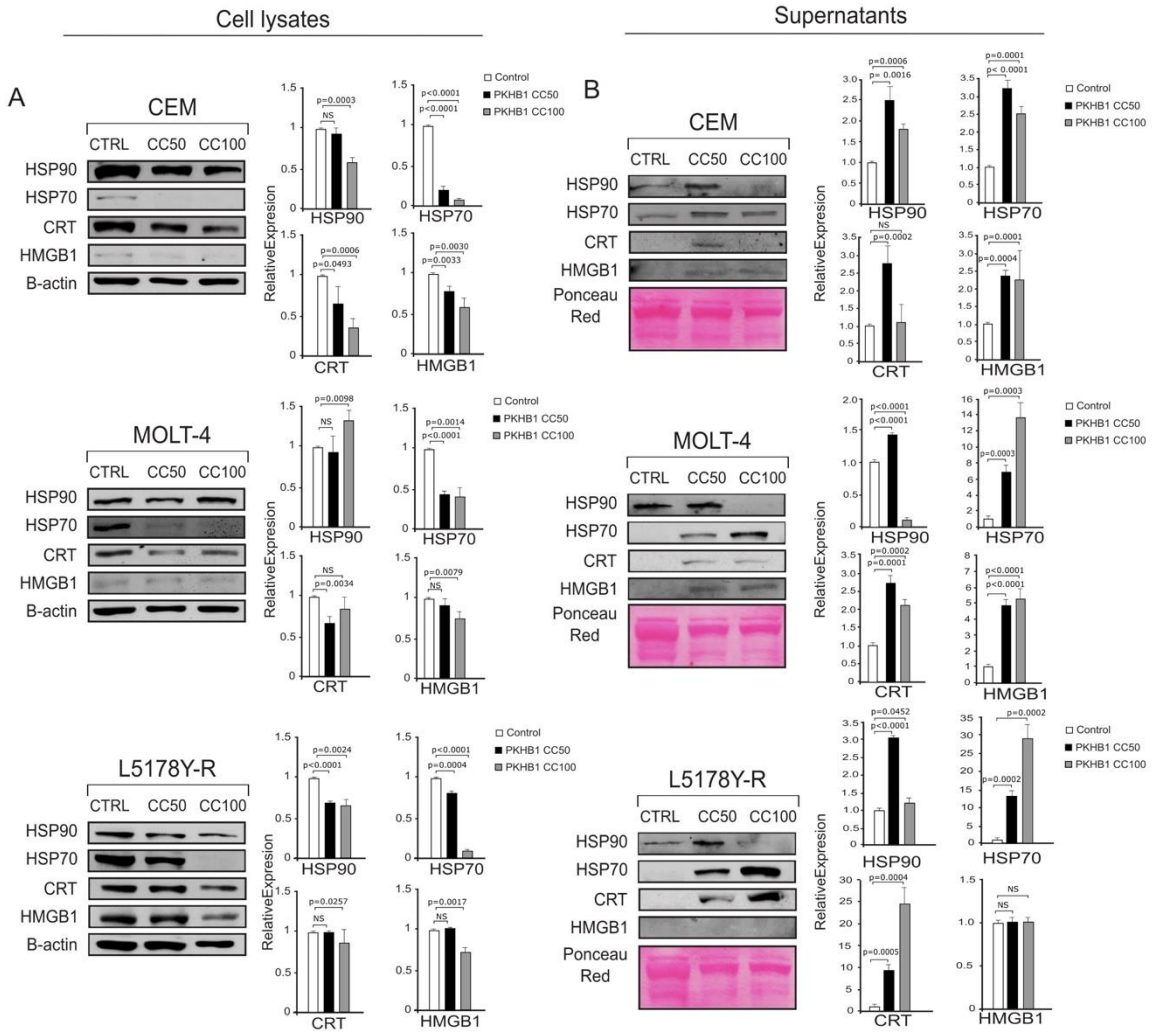

Figure. 22. Expression and release of HSP90, HSP70, CRT and HMGB1 proteins in response to treatment with PKHB1. *Western blot* was performed using **A**. cellular lysates **B.** and Supernatant of CEM, MOLT-4 and L5178Y-R cells untreated and treated with CC50 (200, 350, 150µM) and CC100 (400, 700, 300µM) of PKHB1. Loading control was β-actin (cell lysates) or Ponceau staining (supernatant).

9.11. Release of HMGB1 induced by the treatment of PKHB1 on T-ALL cells

Since HMGB1 was no well detected by *Western blot* (probably because of its low overall expression), an ELISA assay was performed in the three cell lines. The release of HMGB1 varied depending on the cell line studied, as well as the concentration of PKHB1 used. Neither CEM nor L5178Y-R released HMGB1 at CC_{50} (PKHB1 200µM and 150µM, respectively), but its release increased 6-fold compared to control for CEM and 2-fold for L5178Y-R using CC_{100}. On the other hand, MOLT-4 achieved an 8-fold HMGB1 release using CC_{50} (PKHB1 350 µM), while this decreased to 4-fold release using CC_{100} compared to control (Figure 23). Altogether these data indicate that PKHB1 induces HMGB1 release in T cell lymphoblasts, which concentration depended in the type of cell line.

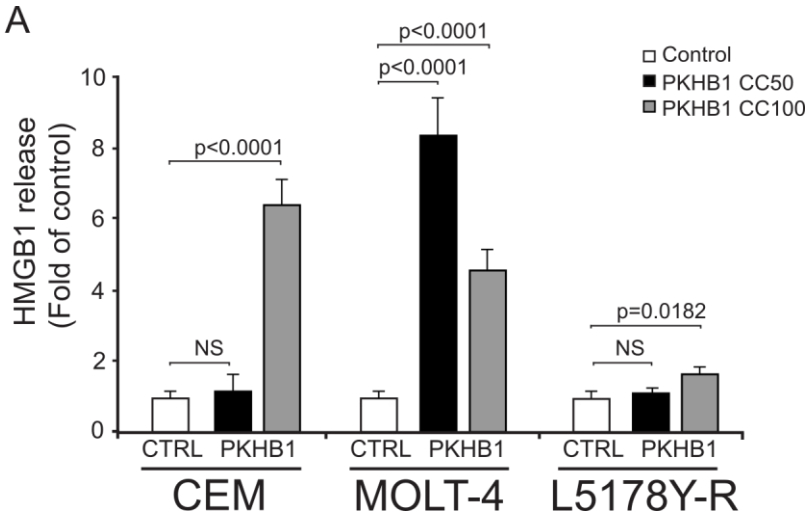

Figure 23. PKHB1 induces HMGB1 release in CEM, MOLT-4 and L5178Y-R cell lines. A. Cells were treated with PKHB1 for 2 h, then 10 0µL of supernatant (without FBS) of each sample was taken to measure HMGB1 by an ELISA test. The charts shown are representative of three experiments, performed in triplicates.

