UNIVERSIDAD AUTONOMA DE NUEVO LEON

SCHOOL OF BIOLOGICAL SCIENCES

STUDY OF THE MECHANISM OF CELL DEATH CAUSED BY PEPTIDES TARGETING CD47 IN LEUKEMIA CELL LINES

by

LUIS GÓMEZ MORALES

In fulfillment of the requirements for the degree of
MASTER OF SCIENCE WITH ORIENTATION
IN IMMUNOBIOLOGY

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For whoever who wants to save their life will lose it,

but whoever loses their life for Me will find it (Mt. 16:25)

To my mom in heaven, I know you'd be proud
(1960-2014)

To José Pablo Gálvez, my friend,
who lost the battle with leukemia
(1991-2014)

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I. ABSTRACT

CD47 activation through the C-terminus of thrombospondin-1 or its derived peptides (4N1K and PKHB1) induce regulated cell death (RCD) in several types of cancer. Recently, it was demonstrated that PKHB1, the first serum-stable CD47 agonist peptide, induce caspase-independent, calcium-dependent RCD in CLL cells, even in those resistant to conventional therapy. Therefore, the objective of the present work was to study the PKHB1-induced cell death mechanism in other types of leukemia. To that end, cell death induction was evaluated by flow cytometry, analyzing phosphatidilserine exposure (Ann-V) and plasma membrane permeability (PI), as well as caspase dependance (inhibitor Q-VD-OPH) or calcium dependance (BAPTA, calcium chelator). The results show that PKHB1 is a better inductor of cell death, compared to 4N1K, and it is selective to different types of leukemia (MEC-1, CEM, Junket, K562, HL-60, L5178Y-R), since it does not kill human peripheral mononuclear cells, nor cells derived from lymphoid organs of healthy mice. PKHB1-induced killing is caspase-independent in all cases. Additionally, in CEM (human T lymphoblasts of acute lymphocytic leukemia, the principal type of childhood cancer) and L5178Y-R (murine T lymphoblasts) cells, death is not modulated by co-culture with chemoprotective bone marrow stromal cells; calcium chelation, however, inhibits PKHB1-induced cell death. Together, the results indicate that PKHB1 is effective in different types of leukemia, and induce caspase-independent, microenvironment-independent calcium-dependent RCD, in leukemic T lymphocytes. These results suggest that PKHB1-induced RCD could be conserved in different types of leukemia, and set the basis for further studies on murine models.

I. RESUMEN

La activación de CD47 por el extremo C-terminal de la trombospondina-1 o péptidos derivados de ella (4N1K y PKHB1) inducen muerte celular regulada (MCR) en varios tipos de cáncer. Recientemente se demostró que PKHB1, el primer péptido agonista de CD47 estable en suero, induce MCR independiente de caspasas, pero dependiente de calcio en células de CLL resistentes al tratamiento convencional. Por ello, el objetivo del presente proyecto fue estudiar el mecanismo de muerte celular que induce el PKHB1 en células de otros tipos de leucemia. Mediante citometría de flujo se evaluó la inducción de muerte celular por la exposición de fosfatidilserina (Ann-V) y la permeabilidad de la membrana plasmática (PI), así como la dependencia de caspasas (inhibidor Q-VD-OPH) o calcio (quelador BAPTA). Los resultados muestran que el PKHB1 es un mejor inductor de muerte que el 4N1K, y es selectivo para células de diferentes tipos de leucemia (MEC-1, CEM, Jurkat, K562, HL-60, L5178Y-R), pues no mata células mononucleares de sangre periférica de donadores sanos ni células derivadas de órganos linfoides de ratón. La muerte inducida por PKHB1 es independiente de caspasas en todos los casos. Adicionalmente, en células CEM (linfoblastos T, de leucemia linfocítica aguda, el principal tipo de cáncer infantil) y L5178Y-R (linfoblastos T murinos) la muerte no es modulada por el co-cultivo con células estromales de médula ósea quimioprotectoras; la quelación de calcio, en cambio, sí inhibe la muerte causada por el PKHB1. En conjunto, los resultados indican que el PKHB1 es efectivo en diferentes tipos de leucemia, e induce MCR independiente de caspasas y de microambiente, pero dependiente de calcio en líneas celulares de linfocitos T leucémicos. Dichos resultados sugieren que el mecanismo de MCR inducido por PKHB1 podría estar conservado en diferentes tipos de leucemia, y sientan las bases para estudios posteriores en modelos murinos.

II. INTRODUCTION

Regulated cell death (RCD) is the process by which the cell succumbs through the activation of a genetically encoded auto-destruction machinery. Mutations in this complex machinery lead to the disruption of homeostasis, generating pathologies associated with either an excess of RCD (e.g., the Alzheimer disease) or resistance to it, such as cancers.

Leukemia comprehends different types of cancer of the blood affecting the cells of the immune system. As these cells are either lymphocytes or myelocytes, according to the speed of its progression, leukemia is classified in four main types: acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), and chronic myeloid leukemia (CML). Together, the different types of leukemia are one of the principal types of cancer worldwide and affect people of all ages. Of special attention is ALL, the neoplasia with the greatest mortality rates in young individuals, of which T cell subtype is the most aggressive. The first-line treatment for all leukemias principally consists of chemotherapies, monoclonal antibodies, and more recently, specific kinase inhibitors, which aim to induce apoptosis, a highly conserved mechanism of RCD, in leukemic cells. However, as mutations in the apoptotic machinery are common, and leukemic cells bear and develop in the bone marrow being protected by stromal cells, these therapies are often ineffective. Lacking treatment options for patients who have developed resistance, their prognosis is poor.

Nevertheless, years of investigation have made clear that the apoptotic machinery is not the only way for the cell to self-destruct; instead, a variety of processes involving partially or totally different cellular components have been described to lead to this outcome. This way, cell death can occur even though apoptosis is altered.

From this perspective, CD47 seems a potential target for the elimination of leukemic cells. CD47 is a transmembrane receptor member of the immunoglobulin family, which activation (e.g., through thrombospondin-1 [TSP1, one of its ligands] or mimetic agonist peptides derived from its C-terminal domain) have demonstrated to induce RCD signaling in different cancer cells. Recently, it was shown that PKHB1, the first serum-stable

soluble CD47 agonist peptide, selectively induces RCD in leukemic cells of patients with CLL, including cells of patients with refractoriness tendencies, and in advanced stages of the disease. It was described that PKHB1 engagement to CD47 induces the sustained activation of phospholipase C gamma-1 (PLCγ1), which was found to be up-regulated in CLL. This event precedes the massive mobilization of calcium ions (Ca^{2+}) to the cytoplasm that rapidly provokes the dissipation of the mitochondrial membrane potential causing the CLL-cell death. This is why the present thesis work focused on determining if the activation of CD47 with PKHB1 induced similar RCD signaling in other types of leukemic cells, as well.

To this end, PKHB1 was synthesized, and cell death was measured after its engagement to CD47 in different types of leukemic cell lines, human peripheral blood mononuclear cells, and primary cultures of mice primary and secondary lymphoid organs. Moreover, potential molecular modulators of cell death were tested to finally show that PKHB1 induces a selective caspase-independent form of RCD in leukemic cells that is not interfered by their co-culture with chemo-protective bone marrow stromal cells. Death is regulated, however, by Ca^{2+} entry into cytoplasm, as it was stated in CLL cells, suggesting that the cell death mechanism triggered by PKHB1 in other types of leukemia is similar to that activated in CLL.

The present thesis work broadens the implication of PKHB1-selectively triggered RCD to different types of leukemia, additionally to CLL. Importantly, RCD also prevails over T cell-ALL apoptotic avoidance, and occurs in a calcium-dependent manner. This was proved not only in human cells, but also in a murine cell line of T cell leukemia, opening the possibility for further *in vivo* experiments that will better illustrate the biological and pharmacological relevance of PKHB1 or other TSP1 mimetic agonist peptides. Furthermore, the identification of molecular actors that regulate Ca^{2+} release in the different types of leukemia, such as the PLCγ1, and their potential activating kinases, will be of major importance to further understand CD47-mediated RCD induction. I am confident that new perspectives will be opened in the field of investigation on anti-cancer alternatives addressing the CD47–TSP1 axis.

III. BACKGROUND

1. Regulated Cell Death

1.1. Killed by accident or by a structured plan?

Death can occur in a fortuitous or a controlled manner. This way, in cell biology, death can be classified for practical ends as accidental cell death (ACD) or regulated cell death (RCD) (Figure 1) (Galluzzi et al., 2014).

ACD is a virtually instantaneous type of cell death provoked by severe physical (e.g., high temperature), chemical (e.g., potent detergents or pH variations) or mechanical (e.g., smearing) damage, same which is insensitive to any type of pharmacological or genetic intervention. This form of cell death does not involve further machinery than the stimulus itself, and, although it can occur *in vivo*, it cannot be prevented or modulated. On the other hand, we refer to cell death as "regulated" (RCD) when we allude to the cases in which the cell utilizes a genetically encoded molecular machinery to destruct itself. From this position, RCD is susceptible of being modulated by the cell, not only by inhibiting the transaction of the lethal signals, but also by improving the cell's capacity to adapt to stress and to recover homeostasis (Galluzzi et al., 2014).

It is important to mention that RCD does not only occur as a consequence of external perturbations to the local environment, but also during embryonic development (Fuchs & Steller, 2011). Likewise, adult individuals can physiologically perform RCD to protect themselves from inner or outer threats, destroying undesired or potentially dangerous cells. Such a case can be observed in the human immune system, where death controls highly rigorous processes of selection that ensures survival of mature and non-auto-reactive lymphocytes (Kindt, Goldsby, & Osborne, 2007). It is when we refer to these types of completely physiological events when we talk about programed cell death (PCD) (Galluzzi et al., 2014).

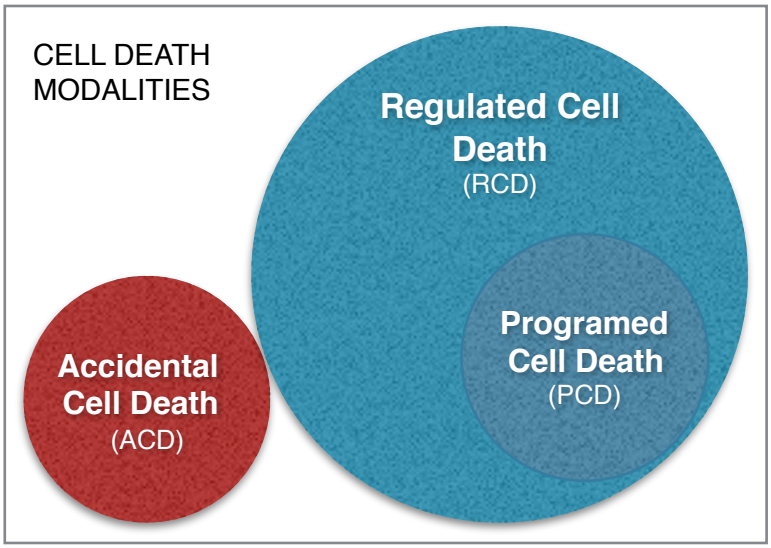

Figure 1. Types of cell death. Cells exposed to extreme physical, chemical or mechanical stimuli succumb in a completely uncontrollable manner, reflecting the immediate loss of structural integrity. We refer to such instances of cellular demise with the term 'accidental cell death' (ACD). Alternatively, cell death can be initiated by a genetically encoded machinery. The course of such 'regulated cell death' (RCD) variants can be influenced, at least to some extent, by specific pharmacologic or genetic interventions. The term 'programmed cell death' (PCD) is used to indicate RCD instances that occur as part of a developmental program or to preserve physiologic adult tissue homeostasis. (Extracted from Galluzzi, *et al*. 2015)

1.2. Defining death: disambiguating apoptosis from Regulated Cell Death (RCD)

This wide classification proposed by the Nomenclature Committee on Cell Death (NCCD) helps to resolve the conflicts that arise when the term "apoptosis" is used as a synonym of RCD, not taking into account molecular or biochemical criteria that are relevant in practice. And, although the apoptosis is indeed a form of RCD, there is a wide variety of other different mechanisms that the cell uses to ensure its demise.

All RCD processes are triggered by an initiator mechanism, which can be an inner or an outer stimulus, that sooner or later activate a molecular machinery that engages the cell to die through activating different executioner mechanisms (Galluzzi et al., 2014). This operation can be stopped and revoked until the cell is driven across the boundaries between life and death, which have been defined as "points of no return" (Kroemer et al., 2009). It has been proposed that these points after which death is imminent can be represented by the massive activation of caspases (cysteine proteases), the loss of the

mitochondrial transmembrane potential (Δψm) and the entire permeabilization of the mitochondrial outer membrane (MOMP), or the outside exposure of phosphatidylserine (PS) residues as an *eat me* signal for adjacent phagocyte cells (Kroemer et al., 2005). However, caspase activation is not limited to cell death pathways (Lamkanfi, Festjens, Declercq, Berghe, & Vandenabeele, 2007), the Δψm can be dissipated by protonophores without progression to immediate cell death (de Graaf et al., 2004), and PS exposure can be reversible [e.g., in blood granulocytes (Yang, Chuang, Chen, & Yang, 2002)]. Hence, as a unique point of no return has not been stablished yet, at least two of these parameters should be fulfilled to determine that a cell is actually committed to die. Other way, a cell should be only considered as *dead* when: (1) its plasma membrane has lost its integrity (*in vitro*), (2) the cell itself and/or its nucleus have fragmented, and/or (3) it has been phagocyted by adjacent cells (*in vivo*) (Kroemer et al., 2009).

Being aware of these key aspects of RCD substantially contributes to its comprehension. The homogeneity in the flux of information not only allows us a better understanding of the process, but also widens the possibilities for addressing current dilemmas of the practice, and permits a rational escalation. In such way, the logical classification of cell death subroutines according to their conserved morphological features, actual biochemical and molecular hallmarks, and considering the scopes and limitations of current technical approaches, takes singular relevance.

Keeping in mind the above mentioned, the apoptosis should be only defined as such when we refer to the widely described mechanism of RCD that is morphologically characterized by the rounding-up of the cell, retraction of pseudopodes, reduction of cellular volume, condensation of the chromatin, fragmentation of the nucleus, little or no ultrastructural modification of cytoplasmic organelles, plasma membrane blebbing, and maintenance of an intact plasma membrane until late stages of the process (Kroemer et al., 2009).

Moreover, the biochemical features of apoptosis include initiator mechanisms that can be either extrinsic (eg. the ligation of the tumor necrosis factor alpha to its receptor on the cell surface) or intrinsic (eg. DNA damage), and the following signaling that will

generally cause the mitochondrial outer membrane permeability (MOMP), owed to the pore-forming activity of pro-apoptotic members of the BCL-2 protein family such as BAK and BAX. This will cause $\Delta\psi m$, and the release of pro-apoptotic proteins such as the cytochrome C from the mitochondria, and the later activation of executioner caspases (Galluzzi et al., 2012). However, it is important to mention that, in particular cases, apoptosis can prescind of some of these features, but still build up its morphological hallmarks, thanks to all of the other molecular participants (Galluzzi et al., 2012).