9.12. ATP release after the treatment with PKHB1.

Another important indicator that ICD is taking place is the release of adenosine triphosphate (ATP) to the extracellular medium. Therefore, a bioluminescence assay was performed in the supernatants of CEM, MOLT-4 and L5178Y-R cells. As depicted in Figure 24, ATP release augmented significantly in the supernatants of cells that were treated with PKHB1 at CC_{50} and CC_{100}. The concentration of ATP released by CEM increased almost 4-fold and more than 2-fold respect to the control at the CC_{50} and CC_{100} respectively, while MOLT-4 and

L5178Y-R cells at its CC_{50} almost reach the 5-fold of ATP release. Only in L5178Y-R treated with CC_{100} PKHB1 ATP release was not significantly different to that of control.

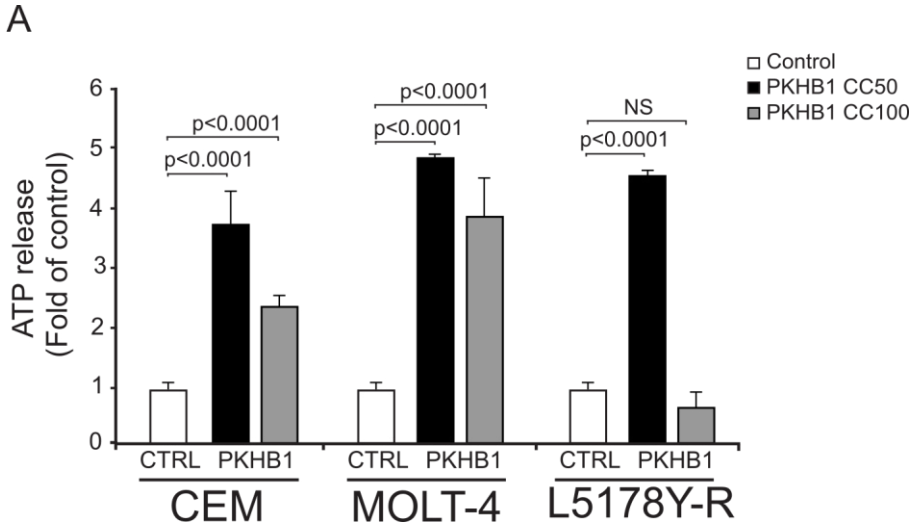

Figure 24. PKHB1 induces ATP release in CEM, MOLT-4 and L5178Y-R cell lines. A. Cells were treated with PKHB1 in medium without FBS (200 µM and 400 µM to CEM, 350 µM and 600 µM to MOLT-4, 150 µM and 300 µM to L5178Y-R) for 2 h, then 100 µL of the supernatant of each sample was taken to measure the ATP by bioluminescence. The chart shown is representative of three similar experiments, performed in triplicate.

9.13. *In vivo* effects of prophylactic vaccine based on DAMPs derived from L5178Y-R treated with PKHB1 and re-challenge with tumor viable cells

Assembling all the previous data, it was demonstrated that PKHB1 was effective to kill neoplastic T cells inducing DAMPs release, which is characteristic of ICD. However, it has been observed that sometimes, although a cell death inductor generates DAMPs release, immunological response and memory does not occur (Garg, Dudek & Agostinis, 2013). Therefore, the gold-standard assay to determine whether a treatment is an inducer of ICD are the *in vivo* vaccination experiments (Kroemer et al.,2013; Bezu et al.,2015; Galluzzi et al.,2016). Thus, the next step was to perform prophylactic vaccinations based in cell lysates derived from PKHB1-treated L5178Y-R cells.

Four groups of mice where assembled for this experiment: i) control group without vaccine; ii) 1.5M vaccine group, with 1.5x 10^6 PKHB1-treated cells; iii) 3M vaccine group, with 3x10^6 PKHB1-treated cells; and iv) 5M vaccine group with 5X10^6 PKHB1- treated cells. The results show that the greater the number of dead cells due to the peptide, the better the response

against the tumor cells inoculated 7 days after the vaccine administration (Figure 25). The vaccination of PKHB1-treated cells rich in DAMPs prevented the development of L5178Y-R tumor. In control group 6 out of 6 mice developed the tumor after the inoculation with viable cells (Figure 25A top-left), while 3 out of 4 mice developed tumor in 1.5M vaccine group (Figure 25A top-right), 3 out of 8 mice developed tumor in 3M vaccine group (Figure 25A down-left), and no-one of the 5M vaccine group (Figure 25A down-right). Also, the survival was graphed for each group compared with the control (Figure 25B), evidencing that prophylactic vaccination also prolongs the mice survival in a concentration-dependent fashion.

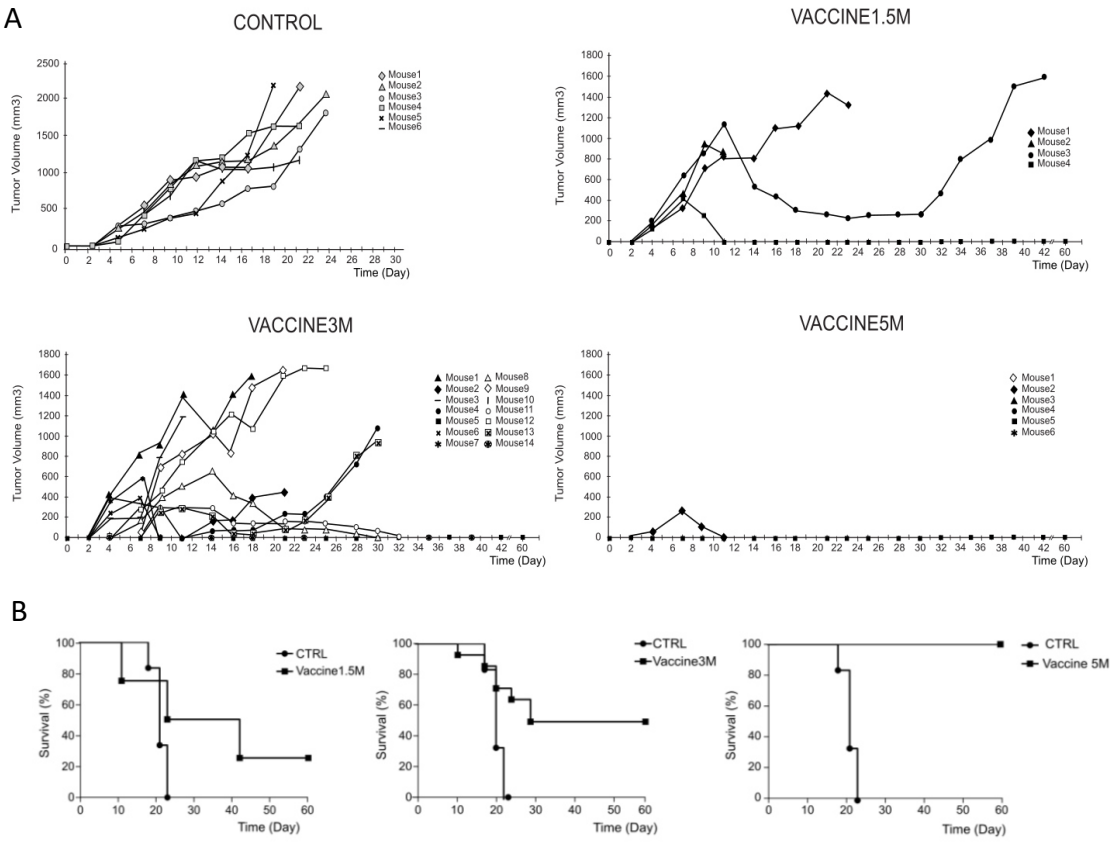

Figure. 25. PKHB1 is able to induce shorth-term immunological memory, through prophylactic vaccination. A. The graphs display the tumor volume (mm^3) through time (days) of the unvaccinated mice (Control) and vaccinated mice. Graphs with the 4 groups, Control (top-left), 1.5M vaccine (top-right), 3M vaccine (down-left), and 5M vaccine (down-right). Vaccine consists of 1.5M, 3M or 5millions of L5178Y-R cells previously treated with PKHB1 (CC_{100}) and re-challenged with live L5178Y (control n=6, PKHB1-treated n=4, 14 and 6, respectively). Each line represents one mouse. **B.** Survival in vaccinated mice over time is represented by the Kaplan-Meier graph.

9.14. PKHB1 gendered Long-term immunological memory through DAMPs release.

Additionally, long-term memory tests were carried out in mice that presented complete tumor regression after PKHB1 treatment, and that were without tumor at least for 30 days. In this assay 1 out 6 mice re-challenged with L5178Y-R viable cells developed the tumor while in the control group 6 out of 6 presented tumor growths (Figure 26A), Their survival was graphed in a Kaplan-Meier curve, showing around 80% survival (Figure 26B).

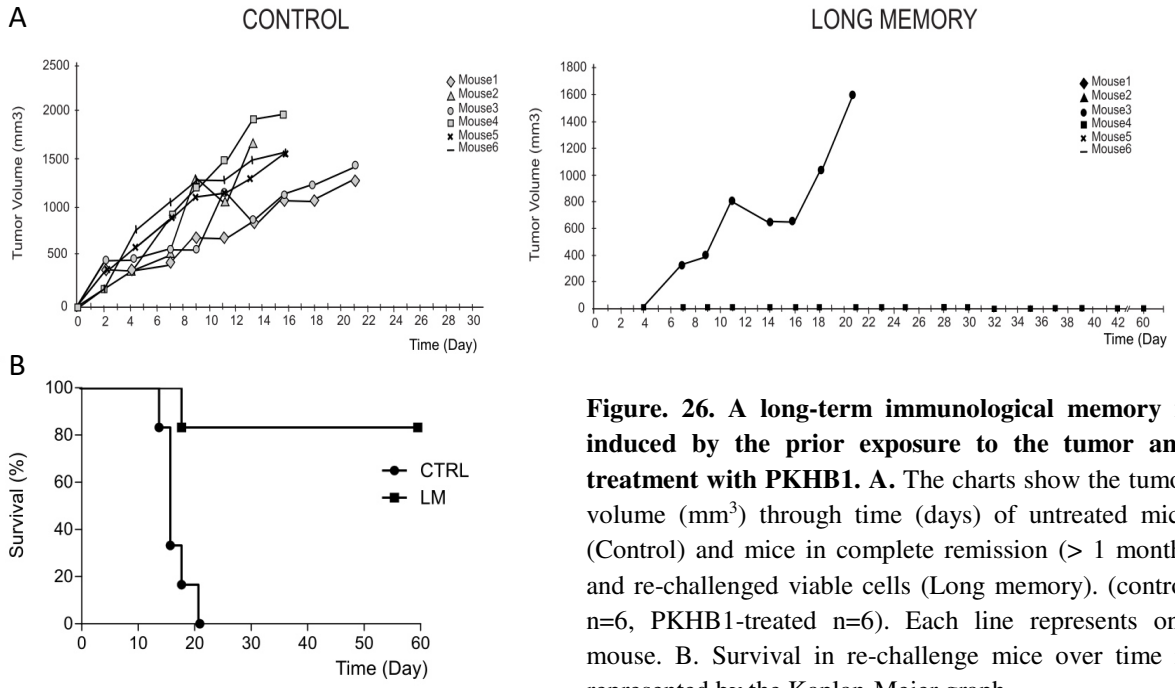

Figure. 26. A long-term immunological memory is induced by the prior exposure to the tumor and treatment with PKHB1. A. The charts show the tumor volume (mm^3) through time (days) of untreated mice (Control) and mice in complete remission (> 1 month) and re-challenged viable cells (Long memory). (control n=6, PKHB1-treated n=6). Each line represents one mouse. B. Survival in re-challenge mice over time is represented by the Kaplan-Meier graph.