Indeed, the extensive knowledge generated from studying apoptosis has led to the identification of various forms of non-apoptotic cell death modalities in several physiological processes or as a response to treatment (Gross & Katz, 2017; Kutscher & Shaham, 2017; Y. Liu & Levine, 2015; Ranjan & Iwakuma, 2016; Vanden Berghe et al., 2014). These other types of RCD can use some modules utilized in apoptosis (such as caspases or different BCL-2 family members) or even leave them aside. Instead, a variety initiators and executioners give dying cells different morphological characteristics (Kroemer et al., 2009). Importantly, the molecular machinery utilized in the different RCD modalities significantly differ from each other, evidencing the extremely complex network that evolution has built to favor those who can, by different means, ensure cell death.

1.3. Non-apoptotic forms of RCD: autophagy, necrosis, and beyond

There is a vast list of non-apoptotic RCD modalities. Nevertheless, together with the traditional morphological classification of cell death (Kroemer et al., 2009), which categorizes apoptosis as type I cell death, two other main types: autophagy (type II) and necrosis (type III) have been widely described.

Autophagy (derived from the greek for *self-digestion*) is normally performed by cells to eliminate old or useless proteins and organelles, and has important functions in cell remodeling through differentiation, stress or cytokine-induced damage. However, although physiological levels of autophagy are essential for the maintenance of cellular homeostasis, cells performing it excessively become engaged into a RCD process

(Glick, Barth, & Macleod, 2010). Autophagic cell death is characterized by an early formation of double-membrane structures termed phagophores, which mature forming autophagic vesicles called autophagosomes. These two-layer vesicles sequester great part of the cytoplasm and organelles, and, canonically, they get degraded by self-cell lysosomal system (Klionsky et al., 2016). Autophagy can initiate as a result of starvation or treatment with rapamycin (sirolimus), which will activate ULK1/2 by inhibiting mTOR, promoting beclin 1 phosphorylation and further phagophore formation (Kang, Zeh, Lotze, & Tang, 2011; Nazarko & Zhong, 2013). Chemically synthesized autophagy-inducing peptides, such as Tat-Beclin 1, can also induce autophagy by mimicking beclin 1, impeding protein aggregation, and pathogen replication *in vitro*, and reducing mortality in mice infected with chikungunya or West Nile virus (Shoji-Kawata et al., 2013).

Specific forms of autophagic cell death such as autosis (Y. Liu & Levine, 2015), have been characterized. Autosis is distinguished by enhanced cell substrate adhesion, focal ballooning of the perinuclear space, and dilation and fragmentation of endoplasmic reticulum. It can be triggered by autophagy-inducing peptides, starvation, and neonatal cerebral hypoxia-ischemia, and neither caspases or regulated necrosis inhibitors, nor genetic deletion of pro-apoptotic Bax, Bak, or necrosis regulatory proteins, are able to block autosis. Pharmacological inhibition or genetic inactivation of the Na^+,K^+-ATP-ase, however, strongly regulates autosis *in vitro* and *in vivo* in cerebral hypoxia-ischemia (Y. Liu & Levine, 2015). Autosis is a very interesting example of a cell death pathway that uses a key modulator in the machinery of cell maintenance to suicide.

On the other hand, and differently to the type I and II cell death, in which morphological features clearly advocate a *bona fide* mechanism willing to maintain homeostasis, type III cell death, characteristic in cells undergoing necrosis, used to be considered as a passive, merely accidental process, that occurred in response to severe physical or chemical damage. Nevertheless, genetic evidence on necrosis regulators as well as the discovery of molecular inhibitors of necrosis contradicts this idea, revealing us the existence of

multiple pathways of RCD pathways that give the cell necrotic characteristics (Vanden Berghe et al., 2014), and that are described below.

1.3.1. Regulated Necrosis

Regulated necrosis is defined as a cell death process that occurs under control of a genetically encoded mechanism that eventually induces plasma membrane permeabilization (PMP), and is morphologically characterized by cytoplasmic granulation, and organelle and cell swelling (oncosis) (Vanden Berghe et al., 2014). A variety of RCD modalities share this morphological features (necroptosis, parthanatos, oxytosis, ferroptosis, ETosis, NETosis, pyronecrosis, pyroptosis), and it has been described that their molecular mechanisms are inter-connected, becoming active by different stimuli. Actors participating in these mechanisms depend on the nature of the stimulus and the cell type, leading to different outcomes according to the particular cellular context. In consequence, it has recently been proposed a way to schematically classify regulated necrosis considering the ordered steps that different cell death mechanisms follow (Vanden Berghe et al., 2014). Tom Vanden Berghe, and colleagues propose to divide regulated necrosis into four steps that define it (Figure 2): when a stimulus (Level 1, the outermost) triggers an initiator (Level 2, which can be blocked by different molecular inhibitors) this last activates one or more of the intracellular mediator mechanisms (Level 3), which effectuate the biochemical processes that execute regulated necrosis (Level 4) and give the cell its morphological and biochemical hallmarks.

Overlapping pathways between different initiators and mediators advises the existence of an intricate yet relatively common interacting-network that eventually affects similar executioner components (Vanden Berghe et al., 2014). These recurrent biochemical mechanisms categorized in levels 2 and 3 suggest that regulated necrosis machinery is a product of evolution, as it has been described that it plays a role in neuronal cell death (Seiler et al., 2008) and as defense against infections (Cho et al., 2009).

Regulated necrosis weaponry comprises the metabolism of oxidation-reduction and of bioenergetics, which control oncosis and PMP, lysosomal membrane permeability

(LMP), and MOMP. Its distribution is extensive, and allocated to different organelles. However, on the following pages attention will be focused on the plasma membrane and the endoplasmic reticulum, which play an active role in cell death induction that is relevant for the present work.

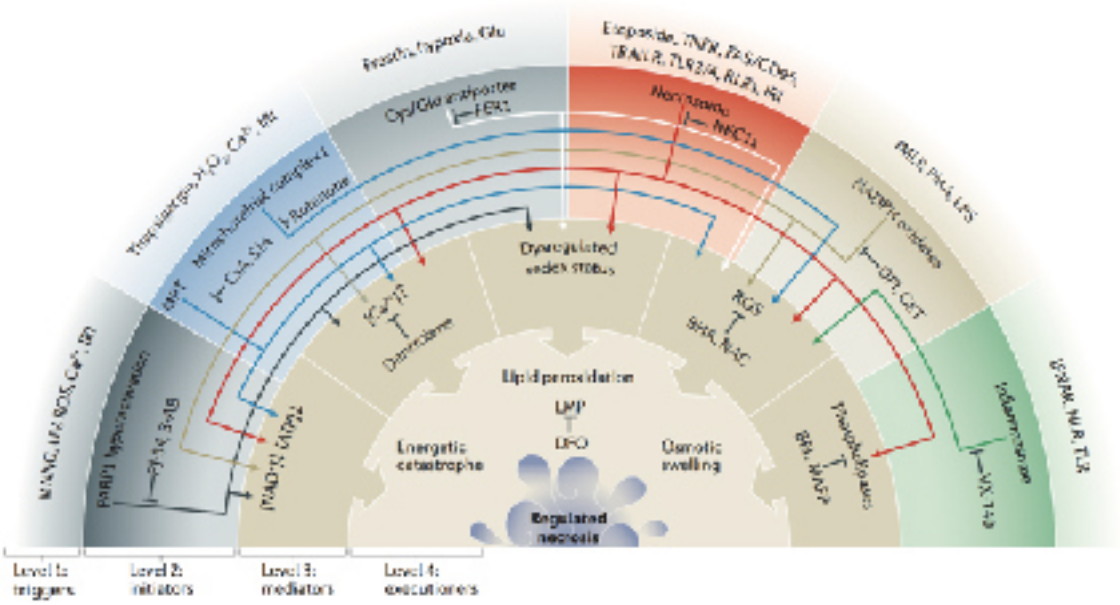

Figure 2. An integrated view of the emerging modes of regulated necrosis. Regulated necrosis can be induced by poly(ADP-ribose) polymerase 1 (PARP1) hyper-activation, mitochondrial permeability transition (MPT), mitochondrial complex I, the Cys/Glu antiporter, the necrosome, NADPH oxidases and the inflammasome. Diverse pathophysiological stimuli can trigger (level 1) each of these initiators (level 2), which can be blocked by the listed specific inhibitors. The colored arrows indicate the established links between the initiator signals and various common intracellular mechanisms that mediate regulated necrosis (level 3), such as NAD^+ and ATP-depletion, Ca^{2+} overload, deregulation of the redox status, increased production of reactive oxygen species (ROS) and the activity of phospholipases. All of these factors are mediators of regulated necrosis, and even at this level, inhibitors such as dantrolene, BHA (butylated hydroxyl anisole), NAC (*N*-acetyl-Cys), BEL (bromoenol lactone) and MAFP (methyl-arachidonyl fluorophosphonate) may interfere with necrotic signaling. Importantly, similar mediators can act downstream of various initiators, through different mechanisms. The complex interconnected effects of the mediators on cellular organelles and membranes results in the activation of processes that execute regulated necrosis (level 4), including cellular osmotic swelling, bioenergetic breakdown that results in energetic catastrophe, lipid peroxidation and the loss of lysosomal membrane integrity through lysosomal membrane per-

meabilization (LMP). Note that feedback loops are not included for simplicity (Extracted from Vanden Berghe, et al. 2015).

1.3.2. The link between the plasma membrane and the endoplasmic reticulum in cell death

Plasma membrane does not only separate the core of the cell from outer environment, it also actively participates in cell homeostasis and cell death. It is made up of phospholipids and embedded proteins that perform diverse tasks that are crucial in these processes. Plasma membrane actively participates in RCD by recognizing lethal signals and mediating outside-in signaling of death receptors, such as the previously mentioned receptor of the TNFa (Guicciardi & Gores, 2009), different toll-like receptors (TLRs) (Salaun, Romero, & Lebecque, 2007), or CD47 (see chapter 3: CD47). In addition, plasma membrane is selectively permeant to certain ions and organic molecules, mediating their flow outside or inside the cell; it also controls chloride, potassium, and calcium ionic channels (Cooper, 2000), actively participating in RCD by modifying cell's inner ionic composition (Y. Liu & Levine, 2015; Martinez-Torres et al., 2015; Vanden Berghe et al., 2014). Furthermore, plasma membrane avails the generation of diverse second messengers (Cooper, 2000) that activate different cell death executioner mechanisms (Brini, Calì, Ottolini, & Carafoli, 2013; Insel, Zhang, Murray, Yokouchi, & Zambon, 2012). Finally, during the last stages of RCD, cell membrane exposes "eat me" signals such as phosphatidylserine and calreticulin to promote phagocytic recognition and phagocytosis *in vivo* (Gardai et al., 2005; Kroemer et al., 2005). Additionally PMP is one of the markers of cell death *in vitro* (Kroemer et al., 2005).

The endoplasmic reticulum (ER) is a membrane-surrounded network of interconnected flattened tubules that controls various processes of cell survival and cell homeostasis. The ER participates in protein folding, protein transportation and lipid biosynthesis. It also controls mitochondrial biogenesis, and is the largest store of calcium ions (Ca^{2+}) of the cell. (Brini et al., 2013)

Ca^{2+} is an extremely regulated and versatile second messenger. At physiological conditions, extracellular Ca^{2+} concentration goes around 1.2 mM, while in the cytosol oscillates the 100 nM (Berridge, 2002; Berridge, Lipp, & Bootman, 2000), generating a gradient greater that 10,000:1. At the ER, Ca^{2+} concentrations oscillates between 100 and 500 μM (Berridge, 2002; Berridge et al., 2000) which is 2-10 fold less than at the outside of the cell, but from 1,000 to 2,000 fold greater that at the cytosolic space. These facts urge two things: *1)* Ca^{2+} mobilization into the cytoplasm ought to be required for vital means, and *2)* a fine regulation must mediate this mobilization in order to avoid an overload.

Indeed, Ca^{2+} mobilization mechanisms ubiquitously control diverse cell processes such as cell proliferation, metabolism, and gene transcription, but also RCD (Plattner & Verkhratsky, 2016). Different proteins control these tasks. Mobilization may be triggered either by signals coming from stimulated receptors on the plasma membrane or by a Ca^{2+} feedback loop mechanism known as "calcium-induced calcium release" (CICR) (Berridge et al., 2000; Pinto et al., 2015). The mechanism of CICR involves the activation of the Ca^{2+} release channels in the ER membrane, principally the ryanodine receptor (activated by Ca^{2+} itself), and the inositol triphosphate (IP_3) receptor (IP_3R), which activates calcium mobilization after its engagement with IP_3, a product of the phosphatidylinositol diphosphate (PIP_2) breakdown by phospholipase C (PLC) (Berridge, 2002; Martínez-Torres, 2013).

Ca^{2+} release into the cytosol via IP_3R and CICR induces the depletion of the Ca^{2+} stores in the ER through the activation of the store-operated Ca^{2+} entry (SOCE) mechanism (Figure 3) (Martínez-Torres, 2013; Pinto et al., 2015). In fact, ER transmembrane proteins that function as calcium sensors in the lumen of the ER (the stromal interaction molecules, STIM-1 and/or STIM-2) will detect low Ca^{2+} levels inside ER, and thus, associate with Orai (1, 2 or 3) proteins to form the calcium-release activated calcium (CRAC) channels, which finally permit the entry of functionally relevant levels of Ca^{2+} (Thierry Capiod, 2013; Martínez-Torres, 2013; Pinto et al., 2015; Xu, Cioffi, Alexeyev, Rich, & Stevens, 2015).