X. DISCUSSION

Recently it was reported that CD47 activation by TSP1-derived peptides, 4N1K and PKHB1, orchestrates a molecular mechanism that rapidly mediates a massive Ca^{2+} mobilization to the cytoplasm, hence causing the death of CLL cells, even those from refractory patients (Martínez-Torres et al.,2015). With this vision, in this work I sought to test whether the CD47 agonists peptides (4N1K and PKHB1) induce a similar effect on different types of leukemia cell lines and their effect *in vivo*. Furthermore, PKHB1 was able to induce CRT exposure in CLL cells (Martínez-Torres et al.,2015), suggesting a possible immunogenic implication.

In the present study, it was found that the expression of CD47 in leukemic cell lines, PBMCs and lymphoid organs are similar (Figure 11). It is well known that CD47 is widely expressed in the plasma membrane of the cells and it has been reported that CD47 is overexpressed in different types of cancer like AML, ovarian, breast, colon, bladder, glioblastoma, hepatocellular carcinoma and prostate cancer (Willingham et al.,2012). However, in this case the expression of CD47 did not affect the cell death induced by the CD47-agonist peptides, as both, 4N1K and PKHB1 peptides, showed a cytotoxic effect against the different leukemia cell lines tested (MEC-1, CLL; Jurkat, CEM and MOLT-4, ALL; K562, CML; HL-60, AML and a murine T cell lymphoblastic tumor cell line L5178Y-R). The half cytotoxic concentration (CC_{50}) for most of the cells was 200µM using PKHB1 and 300µM using 4N1K (Figure 12). These results are comparable with previous studies which used 4N1K in a variety of cancer cell lines (Jurkat and CEM: T-ALL; RAJI and RAMOS: Burkitt's Lymphoma; RPMI-8226 and RPMI-8866: multiple myeloma, MCF7: Breast cancer; HELA, Cervix cancer; A549: Lung cancer; LNCAP: prostate cancer) (Denèfle et al.,2016) and report similar differences in the concentration needed of each peptide to induce cell death on cells of patients with CLL (Martínez-Torres, et al. 2015).

One possibility of the difference between the effectiveness of PKHB1 over 4N1K is because the first one is more stable in serum and resistant to proteolysis due to the addition of synthetic amino acid the $_L$-lysine at the extremes N amino and Carboxyl termini residues of the 4N1K sequence (Sue 2013; Martínez-Torres, et al. 2015; Denefle et al.,2016). On the

other hand, an explanation about why the peptides induce RCD in all types of leukemia cell lines tested could be that they share the expression of certain molecules like PLCγ1, which is overexpressed in CLL (Martinez-Torres et al.,2015) and cutaneous T-cell lymphoma (Vaqué et al.,2014) as well as in other types of cancer like breast cancer (Uhlmann et al.,2010) and glioblastoma (Serao et al.,2011). It was shown that CD47 activation induced the sustained phosphorylation of this enzyme, leading to death of these cells (Martínez-Torres, et al. 2015).

Since PKHB1 showed a better cell death effect in the different types of cell lines, this thesis focused on using PKHB1 (Figure 13). Some of the characteristics found in the cell death induced by PKHB1 in different types of leukemia cells, were a fast caspase-independent cell death that implicates phosphatidylserine exposure together with the plasma membrane permeabilization. These features have been largely implicated with CD47-induced cell death (Mateo et al.,1999; Manna & Frazier 2003; Johansson et al.,2003; Martinez-Torres et al.,2015; Denefle et al.,2016). In addition, Ca^{2+} could be a hallmark of CD47-mediated cell death, as in the different types of leukemia cells tested the use of the calcium chelator, BAPTA, inhibited cell death (Figure 15). This feature was observed recently, where it was shown that CD47 activation by the TSP1-derived peptide, PKHB1, was reported to mediate a massive Ca^{2+} mobilization to the cytoplasm, causing the death of cells coming from patients with CLL, even of those who were refractory to conventional treatments (Martínez-Torres et al.,2015).

Nowadays, some pre-clinical and clinical trials are being carried out using anti-CD47 monoclonal antibodies and fusion proteins like ALX148, that block the interaction of CD47-SIRPα with side effects ranging from headache to death (Nehal et al.,2018). This type of therapies is promising, but they forget that CD47 has multiple functions in the immune system such as leukocyte adhesion and migration, T cell activation and proliferation (Ticchioni et al.,1997; Soto-Pantoja et al.,2015) and that blocking it may not be the best option.

Since PKHB1 was able to induce cell death in cancer cells, the determination of its cytotoxicity and selectivity in non-cancer cells was required. I observed that its cell death was selective as only leukemic but not in healthy lymphoid cells were not susceptible to this type of cell death. PKHB1 does not appear to differentiate between different types of leukemia to induce cell death, but it does so in relation to human and murine PBMC, allowing them to survive. Indeed, although it was cytotoxic to the four T cell lymphoblastic cell lines tested (CEM, MOLT-4, Jurkat, and L5178Y-R), CD4+ and CD8+ human T cells treated with PKHB1 were not affected (Figure 14). As a reference, PKHB1 predecessor (4N1K) is able to kill effector but not central memory $CD4^+$ T cells (Van VQ et al.,2012), in freshly isolated but not in cultured dendritic cells (Johansson, Higginbottom and Londei 2003) or in CD^{5+} but not CD^{5-} B lymphocytes (Martinez-Torres et al.,2015).

In addition, the safety of PKHB1 was determined in primary cells from the bone marrow, spleen, thymus, and lymph nodes of BALB/c mice (Figure 16). Also, healthy BALB-c female mice injected with PKHB1 showed not significant differences in weight, organs weight, cellularity and complete blood count with respect with mice with no injection (Figure 17). Hence, systemic application of PKHB1 did not seemed to affect heart, lungs, kidneys, nor liver, highlighting the interest in using this type of strategies with a therapeutic perspective.

From this perspective, the *in vivo* assay was performed. Immunocompetent mice inoculated with L5178Y-R cells and treated with PKHB1, did not only reduce tumor growth drastically, but eradicated it in the majority of cases. In general, the treatment with PKHB1 improved the quality of life in mice, which reflected an increase in time of survival, and remission on 80% of the cases (Figure 18). That could not be observed with a previous study of immunocompromised mice performed by our research group, where treatment with PKHB1 decreased the tumor growth of CLL cells but did not eradicated the tumor (Martinez-Torres et al.,2015). The lack of complete remission in the previous work may be due to the immune system deficiency. It is worth mentioning that 4N1K peptide was tested *in vivo*, and the intra-vesical administration of the peptide in a bladder cancer model induced with N-butyl-N-(4-hydroxybutyl) nitrosamine show encouraging results since 4N1K-treated mice were alive and muscle invasive disease were prevented (Miyata et al.,2012; 2018). These results in

complement with the ones found through this thesis, indicate that the immune system could be contributing to tumor remission.

Before delving into the role of the immune system in the complete tumor remission, sides effects of the PKHB1-treatment were analyzed, for this, organs, blood and tumors were obtained and data show that PKHB1 does not cause damage to vital organs like lung, heart, kidneys and liver, similar data were reported by Martinez-Torres in a CLL *in vivo* model (Martinez-Torres et al.,2015). Moreover, PKHB1 does not generate lymphocytopenia or thrombocytopenia (Figure 19) contrary to the results observed in a clinical trial of anti-CD47 mAbs, where they observed side effects like anemia, thrombocytopenia and hypertension (Soto-Pantoja et al.,2015). The use of mouse anti-human CD47 antibody, B6H12 *in vitro* showed the stimulation of phagocytosis and tumor burden reduction of a primary human AML xenograft model (Majeti et al.,2009). It was also also shown that using a second antibody like rituximab, in addition to anti-CD47 mAb, helps to the eradication of NHL in xenograft models (Chao et al.,2010). The anti-CD47 mAb, was shown to work in solid tumors (Willingham et al.,2012; Edris et al.,2012). Moreover, the results of pre-clinical trials using Hu5F9-G4 show that Hu5F9-G4 induced potent macrophage-mediated phagocytosis of primary human AML cells *in vitro* and completely eradicated human AML *in vivo*, leading to long-term disease-free survival of patient-derived xenografts. However, the anti-CD47 Hu5F9-G4, show a relapsed disease in 2 out 10 mice in an AML *in vivo* model that did not respond to further Hu5F9-G4 therapy, and blood transfusions could be required during treatment (Liu et al.,2015). Despite these adverse effects, clinical trials using anti-CD47 monoclonal antibody Hu5F9-G4 for solid and hematological malignancies are undergoing (NCT02953509; NCT03248479; NCT02953782; NCT02678338). So far there are no results from these clinical studies, but they are waiting for good results.

One of the predictable side effects of PKHB1, is anemia, since large amounts of CD47 lie in the Rh group of erythrocytes and have been associated with the induction of death of senescent erythrocytes (Soto-Pantoja et al.,2015). Indeed, a low number of erythrocytes were observed in mice treated with PKHB1, however, erythrocytes count is not significantly different from that observed in untreated mice. Since anemia is a common feature of

hematologic cancers, we cannot conclude that PKHB1 treatment induces anemia, since PKHB1 treatment started when the tumor was already visible and ended 30 days later, when the mean erythrocyte turnover rate is 50 days in mice (Shi et al.,2014). Thus, a count to the erythrocytes before and after the treatment should be performed to compare the red blood parameters.

Continuing with the idea that the immune system is involved in the complete tumor regression, the histological slides of the tumor showed that treatment with PKHB1 in tumor-bearing mice induces leukocyte-infiltration to the tumor site (Figure 20) and improves leukocyte-cell number in different lymphoid organs. This is the first report about mobilization of leukocytes into tumor site induced by PKHB1- treatment. However, it is well known that CD47 is involved in PMN cell migration, but control mice did not showed migration by themselves, making me hypothesize that the cell death induced by PKHB1-treatment are helping this leukocyte mobilization. Increasing evidence suggest that immunogenic cell death (ICD) induces an antitumor immune response, increasing tumor infiltration of T cells. ICD stimulates the recruitment of DCs trough DAMPs release. DCs process the tumor antigens and make the antigen presentation to T cells helping to kill tumor cells (Wang et al.,2018).

For these results, the tumor regression, the lymphocytes infiltration and for the previous report, which indicates PKHB1 can induce calreticulin (CRT) exposure in CLL cells (Martínez-Torres et al.,2015). I hypothesized that PKHB1 is able to induce immunogenic cell death (ICD). To check the hypothesis only CEM, MOLT-4 and L5178Y-R cell lines were selected due to ALL is the most common leukemia in Mexico and because the *in vivo* model is homologous to human T-ALL.

Indeed, PKHB1 turned out to be an ICD inductor at least in CEM, MOLT-4 and L5178Y-R cells, since PKHB1 was capable to prompt DAMPs release on these cells. My results demonstrated the CRT exposure on T-ALL cells after the treatment of PKHB1 (Figure 21). Diverse studies in the field of immunology highlight the importance of CRT exposure as a "eat me signal" (Gardai et al.,2005; Obeid et al.,2007; Chao et al.,2010; Inoue & Tani, 2014) that helps antigen up-take by APCs by binding to low density lipoproteinreceptor-related

protein 1 (LRP1, also known as CD91) (Garg et al.,2012). There is a tight correlation between CRT and CD47 expression in cancer cells (Chao et al.,2010). Indeed, Chen and coworkers in 2015 determined in breast cancer cell lines that the treatment with thrombospondin (TSP-1) promoted interaction of TSP-1 with CRT and CD47 and induced cell autophagy and tumor growth inhibition in xenografted mice (Chen et al.,2015). These results support the idea about TSP-1 or peptides derived from TSP-1 can induce cell death through the activation CD47 and its correlated with CRT exposure as it was determined.

Also, PKHB1 was able to induce the release of heat shock protein HSP70 and 90 as well as non-histone high mobility group box-1 protein (HMGB1) and adenosine triphosphate (ATP), other important DAMPs involved in ICD (Figures 22,23 & 24). Fucikova and co-authors reported that the use of doxorubicin and idarubicin increased the expression of HSP70, HSP90, and CRT on leukemic blast of T-ALL and REH cell line (ALL), also these anthracyclines induce the HMGB1 secretion (Fucikova et al.,2011). The release of these molecules is implicated in the activation of the immune system and induction of potent anticancer immunity (Fucikova et al.,2011; Krysko et al.,2012; Rodriguez-Salazar et al.,2017).