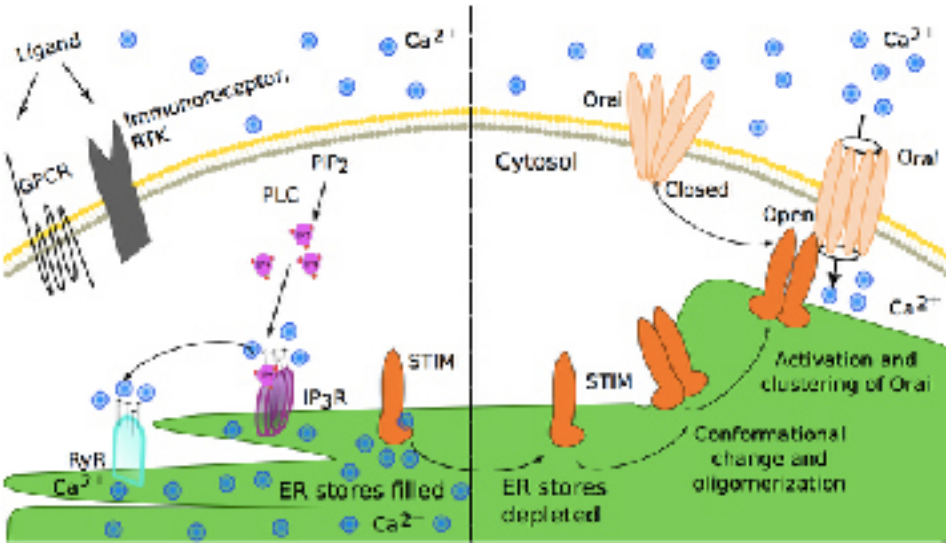

Figure 3. STIM and Orai proteins mediate store-operated Ca^{2+} entry. In cells with replete endoplasmic reticulum (ER) stores, the stromal interaction molecules (STIM) are localized in the membrane of the ER away from the plasma membrane. Depletion of Ca^{2+} stores following the activation of G-protein coupled receptors (GPCR), receptor-tyrosine kinases (RTK), or immunoreceptors, trigger phospholipase C (PLC) activation and Ca^{2+} release from the ER through its receptors. The reduction in the Ca^{2+} concentrations in the lumen of the ER results in dissociation of Ca^{2+} from STIM, its aggregation and conformational change (right). Then, STIM translocates to sections of the ER juxtaposed to the plasma membrane and binds Orai, resulting in the opening of Orai-CRAC (calcium release activated Ca^{2+}) channels. (Extracted from Martínez-Torres, 2013)

Differences in Ca^{2+} mobilization can lead to different outcomes depending on the specific cellular context, physiologic condition, and spatial and temporal circumstances (Berridge et al., 2000; Thierry Capiod, 2013). For example, it has been described that cancer cells have increased cytosolic Ca^{2+} levels compared to normal cells, which is a result of a major rearrangement of Ca^{2+} pumps, sodium-calcium exchangers, and Ca^{2+} channels (Monteith, McAndrew, Faddy, & Roberts-Thomson, 2007; Stewart, Yapa, & Monteith, 2015). These adaptations of cancer cells enhance proliferation and impair cell death (T Capiod, Shuba, Skryma, & Prevarskaya, 2007; Orrenius, Zhivotovsky, & Nicotera, 2003). Actually, many proteins that have been historically described to be remodeled in cancer cells are kinases related to Ca^{2+} signaling, such as the Src and Ras family kinases, which can amplify IP_3 (and thus Ca^{2+}) signaling by increasing the stimu-

lation of different PLCs (Bivona et al., 2003; Rusanescu, Qi, Thomas, Brugge, & Halegoua, 1995; Stith, 2015).

In the previous paragraphs some aspects of cell death have been described, highlight ing the importance of its regulation to maintain homeostasis in a multicellular organism. Remembering that this regulation is only possible thanks to a genetically encoded molecular machinery, it might be intuited that certain genetic mutations could alter this process, resulting in severe physiopathological implications. In one of the most know examples, cancer, this is clearly observed, as the resistance to PCD is one of the hallmarks of the disease (Hanahan & Weinberg, 2011).

2. Leukemia

2.1. A major type of cancer

Cancer is a vastly heterogeneous group of diseases that surge within self-cells. Basically, all multi-cellular organisms are susceptible to originate cancer, and cancer can surge from any of their cells. According to its origin, cancer has been classified in five categories: 1) carcinoma (epithelium); 2) sarcoma, (connective tissue); 3) myeloma, (plasma cells); 4) leukemia (leukocytes originated in the bone marrow); and 5) lymphoma (leukocytes accumulated in the lymph nodes). Complex types of cancers comprising two or more of the above mentioned are also (U. S. National Institutes of Health, 2017).

Different alterations in the organism contribute to the evolution of cancer by making cells to grow uncontrolledly, evade death, and spread over surrounding tissue, provoking organs malfunction. It has been proposed that cancer is the result of ten principal alterations in the cell's physiology that act collectively to confer it capacities that let it survive, proliferate and disseminate. These alterations, known as "the hallmarks of cancer" originate in a multi-step mutation process that is also multi-factorial, and are acquired gradually, favored by a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of these traits by creating the tumor microenvironment. (Hanahan & Weinberg, 2011)

Leukemia, the type of cancer studied in this work, is characterized by the excessive accumulation of certain white blood cells in the blood or the bone marrow, where they impede the correct functioning of other cells (National Cancer Institute, 2013). Similarly to any other type of cancer, leukemia surges as a consequence of the development of molecular characteristics in a leukocyte that distinguish it from their normal equivalents and confer it adaptive advantages that make it accumulate. Such capacities are acquired thanks to the bone marrow, where leukemia is formed.

2.2. The bone marrow microenvironment and chemo-protection

The bone marrow entails a dynamic web of growth factors, cytokines and cells that provide suitable conditions for leukemic cells to grow (Nwajei & Konopleva, 2013). Leukemia develops in this microenvironment composed by hemato-lymphoid cells, which are immersed in a media surrounded by mesenchymal tissue that protects them (Sison & Brown, 2011). Both, cells and compartment will be described in the following paragraphs.

The mesenchymal compartment is composed of the extracellular matrix, common and hematopoietic mesenchymal cells, endothelial progenitor cells, and stromal cells (Ayala, Dewar, Kieran, & Kalluri, 2009). Inside these compartments or "proliferation niches" is common to find cells involved in stem cell maintenance, such as the CXCL12 abundant reticular (CAR) cells (Greenbaum et al., 2013; Sugiyama, Kohara, Noda, & Nagasawa, 2006) and marrow stromal cells (Sison & Brown, 2011).

Bone marrow stromal cells (BMSC) are in this way, mesenchymal cells, and include fibroblasts, adipocytes, endothelial cells, and osteoblasts. These are the principal source of growth factors and cytokines for the hematopoietic cells in normal and pathological conditions (Ayala et al., 2009). These cells not only function as a biological barrier that divides and nurtures the hematopoietic stem cells, in leukemia they also contribute in the potentiation of growth, the angiogenesis and the initiation of the metastatic phenotype by providing them with different cytokines and growth factors (Meads, Hazlehurst, & Dalton, 2008). For example, in leukemias of rapid progression, interleukin (IL)-8 is up-regulated as a result of the increased interaction of CXCL12 (the principal cytokine produced by BMSC) with its receptor (CXCR4) (Scupoli et al., 2008) and promotes angiogenesis in the bone marrow microenvironment (Li et al., 2005). Similarly, growth factors and angiogenic factors produced by stromal cells, such as angiopoietins, the vascular endotelial growth factor (VEGF), and the hepatocytes growth factor (HGF), contribute to the development and proliferation of the leukemic cells (Bellamy, 2001; Konopleva et al., 2002). Its relevance is such, that genetic polymorphisms in microenvironment-related markers, such as the VEGF (Kim et al., 2009; Lozano-Santos et al., 2014) and CX-

CL12 (Dommange, 2006; Peled & Tavor, 2013), have demonstrated to have prognostic value, and have been strongly implicated in resistance of tumors to treatment (Meads, Hazlehurst, & Dalton, 2008).

As mentioned above, the mesenchymal compartment of the bone marrow supports leukemic cell survival by different means. In addition, leukemic cells interact with other cells in the hematolymphoid compartment which is immersed in the mesenchyme. This compartment consists of diverse cell types, including healthy and malign leukocytes. These, as well as every other blood cell, are generated in a cell differentiation process known as hematopoiesis, which takes place in the bone marrow hematopoietic compartment (Ayala et al., 2009).

Hematopoiesis (Figure 4) begins from a small population of pluripotent cells called hematopoietic stem cells (HSCs), which have the ability to differentiate in every blood cell type and are able to regenerate as well. Regenerative HSCs maintain an opened cell cycle that allows continuous production of hematopoietic cells, these are known as long term HSC. The rest of the progeny cannot regenerate, and they differentiate into a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP), from which every other specific blood cell types comes from. The incapacity of common progenitor cells to regenerate is a consequence of maturation of the HSC, which consists in changes in its gene expression; this way starting to define its specific cell type.

The number of each cell type is finely regulated by cell proliferation and PCD, which keep them at homeostatic levels. An imbalance between these processes provokes leukemia, which is characterized by the overproduction of leukocytes or their non-differentiated immature progenitors (blasts). Accumulation of leukemic cells in blood and lymphoid organs hampers oxygen transfer to tissues, bleeding control, and the ability of the host to fight off infections (National Cancer Institute, 2013).

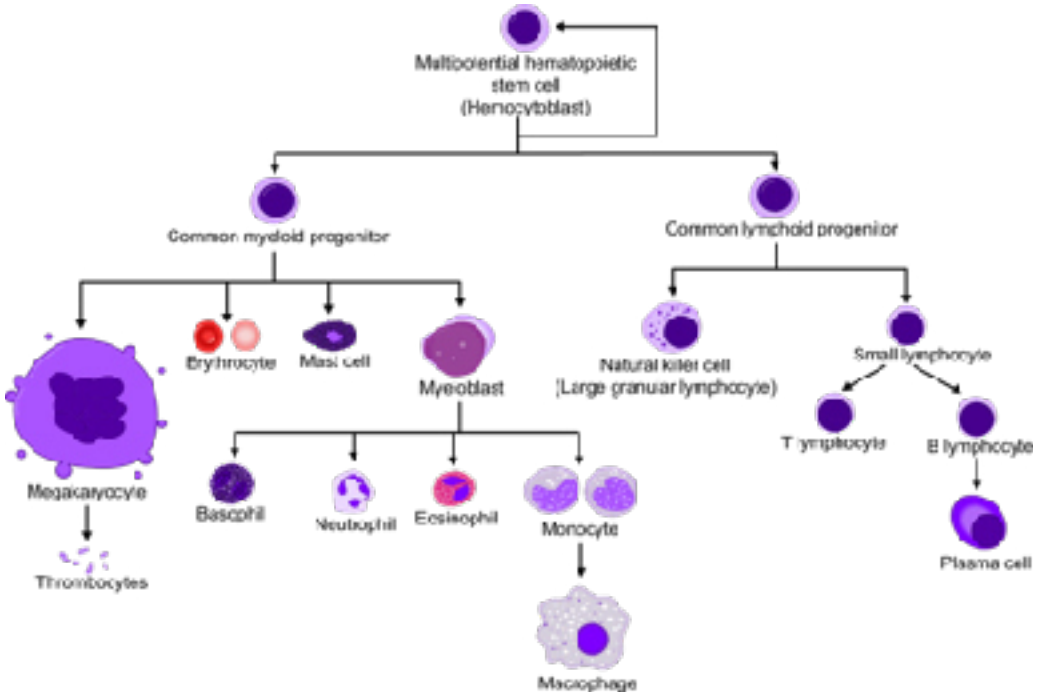

Figure 4. Hematopoiesis. A multipotential hematopoietic stem cell HSC can give rise to another HSC, a common lymphoid progenitor (CLP), or a common myeloid progenitor (CMP), which at the same time mature into every other blood cell lineages.

2.3. A worldwide public health issue

It is estimated that in 2012 around 352,000 people from all over the world were diagnosed with leukemia, differentially according to location (Cancer Recearch UK, 2012). In the United Kingdom, for example, leukemia is the eighth most common type of cancer, with over 8,600 patients diagnosed in 2011 (Cancer Recearch UK, 2012). In comparison, in the United States 52,380 new cases joined the over 327,500 people already living with some type of leukemia or in remission in 2014 (Leukemia & Lymphoma Society, 2015).

In Mexico there are no exact figures regarding the cases of leukemia. However, in recent years Mexican government has recognized and addressed this health issue with the inclusion of some adult hematological malignancies to the National Commission for Social Healthcare, most known as “Seguro Popular” that sums to the already covered pediatric cancers, of which leukemia is the most commonly diagnosed.

Nevertheless, the Mexican National Institute of Statistics, Geography and Informatics (INEGI), estimates that each year around 7,000 children develop leukemia, and 90% of them will die without having received adequate medical attention (INEGI, 2016). In part, this is attributable to the lack of treatments for leukemia in advanced stages, where the disease is frequently diagnosed as a result of the common limitations of human and technical resources, not only in Mexico but Latin America and the Caribbean (Goss et al., 2013).

2.4. Characteristics, epidemiology, diagnostics and prognostics of leukemia

The molecular characteristics of leukemia vary depending on the type of damaged cell, and so do their treatment. Hypothetically there could be as many types of leukemia as cell types in the blood; however, their classification comes according to clinically relevant criteria. This has been achieved by classifying leukemia in four classes comprising the two principal cellular lineages from which the leukemic cell comes from (lymphoid or myeloid) and the speed with which it develops (acute or chronic). This way, the principal types of leukemia are: acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), and chronic myeloid leukemia (CML) (National Cancer Institute, 2013).

Leukemia is commonly mistaken as other diseases since their symptoms include weakness, loss of appetite, fever, queasiness, among other signs of general discomfort, which can be explained by many different infectious diseases. Then, diagnosis of leukemia normally begins with clinic history and routine health check-up, specifically blood work (total blood count and blood chemistry), in which a high leukocyte count is observed. Generally a higher count of lymphocytes correlates with a poorer prognostic. A bone marrow biopsy obtained by lumbar puncture provides a definitive diagnosis. From this biopsy, pathological information is obtained through the tissue immunophenotypification that defines leukemia as lymphocytic or myeloid. (National Cancer Institute, 2013; U. S. National Institutes of Health, 2017)

2.4.1. Acute Lymphocytic Leukemia (ALL)

ALL is a form of leukemia of rapid progression that is characterized by the excessive accumulation of monoclonal lymphocytes in the blood, which impedes the production of normal cells. In general, ALL can originate either from B or T cells. B cell ALL (B-ALL) is the most common type of ALL (~80%), nevertheless T cell ALL (T-ALL) is extremely aggressive and very often lethal (National Cancer Institute, 2013). ALL is the most common type of pediatric cancer (nearly one of each four cases), though it is mostly diagnosed in adults. (National Cancer Institute, 2013; NIH, 2015; PDQ Adult Treatment Editorial Board, 2017a)

A morphological bone marrow assessment represents the first step in the diagnostic pathway, for the primary diagnosis of ALL since ALL, by definition, always presents with bone marrow involvement (Chiaretti, Zini, & Bassan, 2014). Cytogenetic aberrations are commonly encountered, and they are generally related to prognosis (Mrózek, Harper, & Aplan, 2009). For example, translocation of the short arm of chromosome 12 in the long arm of chromosome 21 [t(12;21) (p13;q22)], found in 15-35% of pediatric patients with B-ALL, induces the fusion of genes *ETV6* and *RUNX1* (ETV6/RUNX1 fusion gene); this is related to complete remission after first line treatment, thus, prognosis seems good (Burmeister et al., 2010). On the other hand, translocation t(9;22) (q34;q11), present in around 5% of ALL patients (PDQ Adult Treatment Editorial Board, 2017a), gives rise to the Philadelphia chromosome (Ph), which contains the BCR/ABL1 translocation gene (see Chronic Myeloid Leukemia) and used to correlate with a very short remission duration and median progression-free survival (PFS) before the era of the tyrosine kinase inhibitors (TKIs) (Hoelzer et al., 1988).

Currently the therapy for ALL patients is primordially determined by the age of the patient and the Ph status. In general, treatment principally consists of a standard multi-agent chemotherapy regimen (e.g., CALGB 8811 [daunorubicin, vincristine, prednisone, pegaspargase, cyclophosphamide]) for $Ph^{(-)}$ patients, and chemotherapy (e.g., hyper-CVAD [cyclophosphamide/vincristine/doxorubicin/dexamethasone]) plus TKIs (eg. imatinib) for $Ph^{(+)}$ patients (Medsc, 2016). Complete remission (CR) is achieved in ~80%

cases. However, the reemergence of the disease (relapse) occur in nearly 20% of patients, principally in $Ph^{(+)}$- and T-ALL patients (Locatelli, Schrappe, Bernardo, & Rutella, 2012; PDQ Adult Treatment Editorial Board, 2017a). Lacking treatment options for patients who have developed resistance, their prognosis is poor. Allogenic hematopoietic stem cell transplantation (HSCT) increases their chances of complete remission in these patients, although not all patients are candidates (Medsc, 2016).

2.4.2. Chronic Lymphocytic Leukemia (CLL)

CLL is a chronic leukemia characterized by the accumulation of monoclonal $CD5^+$/$CD23^+$ B-lymphocytes in blood, bone marrow and lymph nodes (Martinez-Torres et al., 2015). CLL is the most common type of leukemia in western countries, constituting around 20-30% of total cases of leukemia (PDQ Adult Treatment Editorial Board, 2016b). Age increases the risk for developing CLL; 90% of the cases occur in people older than 50 years, being 71 years the median age at the moment of diagnosis, although it can also affect younger individuals (Delgado & Villamor, 2014).

Although over 80% of CLL patients are diagnosed in early stages of the disease, the course that CLL will take is highly unpredictable. Nonetheless, some parameters such as the ZAP70 and CD38 expression levels, *IGHV* genes mutational status, presence of soluble CD23, soluble thymidine kinase, some cytogenetic anomalies such as depletions, translocations, trisomies among others, are related with the curse of the disease. (Martínez-Torres, 2013)

Around four out of five cases of CLL present genetic aberrations that are clinically relevant. For example, a deletion in the long arm of chromosome 13 (deletion 13q14), found in over 50% of CLL patients, generates the over expression of BCL-2, and is related to a good prognosis. Deletion 17p13 which is present in around 7% of the patients though, provokes the mutation of the *p53* gene "the guardian of the genome" which regulates various processes of RCD, leading to resistance to chemotherapy and a poor prognosis. (Martínez-Torres, 2013)

Current therapies against CLL generally consist on chemoimmunotherapy (CIT), a regimen of chemotherapy such as fludarabine and cyclophosphamide, which act by interfering DNA replication, and are applied together with Rituximab, a monoclonal antibody targeting CD20, a regulator of cell cycle progression in B lymphocytes (Medscape, 2016a). A recent follow-up of a phase II study of patients treated with this regimen shows that PFS has been achieved at the median follow-up (~6.4 years) in 30.9% of patients, most of them with a mutated *IGHV* status (Thompson et al., 2016). However, there is no accepted cure for CLL, with the exception of HSCT, and it is expected that although treatment with CIT prolongs PFS and survival, all patients will ultimately relapse (Thompson et al., 2016). Moreover, the same treatment in *p53*-deficient patients leads to unfavorable results, as aggressive cells seem to be micro-evolutively selected. Alternative treatment for these and other refractory patients are scarce. Bendamustine has been tested together with immunotherapies in different phase III trials, leading to an improvement of PFS without further compromising quality of life, but still, without reaching complete remission (Cortelezzi et al., 2014; Fischer et al., 2012). Yet, the advent of kinase inhibitors and early data reported with their use in patients with previously untreated disease are promising (J. R. Brown, 2013; Byrd et al., 2016). Moreover, potential peptide-based strategies targeting the transmembrane receptor CD47, tested in early preclinical studies, are currently meaning to overcome disease resistance (Martinez-Torres et al., 2015).

2.4.3. Acute Myeloid Leukemia (AML)

AML is principally characterized by the overproduction of myeloblasts in the bone marrow (PDQ Adult Treatment Editorial Board, 2016a; PDQ Pediatric Treatment Editorial Board, 2016). This is the most common type of adult acute leukemias and its prevalence increases with age, being 70 years the average age of diagnosis (PDQ Adult Treatment Editorial Board, 2016a). The American Society of Cancer (ACS) esteems that there were around 18,800 new AML cases in the United States in 2014, being almost one-third of all cancers in adults older than 20 years (American Cancer Society, 2017).

Immunophenotyping is necessary for its diagnosis, and due to the high heterogeneity of the disease it includes a wide variety of surface markers. The widest used acceptance criteria is that taking over 20% cells expressing precursor phase markers (CD34, CD38, CD117, CD133, HLA-DR), and/or granulocytic (CD13, CD15, CD16, CD33, CD65, cytoplasmic MPO), monocytic (NSE, CD11c, CD14, CD64, CD36), megakaryocytic, (CD41, CD61, CD62) or erythroid (CD235a) markers (van Lochem et al., 2004). This will reveal the type of AML being faced, and the possible treatment and prognosis.

Subdivision of AML also regularly predicts treatment-related mortality, and it gains importance with the age of the patient, as resistance to conventional therapy can be predicted together with some chromosomal anomalies (Papaemmanuil et al., 2016). For example, one of the most common aberration is the t(8;21)(q22;q22), that give rise to the *RUNX1-RUNX1T1* gene. It is rare in elderly patients but frequent in childhood AML, and it belongs to the favorable risk AML subset, with remission rates of over 90% after high dose chemotherapy (Duployez et al., 2014). However, relapse incidence reaches up to 30% in these patients (Duployez et al., 2014) as further secondary genetic lesions impacting on clinical outcome have been described (Krauth et al., 2014).

Acute promyelocytic leukemia (APL) is a relatively rare subtype of AML, accounting for 8% to 15% of all acute non-lymphocytic leukemia patients (Degos & Wang, 2001; Lu et al., 2014). Its treatment with all-*trans* retinoic acid (ATRA) is one the greatest success stories in the history of cancer treatment, as it accounts for a 90% cure rate (Coombs, Tavakkoli, & Tallman, 2015). *In vitro*, it has been shown that ATRA treatment induces maturation of granulocytes which further perform apoptosis (Degos & Wang, 2001). *In vivo*, APL cells obtained from patients engaged on ATRA regimen seemed to be morphologically affected; treatment was also implicated in regulation of cell cycle progression and apoptosis, and possibly also in microvascular endothelial cell functions (Ryningen et al., 2008). ATRA treatment in APL has achieved a diminishment of relapse from 50% to less than 10% in the past two decades (Lu et al., 2014), however treatment for ATRA resistant patients with APL relies mostly on the allogenic HSCT (Medscape, 2015), which is not always feasible.

In the wider picture, between 10% and 40% of newly diagnosed patients with some type of AML will relapse after intensive induction therapy (Thol, Schlenk, Heuser, & Ganser, 2015). And, as a standard treatment protocol has not been stablished yet, few of these patients can aspire to be cured with conventional salvage therapy regarding eligibility for allogeneic HSCT, the treatment with the current highest probability of cure (Thol et al., 2015).

2.4.4. Chronic Myeloid Leukemia (CML)

CML is characterized by an augment in the proliferation of immature granulocytes that not necessarily lose their ability to differentiate (PDQ Adult Treatment Editorial Board, 2017b). Consequently, blood work shows an elevated number of granulocytes and their immature precursors, occasionally including blasts. This type of chronic leukemia stands for at least one out of each six cases of adult leukemias, being 65 years old the median age at the moment of diagnosis (PDQ Adult Treatment Editorial Board, 2017b). Although Mexico lacks of reliable incidence rates for such a disease, specialists regard CML as the most treated leukemia in Mexico, which seems to initiate earlier in the economically active age (between 37 and 40 years old) than in caucasian populations (Cervera et al., 2013).

Philadelphia chromosome (Ph) is a telltale sign of CML, although it is also frequent in adult ALL, and scarcely ever in childhood ALL (PDQ Adult Treatment Editorial Board, 2017b). Patients presenting CML symptoms and immunophenotype but who are $Ph^{(-)}$ are considered as having non-differentiated myelodysplastic syndrome and treated as such, as the curse of their disease is completely different to that of $Ph^{(+)}$ CML patients (Pugh, Pearson, Vardiman, & Rowley, 1985).

As previously mentioned, Ph originates from t(9;22)(q34;q11). Such translocation leads to the fusion of an Abelson murine leukemia viral oncogene homolog (ABL1) with the breakpoint cluster region (BCR) gene. ABL1 codes for a tyrosine kinase protein that is involved in diverse cell processes such as cell division, cell adhesion, cell differentiation, and cellular responses to stress. Its translocation with BCR induces the formation of

an *always on* state-chimeric protein that results in the cell's uncontrolled division (Pane et al., 2002).

CML states can be divided in three main clinical stages. Chronic phase is the first stage of CML, and it is the one in which most patients are diagnosed. This first period is generally asymptomatic and it can take years for it to evolve into accelerated phase, the second stage of CML. Accelerated phase is related to an increase in the expression of BCR/ABL1 and somehow resembles an "acute" form of CML. Although *how* this occurs is not well-understood, it may end in mechanisms favoring the expansion of differentiated leukemia cells (eg. by the activation of metabolic pathways controlled by Src kinases family). Accelerated phase is characterized by a less than 20% myeloblasts count in blood or bone marrow and over 20% count of basophils, cytogenetic anomalies added to Ph, or splenomegaly. The evolution from chronic to accelerated phase is a sign for the disease progression and forewarns blastic crisis, the terminal phase of CML which is often lethal. (Cervera et al., 2013)

Tyrosine kinases inhibitors (TKIs) such as imatinib, nilotinib and desantinib, are the main option to treat CML (Baccarani et al., 2009; Medscape, 2016b). Imatinib accounts for about 65% of complete cytogenetic regression within one year of treatment of chronic phase-diagnosed CML patients (Etienne et al., 2014; Kantarjian et al., 2002). However, its efficiency rates lessen in patients going through accelerated phase or blastic crisis (Cervera et al., 2013). Treatment for these patients incorporate a chemotherapy or interferon regime, and the possibility for HSCT. Nevertheless, long-term response to any treatment is less likely, and only about one or two out of five patients who had undergone HSCT will live for more than five years (Jabbour et al., 2011; Medscape, 2016b).

3. CD47

3.1. A ubiquitously expressed molecule with an important physiopathological role in the cancer and the immune system

CD47 (also known as integrin-associated protein) is a transmembrane protein receptor member of the immunoglobulin family which is widely expressed in different cells. It is the principal receptor of the cellular matrix protein thrombospondin-1 (TSP1), and functions as a counter-receptor for two members of the signal-regulatory protein (SIRP) family (SIRPα and SIRPy). It controls different signaling pathways including cell death, cell differentiation and responses to stress (Soto-Pantoja, Kaur, & Roberts, 2015), and participates in phagocyte recognition of self-cells by interacting with SIRPα (which is highly expressed in these cells), serving as a "don't eat me" signal that inhibits phagocytosis (X. Liu, Kwon, Li, & Fu, 2017). CD47 also modulates several cellular activities such as platelet activation, cell motility and cell adhesion, leukocytes migration, among others.

CD47 was first identified as a 47-50 kDa glycoprotein component of the Rh antigenic group present in erythrocytes, as it was absent in patients with hemolytic anemia $Rh^{(-)}$ (Avent et al., 1988). Two years later Brown and colleagues described a 50-kDa plasma membrane molecule that co-purified with the integrin αvß3 from leukocytes and placenta, and called it "integrin associated protein" (IAP) (E. Brown, Hooper, Ho, & Gresham, 1990). In the following years Brown's group and others showed that other integrins in different cell types also formed a relatively stable complex, probably with the same protein which was presumed to be ubiquitously expressed (E. J. Brown & Frazier, 2001). This molecule was also identical to a cancer antigen, OV-3, which was up-regulated in ovarian cancer cells (Campbell, Freemont, Foulkes, & Trowsdale, 1992). The hypothesis that CD47 was a ubiquitous receptor was demonstrated when CD47's cDNA was cloned and expressed, letting antibodies to CD47 determine that the Rh-related antigen, the IAP, and OV-3 were all the same protein (Lindberg et al., 1994). And, since this transmem-

brane glycoprotein interacts with a variety of molecules it best known by its information-impartial immunological alias: CD47.

Ever since its identification as an ovarian tumor antigen, CD47 has been observed to be up-regulated in different types of cancer, including the different types of leukemia (M. P. Chao et al., 2010; Jaiswal et al., 2009; Majeti et al., 2009). It could be hypothesized that CD47 over-expression serves as a "don't eat me" flag that keeps the innate immune system away from eliminating cancer cells. However, CD47 is recognized as a *Janus* (the two faced-roman god that alludes to its two contrary roles)-like protein since –contrary to its "anti-death" activities on cancer cells– it plays active roles in blocking angiogenesis and direct induction of cell death, indicating that its role and expression differs from one cell type to another, and that it changes in different cellular situations. Then, although cell regulation of CD47 expression and activities crucially control tissue homeostasis, it is clear that differences in its expression are not sufficient to explain its complex physiopathological roles.

It is due to the above mentioned, that in the following paragraphs a wide vision of CD47 interactions will be introduced to eventually present the way this has motivated researchers to propose diverse strategies to treat cancer, emphasizing its potential use on different types of leukemia.

3.2. Structure and interactions

CD47 is an integral membrane protein consisting of an N-terminal IgV-like extracellular domain anchored to the plasma membrane through a highly hydrophobic transmembrane-spanning presenilin domain that ends in a short (3-36 amino acids) alternatively spliced cytoplasmic tail (Fig. 5) (E. J. Brown & Frazier, 2001).

The IgV-like domain is highly glycosylated (which is a common feature of various surface proteins) including five N-glycosylated sites modified with a O-glycosaminoglycan that are not necessary for interaction with SIRPα (Subramanian, Boder, & Discher, 2007), but they are for TSP1 signaling (Kaur & Roberts, 2011). IgV three-dimensional globular structure is maintained by two bisulfide bridges, one of them linking Cys^{33}

from IgV domain itself to Cys^{263} present in the last extracellular spanning domain. This long range disulfide bond is necessary for CD47 interaction with SIRPα and for Ca^{2+} signaling in T-cells (R A Rebres, Vaz, Green, & Brown, 2001).