The release of DAMPs is not enough evidence to ensure the induction of ICD and the gold-standard is the *in vivo* vaccination because DAMPs can suffer structural changes or be neutralized by the cancer cells (Kroemer et al.,2013; Bezu et al.,2015; Galluzzi et al.,2016). To determine that PKHB1 is an immunogenic cell death inducer, *in vivo* assays were performed. Interestingly, PKHB1 activates short and long-term immunological memory and induces a protective anti-cancer response in an immunocompetent T lymphoblast mouse model, since tumor growth was prevented in around 80% of the cases. A similar result was reported by Schiavoni and co-authors where they found that 60% of the C57BL/6 mice with established EG7 lymphoma tumors were cured of their tumors by 40 days when received peritumoral injections of cyclophosphamide (an alkylating agent) and type I IFN for 4 days and were resistant to subsequent re-challenge by the same cells (Schiavoni et al.,2010), indicating the induction of immunological memory.

I observed that increasing the number of cells treated with PKHB1, the better the response against the tumor. This might be the result of the major release of DAMPs (Figure 25), and that PKHB1 is able to induce long-term memory (Figure 26). The potential mechanism contributing to prevent the tumor growth is the effective anti-tumor immune response, which could be explained since tumor cells expose CRT and secrete ATP and HMGB1, this DAMPs stimulated the DCs recruitment into the tumor microenvironment, DCs processes tumor antigen and present the antigen to T cells. Cross-priming of CD8+ cytotoxic T lymphocytes (CTLs) is triggered by mature DCs in an IL-17 and IL-1β-dependent manner and CTLs can kill tumoral cells through the generation of INF-γ, perforin-1 and granzyme B (Lanitis et al.,2017; Wang et al.,2018).

It is important to notice that this is the first study of this kind using this peptide. Previous reports of similar vaccines, based in tumor cells killed with ICD inducers and inoculated into immunocompetent mice that were re-challenged with the same cancer cells, obtained similar results, the prevention of tumor growth. One example is melphalan, an alkylating agent used to treat melanoma, where they injected murine B78 melanoma cells killed by melphalan and 10 days later re-challenged mice with B78 viable cells, obtaining a 40% of mice without tumor (Dudek-Peric et al.,2015). Similar results were obtained by using Doxorubicin as ICD inducer in mouse colon carcinoma (CT26) cell line (Casares et al.,2005). The use of this vaccine helps to stimulate anti-cancer immunity through the maturation of DCs and activation of cytotoxic T cells (Guo et al.,2013) as well as enhancing the cytotoxic activity of NK cells (Showalter et al.,2017). The DCs maturation and cytotoxic T cell activation are a perspective to complement this work.

Immunotherapy is a promising treatment option against cancer (Papaioannou et al.,2016), using host immune defenses against cancer and seeking to endow cancer cells with immunogenicity (Li, 2018). The increased immunogenicity of tumor cells triggers the antitumor immune responses which could offer long-term therapeutic effects (Kroemer et al.,2013). The finding that certain drugs can induce the awakening of the immune response by releasing DAMPs and generating ICD, triggered investigations looking for these type of agents (Casares et al.,2005; Kroemer et al.,2013; Pol et al.,2015; Showalter et al.,2017). Anthracyclines, platinum derivatives, alkylating agents, and proteasome inhibitors are some

chemotherapeutic drugs with vast evidence on triggering ICD (Vacchelli et al.,2014). Other therapeutic modalities that display ICD induction are photodynamic therapy (Tanaka et al.,2016), radiotherapy (Golden & Apetoh, 2015), oncolytic viruses (Diaconu et al.,2012; Yamano et al.,2016), high hydrostatic pressure (Fucikova et al.,2014), and other phytochemical agents such as shikonin (Lin et al.,2015; Yin at al., 2016) and capsaicin (D'Eliseo et al.,2013; Jin et al.,2016).

Altogether, the results showed in this work suggest that the activation of the immune system is a consequence of the treatment with PKHB1, which makes tumor immunogenic through the induction of DAMPs release and the posterior generation of immunogenic memory but the molecular pathway of how PKHB1 induce this type of death through CD47 signaling in the cells is still unclear. However, it would be interesting to determine if this kind of death could be used to treat or prevent metastasis.

Finally, overall the results from this thesis highlight the advantages and the therapeutic potential of CD47 agonist peptide strategies. The model presented in this work represents other perspective to use CD47 as a therapeutic target using the activation of this transmembrane protein through the interaction with TSP-1 derived-peptides. Also, the use of peptides represents an advance in the application of strategies directed to CD47, which has important advantages over the use of monoclonal antibodies such as minor side effects and a lower production cost (Uhlig et al.,2014). Therefore, I believe that both the different pathophysiological functions of CD47 as well as their agonist peptides deserve further investigation and the possibility of being scaled in the near future to clinical phases.

Overall, our results highlight the advantages of the potential therapeutic use of targeting CD47 through peptide-based strategies, such as PKHB1, leading to consider that this peptide could be used in other types of cancers. However, the molecular pathway by which PKHB1 induces this type of death through CD47 signaling remains unclear, as does whether this kind of treatments could be used therapeutically. Therefore, we believe that CD47 agonist peptides deserve further investigation, which might lead to the possibility of being scaled in the near future to clinical phases.

XI. CONCLUSIONS

From the results obtained in the present work we can conclude that:

- The CD47 agonist peptides 4N1K and PKHB1 cause cell death in the leukemic lines studied.
- PKHB1 was more effective than 4N1K.
- PKHB1 did not generate significant cytotoxicity in human and murine peripheral blood mononuclear cells (PBMCs)
- PKHB1 induces caspase-independent, calcium-dependent cell death in malignant cells.
- PKHB1 did not induce significant cell death on primary cultures of bone marrow, spleen, thymus and lymph nodes obtained from BALB/c mice.
- PKHB1 reduces volume and tumor weight, without causing damage in lymphoid and vital organs and improves mice survival.
- The appearance, mood and weight of the mice treated with the PKHB1 were better than the controls without treatment.
- Treatment with PKHB1 induce the recruitment of immune system cells to the tumor site, probably participating in the complete tumor remission.
- The exposure of calreticulin and the release of ATP, HSP70, HSP90, HMGB1 and CRT to the extracellular medium indicate that PKHB1 is able to induce immunogenic cell death in CEM, MOLT-4 and L5178Y-R cell lines.
- Prophylactic vaccination with PKHB1-treated L5178Y-R cells, prevented the establishment of viable L5178Y-R tumors *in vivo*.
- The greater the number of PKHB1-treated L5178Y-R cells, the greater the concentration of DAMPs released, and the greater the protection against the L5178Y-R cells *in vivo*.
- PKHB1 generates immunogenic memory.