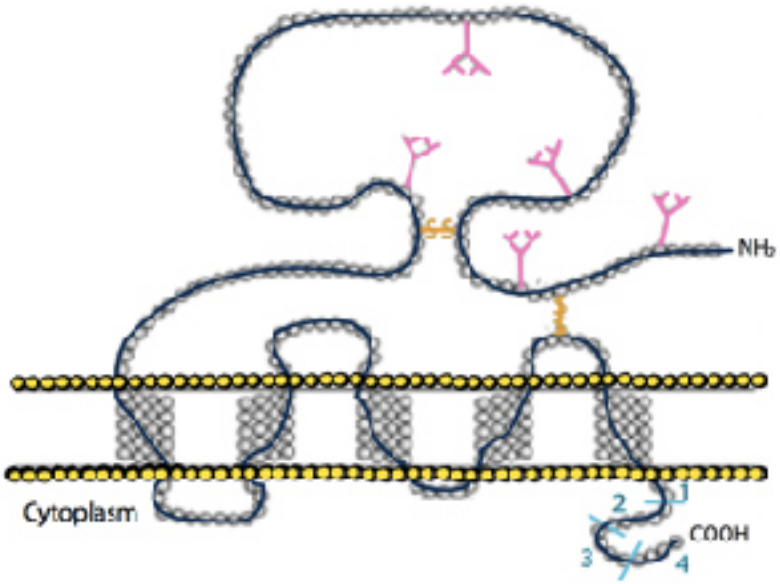

Figure 5. CD47 is a transmembrane protein conformed by an extracellular immunoglobulin variable (IgV)-like N-terminal domain that is highly glycosylated (pink), followed by a five-membrane spanning domain that ends in a cytoplasmic tail where alternative splicing occurs, and that includes the C-terminus. CD47 structure is influenced by disulfide bridges linking the IgV domain to the membrane spanning domain. (Extracted from Martínez-Torres, 2013)

Although it is thought that an additional CD47-interacting molecule that mediates homotypic interaction could exist (Robert A Rebres, Kajihara, & Brown, 2005; Soto-Pantoja et al., 2015), the fact that the two mentioned ligands exert a reciprocal relation with CD47 remains the most accepted option. However, it is important to mention that CD47 interactions with SIRPα and TSP1 do not occur simultaneously. Studies have demonstrated that TSP1 inhibits CD47 interaction with SIRPα and that the CD47-blocking antibody, B6H12, inhibits its interaction with both TSP1 and SIRPα (Isenberg et al., 2009). This highlights the fact that CD47 can perform differently in diverse situations, including those regarding differential interactions with its two major ligands, same which have been largely described and are mentioned below.

3.2.1. CD47–SIRPα

The SIRP family of proteins consists of three different transmembrane glycoproteic receptors. SIRPs consist of three connected extracellular domains, one is an IgV domain that is distal to plasma membrane (D1), and the remaining two are Ig constant domains, closer to the membrane (D2 and D3). Within the SIRP gene cluster, three proteins are coded: SIRPα, SIRPβ, SIRPγ. CD47 is the principal ligand of SIRPα, with which it interacts through its D1 domain. SIRPα is strongly expressed in myeloid cells and neurons. SIRPβ is highly similar to SIRPα in the overall amino acid sequence, including the region containing the residues that interact with CD47. SIRPβ does not interact with CD47 though, probably due to slight differences in the hairpins near CD47-interacting residues. SIRPγ also binds to CD47 although with lower affinity than SIRPα (~9x less) and it is principally expressed in lymphocytes and natural killer (NK) cells (Barclay & Van den Berg, 2014; Martínez-Torres, 2013).

SIRPα realizes various functions in leukocytes, including neutrophils and monocytes transmigration. It was demonstrated that its interaction with CD47 negatively regulates the signaling of some toll-like receptors (TLR), induce inflammatory cytokines secretion and leukocyte oxidative catastrophe (Martínez-Torres, 2013). Importantly, this interaction regulates macrophage-mediated phagocytosis by functioning as a "don't eat me" signal that allows discerning a viable cell from an apoptotic one. This was first described in erythrocytes, and later, its implication in leukocytes and other cells became clear *in vitro* and *in vivo*. Moreover, as CD47 has been found to be up-regulated in different types of cancer, it has been proposed that this signaling favors cancer progression by keeping immune system at bay (Jaiswal et al., 2009), therefore raising as a potential target to treat cancer (Willingham et al., 2012). With the publicity given to such impressive findings, the intracellular activities that control the "don't eat me" signal in phagocytes have been satisfactorily described (Barclay & Van den Berg, 2014; Matozaki, Murata, Okazawa, & Ohnishi, 2009; Per-Arne, 2012).

3.2.2. Thrombospondin-1 (TSP1): the good, the bad and the ugly

Thrombospondins (TSP) are globular proteins discovered in the early 70's by Baenziger and collegues in membranes of human platelets as thrombin-sensitive proteins (Baenziger, Brodie, & Majerus, 1971). They are a family of five multifunctional proteins that can be divided in two subgroups: one of homo-trimers, formed by TSP1 and TSP2, which have pro-collagen–like tail and repetitive regions; and a second group of pure homo-pentamers, including the rest of TSPs (TSP3,TSP4 and TSP5) (Martínez-Torres, 2013).

3.2.2.1. The good: Well-known activities of TSP1

TSP1 mediates various forms of cell–cell and cell–matrix interactions including plate aggregation and angiogenesis. It is a ligand for several receptors, encompassing a variety of integrins, CD36, and CD47. Importantly, TSP1 is the main soluble ligand of CD47; this interaction controls cell's fate, cell viability, and cell's resistance to stress through outside-in signaling that alters cell cycle and cyclic nucleotide signaling, integrins and growth factors signaling (Roberts, Miller, Rogers, Yao, & Isenberg, 2012).

TSP1 interaction with CD47 has several physiological and pathophysiological implications, playing diverse roles in the cardiovascular and the circulatory system. TSP1–CD47 interaction controls vasodilation and chemotaxis in platelets and endothelial cells by inhibiting the soluble guanylyl cyclase (sGC) (Isenberg et al., 2009), the enzyme sensing and activating nitric oxide (NO) production. sGC inhibition consequently impedes NO generation. NO regulates blood flow and pressure by diminishing the amount of platelet adhesion molecules among other mechanisms. Blocking this signaling has a direct impact in angiogenesis, since vascular functions derived from the activation of NO production are inhibited (Martínez-Torres, 2013; Pyriochou et al., 2007).

Moreover, TSP1–CD47 interaction regulates various aspects of the innate and the adaptive immune system (Martínez-Torres, 2013). TSP1 is largely produced by several types of immune cells such as monocytes, dendritic cells (DC), and macrophages (Theocharides et al., 2012). Meanwhile, some T cells express it on their surface (Sarfati,

Fortin, Raymond, & Susin, 2008). Besides, it has been observed that TSP1 levels rapidly rise in damaged tissue, result of inflammatory responses (Isenberg et al., 2008). Together with each cell variable CD47 expression, TSP1 production and secretion influences the interaction between both proteins, and thus, CD47 signaling.

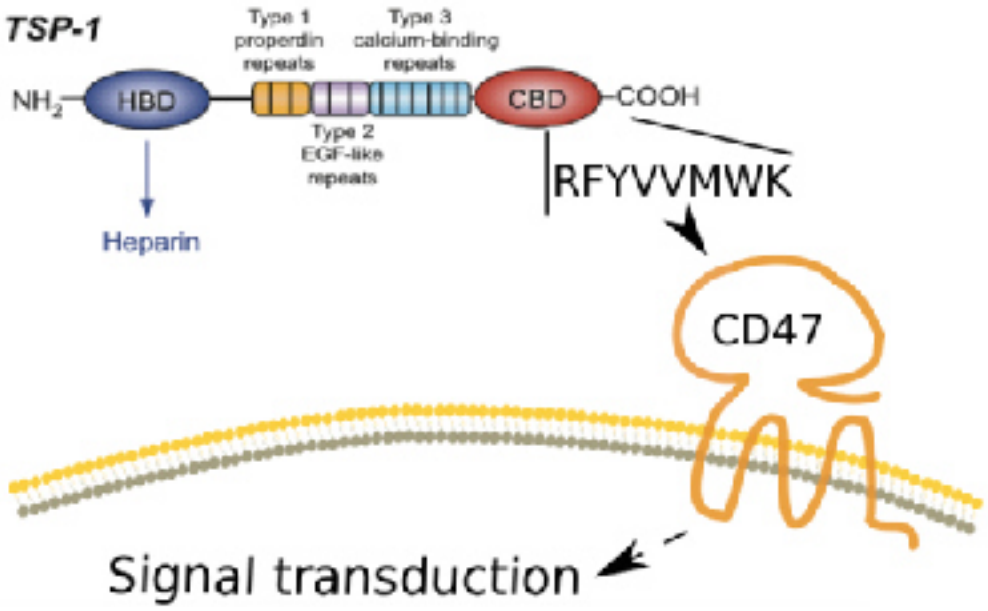

Figure 6. The C-terminal domain of thrombospondin-1 interacts with CD47. Thrombospondin-1 (TSP1) is a large homo-trimeric protein that interacts with several ligands. However, its C-terminus contains the RFYVVMWK peptide specifically interacting with CD47. (Martínez-Torres, 2013)

3.2.2.2. The bad: The history told by peptides

Although of pivotal relevance, CD47 interaction with TSP1 remains exceedingly challenging to crystallize. Nevertheless, TSP1–CD47 interaction is clear. Since the beginning of the 1990s the group of Frazier had observed that human TSP1 includes a cell attachment site that was near to that for the integrins (the Arg-Gly-Asp or RGD sequence) at its carboxy-terminal domain (Prater, Plotkin, Jaye, & Frazier, 1991). This cell binding domain (CBD) was cloned and expressed on *Escherichia coli* from a cDNA construct that began downstream of RGD sequence, but had binding activity even when RGD was left out (Kosfeld, Pavlopoulos, & Frazier, 1991). Later on, and closely in time

with Campbell and colleagues' discovery of the over expression of CD47 (which by that time they named OA3) on ovarian cancer cells (Campbell et al., 1992), others found that the CBD of TSP1 has a binding partner in many normal and transformed cells (Prater CA, et al. 1991; Li WX, et al. 1993; Asch AS et al. 1993). Using overlapping synthetic peptides representing the total residues of the CBD, Kosfeld and Frazier identified and further dissected two minimal cell binding sequences: 7N3 (IRVVM) and 4N1 (RFYVVMWK), related one to the other by sharing the VVM motif, and that seemed to compete for the binding site (Kosfeld & Frazier, 1993). This binding site was on an integral membrane protein of ~50-kDA present on the surface of the cell (Gao & Frazier, 1994). It was CD47.

Since then, a soluble version of the 4N1 peptide, 4N1K (KRFYVVMWKK), was used at first to mark and purify CD47 (Chung, Gao, & Frazier, 1997; Dorahy, Thorne, Fecondo, & Burns, 1997; Gao & Frazier, 1994), but later discoveries showed that the 4N1K interaction with CD47 could mimic that of TSP1, inducing cell signaling (Green et al., 1999; Kanda, Shono, Tomasini-Johansson, Klint, & Saito, 1999), including RCD activation (Mateo et al., 1999). A study corroborated the VVM motif interaction with CD47 using mutants of TSP1 lacking this motif, which were unable to induce the activation of CD47 (McDonald, Dimitry, & Frazier, 2003). Years later, with the X-ray structure of the CBD of TSP1 already available, Floquet and colleagues made a molecular modeling of the TSP1–CD47 interaction (Floquet, Dedieu, Martiny, Dauchez, & Perahia, 2008). They showed that the 4N1 sequence in TSP1 is normally hidden within a hydrophobic pocket where interaction is prevented; however, when in close proximity to CD47 and the phospholipid bilayer, this hydrophobic cleft opens, allowing its recognition by CD47 (Floquet et al., 2008). The interaction between the 4N1K and CD47 is in this way a well-documented story. Nevertheless, direct evidence of this interaction and its specificity still awaits to be reported.

It should not be forgotten that the larger soluble version of the 7N3 peptide (FIRVVMYEGKK) was described simultaneously with the 4N1K (Gao and Frazier 1994). Of note is that, out of the eight articles available to date at PubMed in which 7N3

was used (excluding those in which peptides were discovered), in five it has been utilized alone to mimic TSP1 activities (Csányi et al., 2012; Kaur et al., 2010, 2014; Kaur & Roberts, 2011; Sipes, Krutzsch, Lawler, & Roberts, 1999) and only three used the 4N1K as well (Congote & Temmel, 2004; Isenberg et al., 2006, 2007). In addition, the 7N3 has been more largely used to inhibit TSP1 interaction with CD47 by competing for the binding site. Direct induction of CD47 signaling through 7N3 has not been reported yet though. This might be explained by the recent study describing that the VVM motif in the 4N1K also requires the intact "FYV" overlapping sequence (FYVVM) not only to strongly bind to CD47, but to induce CD47-mediated RCD of CLL cells (Denèfle et al., 2016). The 7N3 does not share this sequence with the 4N1K, thus, it is possible that although it can interact with CD47, 7N3 will not activate CD47-mediated RCD, and perhaps a bouquet of other signals. Maybe this is why the 4N1K has been the preferred choice to mimic TSP1 signaling through CD47, with the advantage of not using the complete and CD47-unspecific ~155-kDa TSP1 which, as already mentioned, is a ligand of several receptors.

3.2.2.3. The ugly: Peptide skeptics

TSP1 mimetic agonist peptides, such as the 4N1K, showed to directly induce CD47 signaling many years ago. Surprisingly, the potential of their applications were not explored during the twentieth century, probably because of the major drawbacks associated with their use as therapeutic tools, such as poor metabolic stability, poor selectivity, poor oral bioavailability, immunogenicity, and difficult syntheses. Indeed, peptides have long been considered only as biological tools by the pharmaceutical industry; however, many recent solutions have been developed by academic scientists in order to overcome these limitations, and to date, more than 150 peptides are used as drugs (Karoyan and Ayoub, 2017). Moreover, the lack of direct evidence for the TSP1–CD47 interaction (eg. crystallization) still leaves doubts on whether TSP1-derived peptides are specific to CD47, specially when a CD47 knock-out model is not used as a control. Authentic silencing techniques of mammalian cells, however, were easily accessible only after the discovering of

the small interference RNA (siRNA) in 1998 (Fire et al., 1998), which was first used in a mammalian cell until two years after (Elbashir et al., 2001).

Before the era of reliable silencing (and even currently) the most used model to study CD47 signaling were the so-called CD47-*null* T-cells, JinB8. These cells are a subpopulation of Jurkat T-cells selected by one anti-CD47 monoclonal antibody (BRIC-126) after their exposure to a randomly-mutagenic compound (ethyl-metanesulfonate). JinB8 cells were inferred as CD47-*null* and presented as such because fluorescently-labeled BRIC-126 was not detected by flow cytometry (Reinhold, Green, Lindberg, Ticchioni, & Brown, 1999). Some are skeptic of the 4N1K–CD47 interaction since the 4N1K has been reported to have an activity on JinB8 (Barazi et al., 2002; Leclair, Lim, Martiny, Dauchez, & Perahia, 2014). However, researchers using these cells rarely inform on their absence of CD47 (eg. Miller et al. 2013; Kaur et al. 2013), or do it by flow cytometry using one single antibody (generally BRIC-126), though abstaining to report it by Western Blot (eg. Barazi et al. 2002; Leclair & Lim, 2014; Azcutia et al. 2017). In fact, only one study have detailed JinB8's CD47 absence in their research by different technical approaches (Kaur et al., 2014).

The lack of dependable proofs on CD47's absence in JinB8, together with the evidence of 4N1K action on these cells, suggests the possibility for them to display a deviant form of CD47 that is not recognized by some antibodies but activated through 4N1K in certain conditions. New CD47 knock-out cell models obtained by reliable silencing techniques, such as the already available and widely used CRISPR/Cas9 targeted genome editing, are urgent to study the *in vitro* roles of 4N1K and their derivates, consequently impacting on the understanding of the TSP1–CD47 interaction and the potential uses of this kind of peptides.

3.3. Flag or not, a target

As previously mentioned, two principal functions of CD47 have been identified. On the one hand, CD47 interaction with SIRPα serves as a "don't eat me" signal limiting self-phagocytosis but contributing with carcinogenesis. On the other hand, its interaction

with the TSP1 C-terminus regulates different signaling pathways controlling cell differentiation, cell migration and cell death. Both functions awaken a clear interest to study CD47 as a target to treat cancer.

Accompanied by the discovery of CD47 up-regulation in different types of cancer and the later discoveries showing that antibodies directed to this receptor stimulate macrophage-mediated clearance of tumors (M. P. Chao et al., 2010; Majeti et al., 2009; Willingham et al., 2012), the interest in CD47 as a therapeutic target to treat cancer gained increasing attention. A substantial number of the last ten years research on CD47 report the effects of blocking or mimicking the CD47–SIRPα axis as a target to treat cancer (Figure 6) (Mark P Chao, Weissman, & Majeti, 2012). The vast information gathered together in recent years has resulted in the fact that today, the process known as "programmed cell clearance" that consists in blocking CD47 with monoclonal antibodies or SIRPα-Fc to impede SIRPα signaling, is currently undergoing different clinical trials (NCT02216409; NCT02096770; NCT02663518; ISRCTN28039294).

With the publicity surrounding the good results of targeting CD47 as a strategy to treat cancer, a misunderstood idea of CD47 as a passive "don't eat me" flag on cancer cells has spread. However, as mentioned previously, CD47 regulates signaling pathways of vital importance, and said idea must fade away. Interesting results showing that miming the SIRPα–CD47 interaction with monoclonal antibodies induces tumor elimination dependent of the recruitment of CD8+ T cells to the tumor site (X. Liu et al., 2015) may be the best example to support this urgency. Instead, understanding the underlying mechanisms triggered by CD47 cross-linking should be noticed and considered to predict unwanted results. For example, when used systemically blocking CD47 would also block some vital anti-tumor functions in the cell, such as that of anti-angiogenicity controlled by CD47 interaction with TSP1. Furthermore, antibodies against CD47 will find a great reservoir of CD47 on erythrocytes, platelets, and endothelial cells; thus anemia, thrombosis and hypertension are all predicted secondary effects. This has already been observed in mice treated with mIAP-301, in which arterial and diastolic pressure was drastically boosted (Bauer et al., 2010).

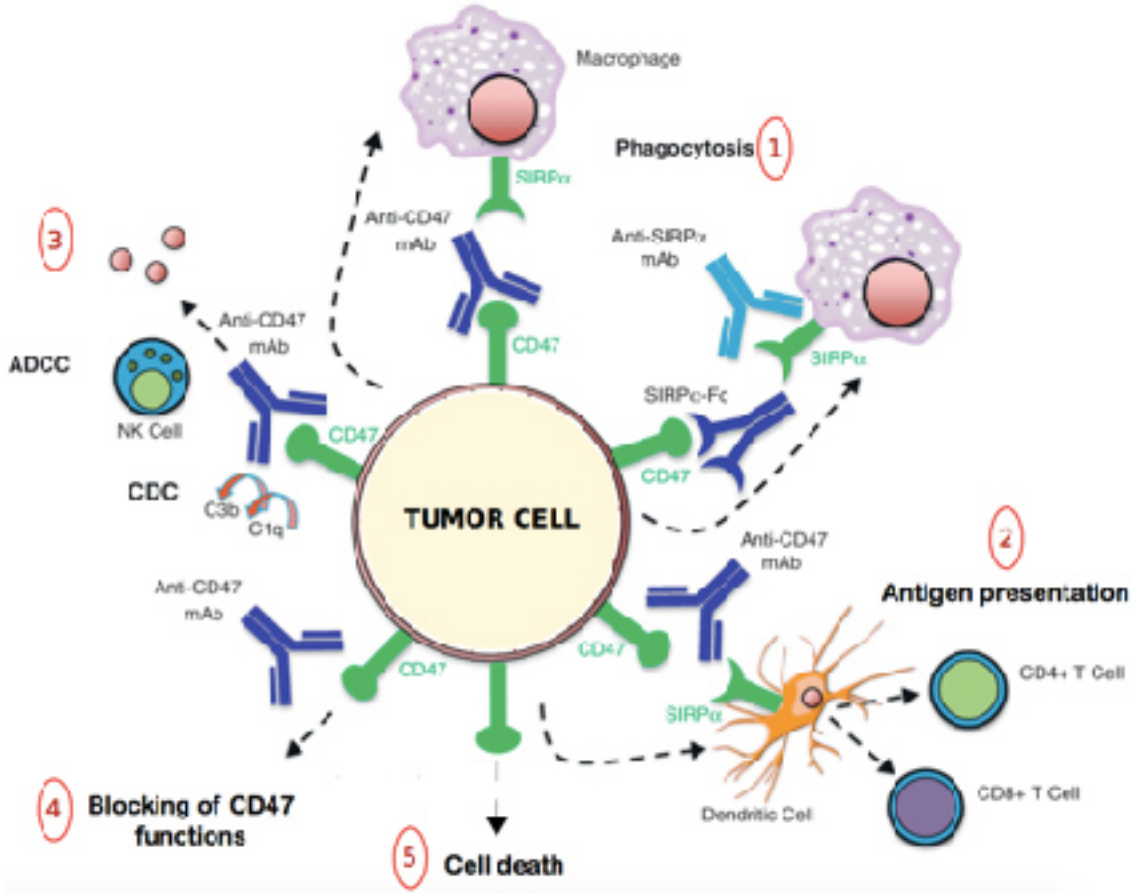

Figure 7. CD47 is used as a target to eliminate cancer cells. Therapeutic targeting of CD47 using monoclonal antibodies (mAb) can induce the elimination of cancer cells through multiple mechanisms. 1) Through phagocytic uptake of tumor cells by macrophages: by inhibiting the CD47–SIRPα interaction with a blocking anti−CD47 mAb, a blocking anti−SIRPα mAb, or a recombinant SIRPα protein (shown as a bivalent Fc−fusion protein). 2) Anti−CD47 antibodies can stimulate an anti−tumor adaptive immune response leading to the phagocytic uptake of tumor cells by DCs and subsequent antigen presentation to CD4 and CD8 T cells. 3) By NK cell− mediated ADCC and CDC induction and tumor cell elimination: an anti−CD47 antibody can eliminate tumor cells through antibody Fc−dependent mechanisms. 4) Function blocking of CD47 may also promote tumor reduction by blocking several of its actions in tumor cells. 5) Finally, CD47 stimulation can directly induce RCD.

3.3.1. Direct RCD induction

Direct RCD induction of cancer cells has been observed since several years ago. Mateo and coworkers used the immobilized B6H12 antibody, TSP1 and the 4N1K peptide to induce RCD in CLL cells (Mateo et al. 1999). Few months later, Petersen and col-

leagues published an article in which they demonstrated that death could also be induced by soluble antibodies (Ad22 and IF7) in Jurkat T cells (Petersen et al. 1999). Time after, many researchers continued using several soluble anti-CD47 antibodies to induce RCD in various types of cancer such as ALL (CEM), breast cancer (MCF-7), and AML (NB4-LR1), among others (Martínez-Torres, 2013). Likewise, TSP1 and the 4N1K peptide attached to the plate proved their ability to induce RCD of leukemic and breast cancer cells (Manna & Frazier, 2004). All of these cases of CD47-mediated cell death conserve punctual characteristics: they are rapid, caspase-independent processes that present mitochondrial membrane depolarization without releasing pro-apoptotic proteins, and induces ROS production, phosphatidylserine exposure, plasma membrane permeabilization, and null DNA fragmentation nor chromatin condensation (Martínez-Torres, 2013).

Interestingly, a recent study demonstrate that contrary to its inhibition, CD47 activation by TSP1-mimetic agonist peptides induce RCD in cells of patients with CLL. CD47 agonists also reduced tumor growth in NSG mice transplanted with CLL cells. This activation provokes downstream signaling of CD47 in CLL cells that leads to the activation of phospholipase C gamma-1 (PLCγ1), which resulted to be progressively over-expressed according to the CLL advance, contrastingly to CD47, which was present at basal levels. It was demonstrated that PKHB1-triggered the sustained activation of PLCγ1, which regulates cytosolic Ca^{2+} levels in the cell, causes a massive intake of Ca^{2+} in the cytoplasm that damages mitochondria and activates serpases, which depolarize actin from the cytoskeleton, finally causing the CLL cell death (Martinez-Torres et al., 2015).

The mentioned work is a breakthrough in the study of CD47-mediated RCD, as it widens the outlook of the promising alternatives that targeting CD47 could have in the treatment of cancer, especially those involving the cardiovascular or the immune system, such as the different types of leukemia.

IV. JUSTIFICATION

Leukemia is a major global issue for public health. The aim of current treatments is to lead the leukemic cell to die by apoptosis, a regulated cell death (RCD) mechanism, using chemotherapies, monoclonal antibodies and kinase inhibitors. However, chemotherapies are not specific to cancer cells, killing other cell populations such as healthy blood cells. Furthermore, patients with deficiencies in certain components of the apoptotic machinery (eg. transcription factor *p53*) are unsusceptible to these treatments since resistant leukemic cells are selected in a microevolutionary process of survival of the fittest that causes extremely aggressive side effects and the inexorable reemergence of the disease. Fortunately, apoptosis is not the only form of RCD. Our cells are armed with exceptional weaponry of self-destruction, genetically encoded to guide the organism back to homeostasis. One of these weapons is the activation of CD47 through the C-terminal end of thrombospondin-1 (TSP1). CD47 agonist peptides (4N1K and PKHB1) have demonstrated to specifically kill different types of cancer cells, including those of patients with chronic lymphocytic leukemia (CLL), and even of those patients with *p53*-deficiency. The mechanism triggered by these peptides in CLL was shown to be a non-apoptotic instance of RCD that is independent of the microenvironmental stimuli, yet dependent on an executioner mechanism of cytoplasmic Ca^{2+} mobilization. However, leukemia is a highly heterogeneous disease, and the RCD mechanism activated by PKHB1 in other types of leukemia has not been demonstrated, limiting its potential therapeutic use.

V. HYPOTHESIS

CD47 agonist peptides selectively induce caspase-independent regulated cell death in different types of leukemia cell lines, independently of their interaction with bone marrow stromal cells, but dependent on intracellular Ca^{2+} augmentation.

VI. OBJECTIVES

General Objective

To study the mechanism of cell death induced by CD47 activation on CEM, Jurkat, HL-60, K562, and L5178Y-R cell lines.

Specific Objectives

1. Perform the chemical synthesis of a CD47 agonist peptide.
2. Analyze whether the treatment with different doses of CD47 agonist peptides (4N1K and PKHB1) induce death in different types of leukemia cell lines.
3. Evaluate whether PKHB1-induced cell death in different types of leukemia is caspase independent.
4. Determine whether co-culture of T cell-derived leukemic cells with bone marrow stromal cells affects PKHB1-induced killing.
5. Evaluate whether the type of death induced by PKHB1 in T cell-derived leukemic cells is Ca^{2+}-dependent.

VII. MATERIAL AND METHODS

7.1. CD47 agonist peptides

Design, synthesis, and production of CD47 agonist peptides 4N1K and PKHB1, as well as corresponding affinity tests were realized at the Laboratory of Biomolecules of the Oncodesign research center in Villebon Sur Yvette, France, under the supervision of Pr. Philippe Karoyan.

7.1.1. Manual Solid Phase Peptide Syntheses (SPPS)

Manual SPPS were realized on a 0.30 mmol scale on chlrotrytil resin beads. Amino acid Fmoc group was split off by treatment with piperidine/dimethyl formamide (DMF, 1:4) (1 × 1 min, 1 × 10 min). Washing steps between deprotection and coupling were carried out with DMF (3 × 1 min), isopropanol (IPA, 3 × 1 min), and DMF (3 × 1 min). Activation step was carried out with Fmoc–amino acids (1.2 mmol, 4 equiv.), HBTU (1.2 mmol, 4 equiv.) as coupling agent, HOBt (1.2 mmol, 4 equiv.) as auxiliary nucleophile, and diisopropylethylamine (DiEA, 2.4 mmol, 8 equiv.) as base. The activated amino acid was then transferred to the resin where the coupling was performed for 1-18 h. Supported coupling reactions were monitored by classical Kaiser test (solution kit from Sigma-Aldrich). When elongation of the peptide chain was completed, a washing step with methanol was added after the final N-terminal Fmoc removal in order to totally shrink the resin under vacuum.

7.1.2. Final Side-Chain Deprotection and Cleavage from the Resin

The crude peptides were cleaved from the solid support by treatment with a cleavage cocktail: trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS) (95/2.5/2.5, 10 mL). The reaction mixtures were shaken for 3 h, and then precipitated 3 times using cooled diethyl ether (3 × 30 mL), recovered after centrifugation (3 × 5 min, 7800 rpm). Diethyl ether was removed (washed 3 times), and then the peptide pellets were dried under nitrogen flow. The resulting crude peptide was dissolved in aqueous 0.1% (v/v) TFA. Pu-

rification was conducted on a reversed-phase HPLC Prep C18 column, eluting with 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) as described earlier.

7.1.3. Cells Treatment with Peptides

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

7.2. Cell cultures

Leukemia cell lines used in this work were MEC-1 (CLL), CEM (ALL), Jurkat (ALL), HL-60 (AML), K562 (CML), and L5178Y-R (murine leukemia). They were maintained following standard protocols proposed by the American Type Culture Collection (ATCC) using complete medium (RPMI 1640 supplemented with 10% fetal calf serum [CORNING Cellgro®], 2 mM L-glutamine and 100 U/mL of penicillin-streptomycin [GIBCO® by Life Technologies™]) and incubated at 37ºC in a controlled humidified atmosphere with 5% ± 0.5% de CO_2. Washes were performed using phosphate saline buffer (PBS, pH 7.2) 1X (GIBCO™) after retiring medium from the container. Cell count was performed using trypan blue (0.4% SIGM-ALDRICH®), a Neubauer chamber, and an optic microscope as proposed by the ATCC's standard protocols.

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors using Histopaque®-1077 (Sigma Aldrich®) following the protocol suggested by the supplier. Primary cultures of mouse lymphoid organs (bone marrow, thymus, spleen, and lymph nodes) were obtained as follows. Healthy control mice were chloroform-anesthetized, and sacrificed by cervical dislocation. Bone marrow cells were obtained from intact femurs, isolated by carefully removing surrounding muscle, cutting off the epiphyses, and flushing diaphyses with PBS using 1 cc syringe and a 12.7 mm 29-gauge pen needle. Spleen, thymus, and lymph nodes were isolated in separate sterile Petri dishes; spleens were injected with PBS for several times until the organ turned

pale, while thymuses and lymph nodes were disintegrated using a sterile syringe plunger and PBS. Each cellular extract was collected in separate sterile 15 mL centrifuge tubes, centrifuged, counted in a Neubauer chamber, and seeded for treatment in complete medium.