XII. PERSPECTIVES

The perspectives of the present work are:

- The development of new peptides that could be used at lower doses to improve the possibilities of being applied in human trails.
- Testing the potential of CD47 agonist peptides in other *in vivo* models of different types of cancer.
- To test PKHB1 in combination with chemotherapeutic agents *in vitro* and *in vivo*, to determine if PKHB1 could be used in combinatory therapies.
- Continue with the characterization of the mechanism of cell death induced by CD47. The mechanism described through this thesis indicates that CD47 activation induces cellular Ca^{2+} overload, CRT exposure, HSP-70 and HSP-90 release, ATP release, HMGB1 liberation, these DAMPs stimulate the recruitment of DCs. DCs process the tumor antigens and present antigens to T cells, helping to kill tumor cells. Thus, the infiltration of T cells into the tumor site can be explained by the exposure and secretion of CRT and the secretion of ATP and HMGB1 by the dying cells, which stimulate DCs recruitment into the tumor microenvironment, antigen processing and presentation to T cells which then infiltrate to the tumor site and leukemic cell death.
- Perform ex vivo assays to complete the ICD description, such as DC maturation analyses and the evaluation of the presentation to T cells.

XIII. BIBLIOGRAPHY

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XIV. ELECTRONIC BIBLIOGRAPHY

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XV. BIBLIOGRAPHICAL ABSTRACT

Ashanti Concepción Uscanga Palomeque

Candidate for the degree of:

Doctor of science with orientation in immunobiology.

Thesis: cell death effect of CD47-agonist peptides on different types of leukemia and in a murine model.

Research Field: Health Sciences.

Personal information: Born in Monterrey, Nuevo León, at December 5th of 1988.

Daughter of Luis Arturo Uscanga León and María Luisa Palomeque Garza.

Education:

Graduated from the Universidad Autonoma de Nuevo Leon.
Graduated degree: Chemistry Bacteriology and Parasitology 2006-2010. Ranked as 1st place among 160 students in my class, with a score of 95.82.
Title of research work: Cytotoxic effect of aqueous extracts of plants *Hemiangium excelsum*, *Cuphea aequipetala, Acalypha mexicana* and *Tabernaemontana australis* in cancer cell lines.

Post graduate Degrees: Master's in science with a specialization in Immunobiology. 2012-2014. Ranked as 1st place among 4 students in my class with a score of 95.38.
Title of research work: Cytotoxic effect of aqueous and methanolic extracts of *Cuphea aequipetala*, "Hierba del cancer" on cancer cell lines and a murine model.

Awards:

- Recognition as first place in the "V National Symposium of Pharmaceutical Sciences and Biomedicine and the III National Symposium of Applied Microbiology. With the poster exhibition of the work: PKHB1 induces tumor regression through regulated cell death and stimulation of the immune system. Monterrey, Mexico. 2018.
- - Obtaining the first place in the poster exhibition with the work: Effect of CD47-agonist peptides on cell death of different types of leukemia. In forum seminar research advances in the Graduate School of Biological Sciences. UANL. 2016.
- Conacyt National Scholarship during PhD degree, 2015-2018.

- Recognition as an Honor Student of the Master's degree, 2012-2014. Ranked as 1st place with a score of 95.38.
- Conacyt National Scholarship during Master's degree, 2012-2014.
- Recognition from the Nuevo León State Congress as a tenacious, dedicated and disciplined person in the professional development being an example to young generations, 2011.
- Recognition from the UANL with the Academic's Merit, 2010.
- Recognition as Honor student. Ranked as 1st place among the undergraduate students with a score of 95.82, 2006-2010.
- Treasurer of the Student Board in the School of Biological Sciences during 2009-2010.

Additional Courses:

- Molecular Biology of the Cell Course. Pasteur Institute, Paris, France. 2017.
- Assistance to course Animal models for laboratory research, FCB-UANL. 2017.
- Assistance to the course Biological risk and biosafety (BSL-3), FCB-UANL, 2017.
- First theoretical and practical course of flow cytometry, FCB-UANL. 2014.
- Theoretical Course about Autoimmunity, FCB-UANL. 2012.
- Biosafety Course, FCB-UANL. 2012.
- First Aid Course, Red Cross Monterrey Mexico. 2008.
- Assistance to BioMonterrey08, an International Congress and Exhibition of Biotechnology. October 2008.
- English courses in Harmon Hall. Centro Lingüístico GV, A.C.2004-2007 Certificate.

Training of human resources:

- Member of the evaluating jury of the final learning integration products of the Bioethics subject. 2018.
- Member of the evaluation committee of bachelor thesis, as external advisor of the work: Analysis of the cytotoxic and antitumor effect induced by the activation of CD47 in the cell line L5178Y-R. 2017. Presented Kenny Misael Calvillo Rodríguez.

Publications:

Congress Presentations

- Poster exhibition at the 21 State Health Research Contest. with the work: the activation of CD47 by the PKHB11 peptide: A promising treatment against leukemia. Monterrey, Mexico. 2018.
- Poster exhibition in 11th European Workshops on Cell Death, with the work: CD47 agonist peptide, PKHB1, induces selective cell death in leukemia cell lines and induces tumor regression *in vivo*. Fuggi, Italy, 2018.

- Oral exposition of the work: CD47 agonist peptide, PKHB1, induces immunogenic cell death in leukemia cell lines and tumor regression *in vivo*. In the 9th *Young researchers in life sciences* conferences, in Paris, France. 2018.
- Oral exposition at the "V National Symposium of Pharmaceutical Sciences and Biomedicine and the III National Symposium of Applied Microbiology. With the work: CD47 agonist peptide, PKHB1, induces immunogenic death in acute T cell lymphocytic leukemia cells. Monterrey, Mexico. 2018.
- Poster exhibition at the "V National Symposium of Pharmaceutical Sciences and Biomedicine and the III National Symposium of Applied Microbiology. With the work: PKHB1 induces tumor regression through regulated cellular death and immune system stimulation. Monterrey, Mexico. 2018.
- XXIX National Congress of Medicine Research. Department of Medical Science. UANL. Oral exposition of the work: PKHB1 induces tumor regression through regulated cell death and stimulation of the immune system. Monterrey, Mexico. 2017.
- Poster Exhibition in forum seminar research advances in the Graduate School of Biological Sciences, UANL. 2017.
- Oral exposition of the work: Effect of CD47-agonist peptides on cell death of different types of leukemias. In forum seminar research advances in the Graduate School of Biological Sciences. UANL. 2015
- Poster Exhibition in the 1st International Congress of Molecular Biomedicine. Title of the work: Cytotoxic effect of aqueous and methanolic extracts of *Cuphea aequipetala*, "Hierba del cancer" on cancer cell lines and a murine model. Mazatlan Sinaloa, México, 2015.
- Poster Exhibition in the International Symposium on Immunotherapy in cancer and infectious diseases. Title of the work: Cytotoxic effect of aqueous and methanolic extracts of *Cuphea aequipetala*, "Hierba del cancer" on cancer cell lines and a murine model. Internationalization Center. UANL. 2014
- XXVII National Congress of Medicine Research. Department of Medical Science. UANL. Oral exposition of the work: Cytotoxic effect of aqueous extracts of plants *Hemiangium excelsum*, *Cuphea aequipetala, Acalypha mexicana* and *Tabernaemontana australis* in cancer cell lines. 2013.
- Poster Exhibition. Research Progress. Title of the work: Cytotoxic effect of aqueous and methanolic extracts of *Cuphea aequipetala*, "Hierba del cancer" on cancer cell lines and a murine model FCB-UANL. 2013.

Scientific articles:

- Research paper derived from Master Degree. In process to submission.

- Zapata-Benavides Pablo*; **Uscanga-Palomeque Ashanti Concepción***, Saavedra-Alonso Santiago; Zamora-Ávila Diana Elisa; Franco-Molina Moisés Armides; Arellano-Rodríguez Mariela; Manilla-Muñoz Edgar; Martínez-Torres Ana Carolina;

Trejo-Ávila Laura M.; Rodríguez-Padilla Cristina. Inhibitory effect of *Cuphea aequipetala* extracts on murine B16F10 melanoma *in vitro* and *in vivo*.

- Research papers derived from PhD Degree. Manuscript submitted in process to review.

- **Ashanti C. Uscanga-Palomeque**; Luis Gómez-Morales; Kenny M. Calvillo-Rodriguez; Thomas Denefle; Diana E. Caballero-Hernández; Santos A. Susin; Philippe Karoyan; Ana C. Martínez-Torres; Cristina Rodríguez-Padilla. CD47 agonist peptide, PKHB1, induces immunogenic cell death in T-cell acute lymphoblastic leukemia.

- Philippe Karoyan,* Ana Carolina Martinez-Torres,* **Ashanti Concepción Uscanga-Palomeque**, Monika Kaminska, Luis Gómez-Morales, Elodie Pramil, Valérie Linhard, Thomas Denèfle, Akankasha Gangar, Kenny M. Calvillo-Rodríguez, Diana E. Caballero-Hernández, Hélène Merle-Béral, Didier Grillot, Nicolas Anceliin, Santos A. Susin, Cristina Rodríguez-Padilla, Laurent Devel.CD47/TSP1 4N1s-Derived Peptide Interaction Evidenced by Ligand-Directed Chemistry. Manuscript in preparation.

- Colaboration paper. Accepted with minor revisions., PeerJ., 2018

- Ana Carolina Martinez-Torres; Luis Gomez-Morales; Alan B. Martinez-Loria; **Ashanti C. Uscanga-Palomeque**; José Manuel Vázquez-Guillen; Cristina Rodriguez-Padilla. IMMUNEPOTENT CRP is cytotoxic in non-small cell lung cancer cell lines through ROS production.
  - Colaboration paper. In process to submission.

- Marilena Antunes-Ricardo, Annia Hernández-Reyes, **Ashanti Concepción Uscanga- Palomeque**, Cristina Rodríguez-Padilla, Ana Carolina MartínezTorres*, Janet Alejandra Gutiérrez-Uribe*. Isorhamnetin glycoside isolated from Opuntia ficus-indica (L.) MilI induces apoptosis in human colon cancer cells through mitochondrial damage. Manuscript in preparation

Divulgation articles:

- Martínez-Torres Ana Carolina, **Uscanga-Palomeque Ashanti Concepción**, Morales-Gomez Luis, Rodríguez-Padilla Cristina. (2017). CD47: a promising therapeutic target against cancer. Ciencia y Desarrollo. Vol. 43, pag. 12-17.

Book Chapter:

- Ana Carolina Martínez-Torres, **Ashanti Concepción Uscanga-Palomeque**, Cristina Rodríguez-Padilla. (2016). In vitro cytotoxic activity against tumor cells. In C. Rivas-

Morales, M.A. Oranday-Cárdenas & M. J. Verde-Star (Eds.), Medically important plants research. (pp. 177-205). Barcelona OmniaScience.

Professional Experience:

Scientific summer, about nucleic acid extraction and PCR techniques for the creation of a tumor cancer bank. PROVERICYT. Biological Science School UANL, 2006.

Scientific summer, about cellular protein extractions, Western blot, Transformations and cloning techniques. PROVERICYT. Biological Science School UANL, 2010.

Bachelor Thesis in the Molecular Cancer Biology Department. 2011-2012.
Title of research work: "Cytotoxic effect of aqueous extracts of plants *Hemiangium excelsum*, *Cuphea aequipetala, Acalypha mexicana* and *Tabernaemontana australis* in cancer cell lines".

Internship: Nogalar Surgical Center. Collection and processing of human samples (blood, urine, and stool) for different types of analysis. 2011

Graduate student of Master in Science with specialty in Immunobiology at the Department of Biological Science, UANL. 2012-2014
Title of research work: "Cytotoxic effect of aqueous and methanolic extracts of *Cuphea aequipetala*, *Hierba del cancer* on cancer cell lines and a murine model".

Chemist of clinical analysis, blood bank and professional staff: Nogalar Hospital, Swiss Hospital, Microbiology Department in Regional Center for Infectious Disease Control. Department of Medical Science, UANL. Processing blood, urine and stool samples for different types of analysis. 2014-2015.

Associated researcher: In charge of cell bank, confocal microscope and flow cytometer. Laboratory of Immunology and Virology. FCB, UANL. 2018.