7.3. Cell death analysis by flow cytometry

Flow cytometry was performed as follows: 100 µL of a cell culture solution with 10^6 cells/mL were harvested in a round bottom 96 well plate (Corning Inc. Costar®, USA), treated, and incubated in a controlled humidified atmosphere with 5% ± 0.5% CO_2. Cells were collected in microcentrifuge tubes, and centrifuged at 1600 rpm per 10 min. Supernatant was then extracted using a vacuum pump and the mixture containing the fluorescent markers were added accordingly to manufacturer's instructions. Samples were analyzed by fluorescence-activated cell sorting (FACS) in a BD Accuri™ C6 flow cytometer, and the resulting cell population was analyzed by using the FlowJo software.

7.3.1. Phosphatidylserine exposure and plasma membrane permeability

To assess phosphatidylserine (PS) exposure, and plasma membrane permeability (PMP), cell lines were stained with allophycocyanin (APC)-labeled annexin-V (Ann-V) (Molecular Probes® by Life technologiesTM) and propidium iodide (PI) (Molecular probes® by Life Technologies™), respectively. Cells were harvested by triplicates for each treatment with 4N1K, PKHB1 or etoposide (ETO 100 µM) (Enzo® Life Sciences), and incubated for 2 h in the case of peptides, or 24 h for the ETO, at 37 ºC in 5% CO_2. Cells were recovered in microcentrifuge tubes and centrifuged at 1600 rpm. The supernatant was discarded using a vacuum pump leaving a withe pellet that was dissolved in 100 µL of a mix containing Ann-V binding buffer (ABB) (10mM HEPES, 140mM NaCl, 2.5mM CaCl2, pH 7.4), Ann-V–APC (2.5 µL/mL of ABB) (Molecular probes®) and PI (0.5 µg/mL) (Molecular probes® by Life Technologies™). Tubes were incubated for 20 min 4º C, and the samples were submitted to FACS.

7.3.2. Co-culture of leukemic and bone marrow stromal cells

Co-culture experiments were realized as further described in (Kenny Calvillo, et al. 2016). In brief: a primary culture of bone marrow stromal cells (BMSC) coming from control BALB-C mice was maintained as described elsewhere (CITA). Cells were obtained by perfusion of the bone marrow, maintained in RPMI medium added with 20% FBS and enriched with pyruvate and non-essential amino acids, and let them attach to the flask. Medium was replaced every two days, selecting the cells that were attached to the flask. These were taken as BMSC and were maintained for no longer than two weeks after mouse was sacrificed. BMSC were detached with trypsin and $1x10^4$ cells in 100 µL were harvested in 96 flat bottom well plates, letting them attach overnight. The following day medium was replaced by 100 µL of 10% FBS RPMI medium with $5x10^4$ CEM or L5178Y-R cells, and let them interact for 24 h. Finally, cells were treated for 24 h with ETO (75 µM) or 2 h with PKHB1 (200 µM for CEM or 150 µM for L51782Y-R), and cell death was measured by FACS (excluding the non-leukemic sized population if necessary).

7.3.3. Inhibition of cell death

To determine if cytotoxicity that peptides caused required the activation of caspases, the general inhibitor of caspases Q-VD-OPH (QVD, 10 µM) (BioVision) was added 30 minutes before treatment. The calcium chelator BAPTA (5 mM) (Enzo) was added 40 minutes before treatment to asses the dependence of calcium on cell death. In both cases, pretreated cells were incubated at 37º C in 5% CO^2 (30 or 40 min, respectively), treated, re-incubated. Cell death was measured by the Ann-V–APC/PI staining.

7.4. Statistical analysis

All the data were statistically analyzed using *t*-tests (parametric) and Mann Whitney tests (non-parametric) performed in GraphPad Prism statistics software.

VIII. RESULTS

8.1 Chemical synthesis of PKHB1

As mentioned in previous sections, CD47 triggering by TSP1-derived agonist peptides represents a promising approach to selectively kill cancer cells since the 4N1K decapeptide is known to trigger RCD in a variety of solid and aqueous cancers. Moreover, it has been recently reported that PKHB1, a novel, soluble, serum-stable, TSP1-derived decapeptide, conceived by the replacement of the N- and C-terminal L-lysins of the 4N1K by their D-counterparts, can induce RCD at greater amounts than the 4N1K in CLL, ALL, breast, lung, and colon cancer cells (Martinez-Torres, 2015; Denèfle et al. 2016). Therefore, PKHB1 (kRFYVVMWKk, Fig. 8A) was synthesized to test its ability to induce RCD of human lymphocytic and myelogenous leukemia, as well as a mouse leukemia cell line, and study its mechanism of action.

Solid phase peptide synthesis (SPPS) was performed as described in the Materials and Methods section to obtain over 95% pure PKHB1, determined by analytical liquid chromatography mass spectrometry (collaboration with Pr. Philippe Karoyan) (Fig. 8B).

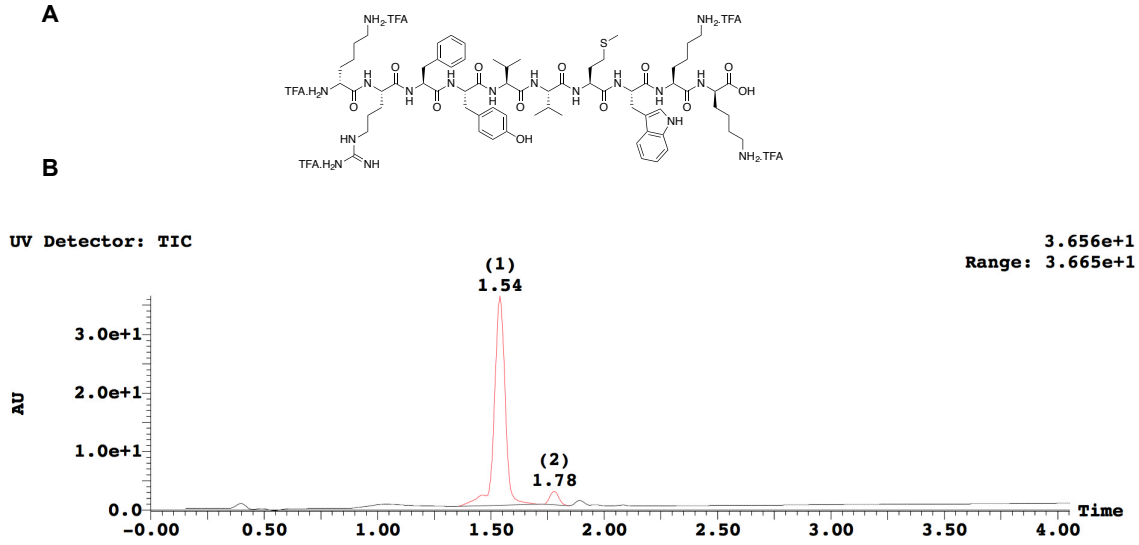

Figure 8. PKHB1, a TSP1 derived peptide, was obtained with >95% purity. A. Chemical structure of PKHB1. **B.** Chromatogram showing analyte concentration (in arbitrary units, AU), at different retention times (RT). Note that the majority (95%) of the analyte is retained at 1.54 minutes, PKHB1 predicted RT.

8.2. CD47 agonist peptides selectively kill different types of leukemia cell lines

Having successfully synthesized PKHB1, cell lines derived from different types of leukemia were used to analyze the effects that the engagement of PKHB1 or 4N1K to CD47 has in cell death induction. Since it was reported previously that CD47 agonist peptides induce RCD of CLL cells (Sarfati, et al. 1999; Martínez-Torres, et al. 2015; Denèfle, et al. 2016), MEC-1 cell line, derived from this type of leukemia, was used as a positive control. First, we tested the cytotoxicity of 4N1K and PKHB1 on MEC-1, CEM, and L5178Y-R cells. As seen in Figure 9A, both 4N1K and PKHB1 induce the cells' phosphatidylserine (PS) exposure, tracked by the allophycocyanin (APC) attached to annexin-V (Ann-V, which specifically binds to PS residues on the exterior of the plasma membrane), and plasma membrane permeabilization (PMP), deduced by propidium iodide (PI) internalization and DNA staining in MEC-1, CEM and L5178Y-R cells. Figure 9B shows that increasing peptide concentration also augments the amount of cells displaying these characteristics of cell death. Note that after 2 h of treatment with 300 µM 4N1K or 200 µM PKHB1, around half of MEC-1 or CEM cells were Ann-V$^+$/PI$^+$.

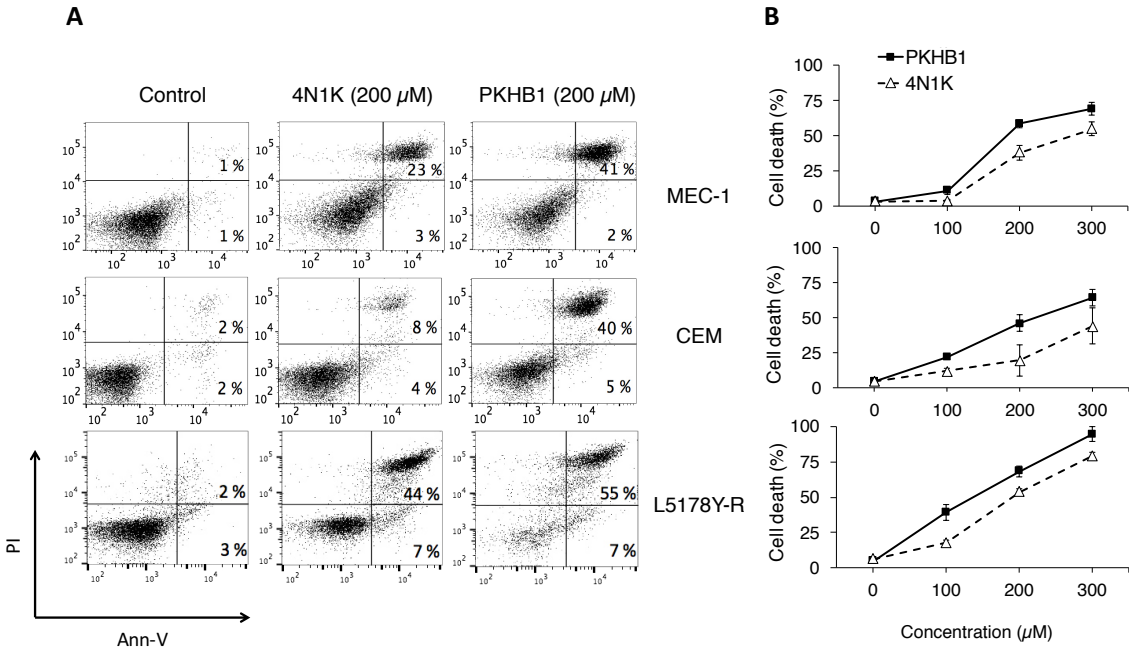

Figure 9. CD47 agonist peptides induce PS exposure and PMP on MEC-1 and CEM cells. A. Each of the representative dot plots show 10^4 MEC-1, CEM or L5178Y-R cells (dots) with negative or positive

Annexin-V-APC (Ann-V, left or right side of the plot, respectively) and/or propidium iodide (PI, lower or upper side of the plot, respectively) staining after 2 h left alone (Control) or treated with 200 µM 4N1K or PKHB1. **B.** Cell death is quantified by the amount of Ann-V^+/PI^+ staining of 10^4 cells at different concentrations of 4N1K or PKHB1 and represented in line charts as the means (±SD) of triplicates of at least three independent experiments.

Noticing the similar results obtained in the three cell lines, and being aware of the predicted therapeutical advantages that PKHB1 would have over 4N1K (Martínez-Torres et al. 2015; Denèfle, et al. 2016), we continued using this peptide for further analyses on other leukemia cell lines. These experiments showed a similar behavior of Jurkat (ALL), HL-60 (AML), and K562 (CML), in which 2 h of incubation with increasing peptide concentrations also enlarged the levels of cells displaying Ann-V^+/PI^+ staining, that was around 50% at the 200 µM concentration for the human leukemia cell lines, and at the 100 µM concentration for the murine analogue (Figure 10).

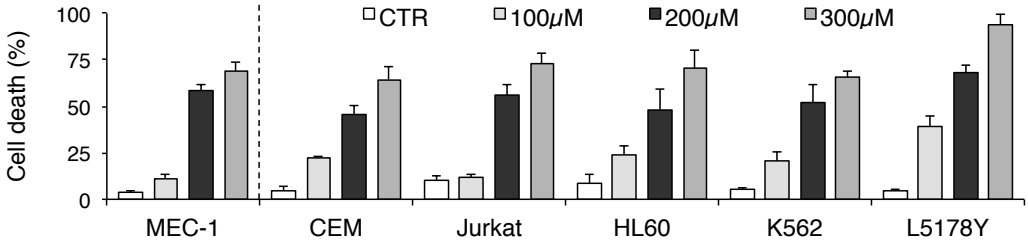

Figure 10. PKHB1 kills different types of leukemia cell lines. A PS exposure and PMP in MEC-1, CEM, Jurkat, HL60, K562, and L5178Y-R cells at different concentrations of PKHB1. B. Column charts show the means (±SD) of triplicates of at least three independent experiments.

Previous studies propose PKHB1 selectivity to induce RCD of CLL cells since it spares non-leukemic B cells, and T cells. (Martínez-Torres et al. 2015; Denèfle, et al. 2016) To confirm this selectivity, we tested PKHB1 cytotoxicity on a greater panel of non-cancerous cells. First, we challenged total peripheral blood mononuclear cells (PBMCs) of healthy donors (n=2) with PKHB1 and compared its effect on cell viability to that induced by the etoposide (ETO), a widely used chemotherapy. A paired *t*-test ($p < 0.001$) showed no significant difference between the viability of non-treated controls and cells incubated with neither of the two different agents (Figure 11 A). In addition,

indirect cell viability assays performed on primary cultures of lymphoid organs of control BALB-C mice displayed no significant difference between the viability of cultures incubated alone and with PKHB1 (Figure 11 B-E), different to that of those cells treated with the ETO, which viability was significantly reduced, as corroborated by a two-way *t*-test and a Mann-Whitney test.

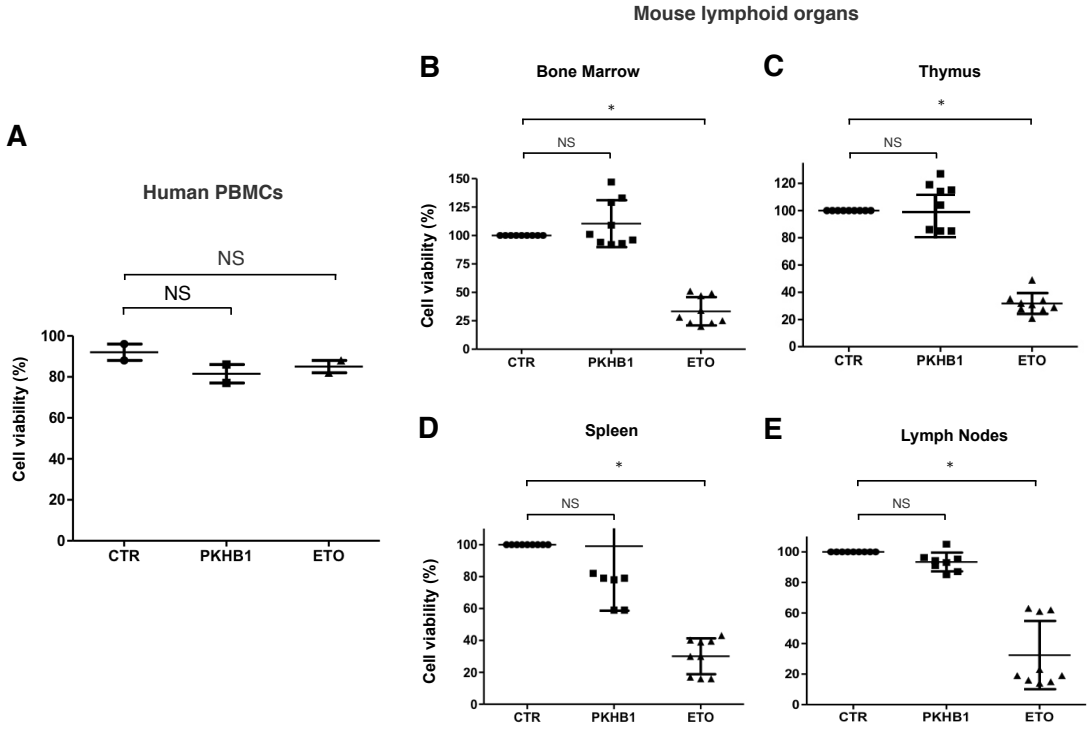

Figure 11. PKHB1 does not affect viability of non-cancerous cells. A. PKHB1 does not affect viability of human PBMCs. $4x10^6$ cells/mL of fresh PBMCs coming from healthy donors (n=2) were cultured in 96 well plates and left alone (CTR) or in presence of ETO (200 µM, 24 h) or PKHB1 (200 µM, 2 h). Cell viability was measured by the AnnV/PI method. Data represents the mean percentage of living cells per triplicate of each donor. **B-E.** PKHB1 does not affect viability of cells coming from mice primary, and secondary lymphoid organs. $4x10^6$ cells/mL coming from the bone marrow (**B**), thymus (**C**), spleen (**D**) or lymph nodes (**E**) of control BALB-C mice (n=9) were cultured in the same conditions as in **A**. Cell viability was measured by the MTT assay and is represented by the normalization of the absorbance emitted by the non-treated control over itself (CTR) or the absorbance from cells treated with ETO or PKHB1 over control. Statistical analyses of all the experiments were performed using paired student *t*-tests.

8.3. PKHB1 induces caspase-independent cell death on different types of leukemia cells

The ability of PKHB1 to induce PS exposure together with the PMP suggest that this peptide would favor phagocytic engulfment of engaged/death cells *in vivo*. Along with this, its selectivity to reduce viability only in leukemic cells advices a special molecular machinery of cell death, absent in non-leukemic cells. Following this rationale, and knowing that caspases are the main effectors of the canonic way of RCD (apoptosis), we proceeded to evaluate if the activity of these enzymes was necessary for the PKHB1 induction of cell death in different types of leukemia. To that end, we pre-incubated the cells with the broad-spectrum caspase inhibitor Q-VD-OPH (QVD), and used ETO as a positive control for caspase-dependent intrinsic apoptosis. As observed in Figure 12, the ETO is cytotoxic on CEM, Jurkat, HL-60, and L5178Y-R cell lines; however cell death is almost fully withdrawn by pre-incubation of cells with QVD. Furthermore, similarly to MEC-1 cells which have dysfunctional TP53 (a necessary first-step component of intrinsic apoptosis that is commonly mutated in leukemias) the ETO was unsuccessful to kill K562 cells as well. Contrastingly, PKHB1 was effective to kill every cell line tested despite previous incubation with QVD, in which cases student *t*-tests showed no significant difference between the cells undergoing cell death in both conditions.

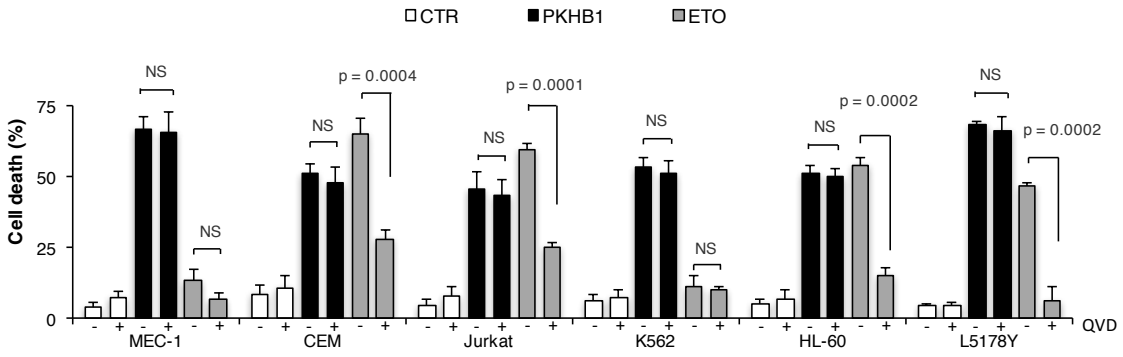

Figure 12. PKHB1 induces caspase-independent cell death on different types of leukemia cell lines. Cell death was measured by the Ann-V^+/PI^+ staining of 10^4 leukemic cells left alone (CTR) or treated with ETO (MEC-1: 200 µM; CEM: 10 µM; Jurkat: 200µM; K562: 200 µM; HL-60: 10 µM; L5178Y-R: 25 µM; 24 h) or PKHB1 (200 µM, 2 h) in presence (+) or absence (-) of broad-spectrum caspase inhibitor

Q-VD-OPH (QVD). The charts represent the means (±SD) of triplicates of at least three independent experiments.

8.4. PKHB1 kills leukemic T cells independently of their interaction with bone marrow stromal cells

One important aspect that must be taken into account for death induction of leukemic cells is the implication of the leukemia microenvironment where these cells bear and develop, in great part due to the stimuli provided by stromal cells (Meads, Hazlehurst, & Dalton, 2008). It has been previously demonstrated that different murine bone marrow stromal cells (BMSC) confer chemo-protection to leukemic cells (Kurtova, et al. 2009). Regarding this fact we standardized a co-culture of primary BMSCs coming from control mice that protects ETO-sensible leukemic cells from the effects of this chemotherapy on cell viability. Under this culture conditions, PKHB1-induced killing was not significantly different to that of CEM, HL-60, K562 and L5178Y-R cells cultured alone (Figure 13), suggesting that this peptide efficacy on different types of leukemia is not influenced by the bone marrow microenvironment.

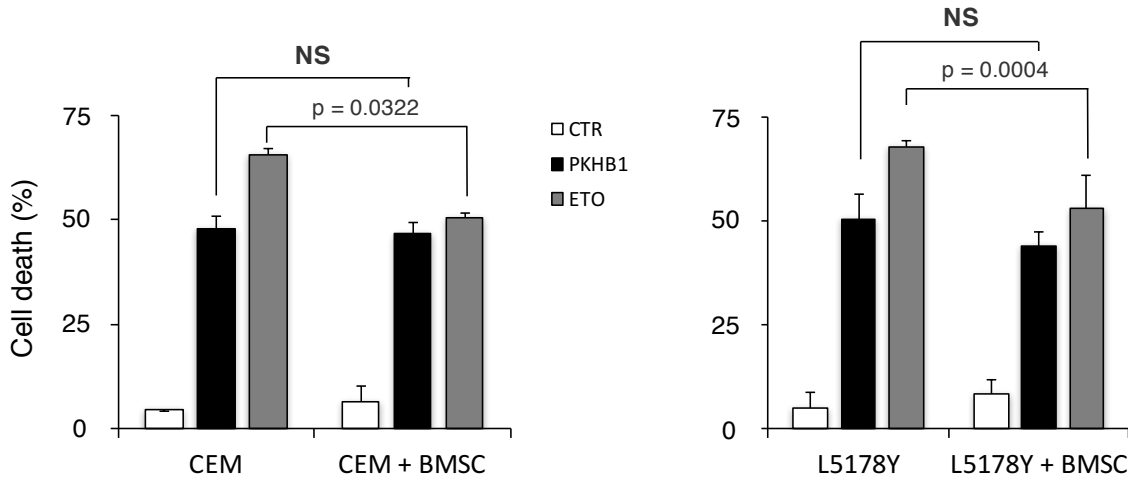

Figure 13. Co-culture with BMSC does not influence PKHB1-induced death of leukemic cells. $5x10^5$ cells/mL of different types of leukemia cells were placed alone or over a culture with $1x10^5$ BMSC/mL formerly adhered to 96-well plates, and left in contact for 24 h. After the incubation time, cells were re-incubated alone (CTR) or treated with corresponding half lethal doses of PKHB1 (2 h) or ETO (24 h). The charts indicate the mean (±SD) percentage of cell death in each case, measured by the Ann-V^+/PI^+ staining of 10^4 cells. Statistical analyses of all the experiments were performed using student *t*-tests.

8.5. Extracellular Ca^{2+} chelation regulates PKHB1-induced T-cell death

At this point we had observed that the induction of cell death by PKHB1 in different types of leukemia cells was not regulated by caspase activation, and presumably by any other apoptotic modulator provided by the tumor microenvironment. Peptide selectiveness to kill leukemic cells and its ability to induce PS exposure, however, hinted that a different form of RCD was activated due to this peptide. The rapidness of cell death, together with the morphological characteristics observed in cells treated with PKHB1, such as granulation of the cytoplasm (data not shown), and the double Ann^{+}/PI^{+} staining suggested regulated necrosis. Cytoplasmic Ca^{2+} overload remains a key executor mechanism of several modalities of RCD, including regulated necrosis (Vandenberghe et al. 2015), and there is evidence that CD47 engagement causes Ca^{2+} mobilization into the cytoplasm of CLL cells to induce RCD. (Martínez-Torres, et al. 2015) Therefore we aimed to determine if the type of death induced by PKHB1 in the other types of leukemia depended on the availability of Ca^{2+}, as demonstrated in CLL.

It is known that different signals induce the endoplasmic reticulum to empty its stores of Ca^{2+} into the cytoplasm, resulting in the induction of the Ca^{2+} release-activated calcium (CRAC) mobilization mechanism. This permits the entry of the large quantities of Ca^{2+} from the extracellular space to the cytoplasm, necessary to execute cell death (Thierry Capiod, 2013; Martínez-Torres, 2013; Pinto et al., 2015; Xu, Cioffi, Alexeyev, Rich, & Stevens, 2015). For that reason, we decided to evaluate the ability of PKHB1 to kill leukemic cells in the presence of BAPTA, a Ca^{2+} chelator. As shown in Figure 14, cells pre-incubation with BAPTA inhibited PKHB1-induced cell death almost completely in T cell-derived leukemic cells, insinuating that Ca^{2+} entry into the cytoplasm is necessary for these types of cells to die after PKHB1 ligation.

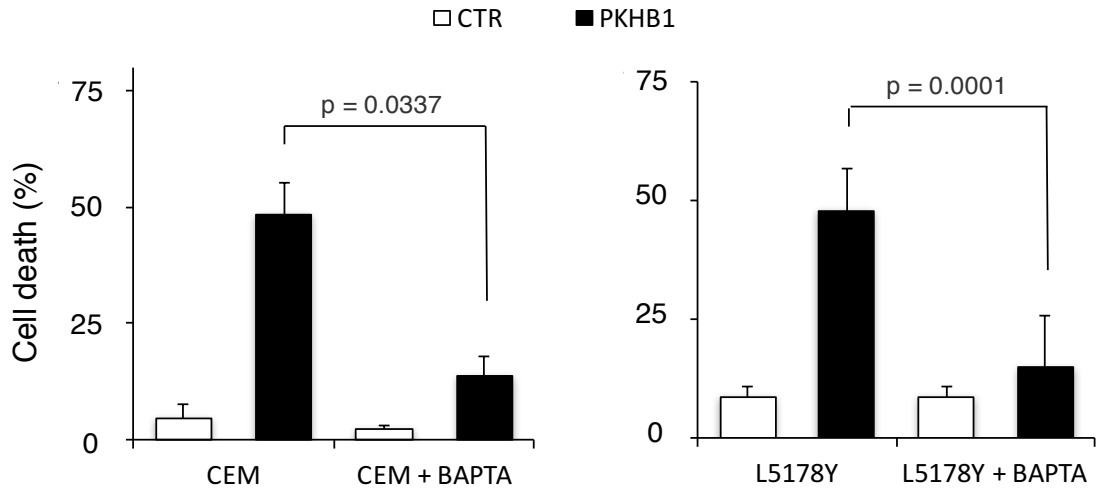

Figure 14. Extracellular Ca^{+2} chelation inhibits PKHB1-induced cell death in different types of leukemia. Cell death was measured by the $AnnV^{+}/PI^{+}$ staining of $5x10^5$ cells/mL left alone or treated with PKHB1 (200µM or 100µM in the case of L5178Y-R cells) for 2 h, in presence of vehicle or 5 mM BAPTA. Statistical analyses of all the experiments were performed using student *t*-tests.

IX. DISCUSSION

9.1. The role of TSP1 in leukemia and its potential use

Not delving into the fact that TSP1 plays a role in the progression of different types of cancer (Sargianndou, 2001), the evidence that it does so in leukemia is clear. For years it has been observed that TSP1 expression is a potential prognostic factor for CLL, as patients display significantly lower levels of TSP1 in total blood, plasma, and urine, related to the disease progression (Martínez-Torres, AC, 2013). Moreover, deficiencies in TSP1 transcription, such as that aroused by KMT2A/ELL translocation (t[11;19] [q23; p13.1]) (Zhou, et al. 2009) are common in acute forms of leukemia (Chiaretti, Zini, & Bassan, 2014; Mrózek, Harper, & Aplan, 2009; Panagopoulos et al., 2015). In contrast, long-term restoration of blood TSP1 levels of children with ALL is achieved with long-term low-dosing maintenance chemotherapy, a crucial step for the complete eradication of leukemia of children with ALL (Andre *et al*. 2015). Furthermore, up-regulation of TSP1 (along with c-MYC down-regulation) in patients with acute promyelocytic leukemia (APL, a specific type of AML) appears to be one major actor in the great success-story of ATRA treatment (Coombs, Tavakkoli & Tallman 2015).

Research on TSP1 regulation on different types of leukemia, though, mostly relies on investigating its anti-angiogenic properties. However, as experimentation procedures scarcely include its role as death inductor it should not be discarded that the anti-cancer attributes of TSP1 could also be due to its capacity to activate CD47-mediated RCD. One example that reflects this idea is that reported by Saumet *et al.* who used recombinant fragments of TSP1 and showed a strong dose-dependent cell death activity of its CBD in APL cells that is independent of ATRA treatment (Saumet et al. 2005), which is believed to influence leukemic cell elimination only by inducing their maturation and impairing angiogenesis.

In this context, using TSP1 as a trigger for direct RCD induction of leukemic cells seems a coherent way to attack these cells, in which down-regulation of TSP1 seems to be a constant in the equation in the leukemic cell machinery.

9.2. Importance of TSP1–derived CD47 agonist peptides as leukemic-cell death inductors

In order to overcome the difficulties to obtain and study purified proteins as large as TSP-1 (~155kDa), different approaches such as chemical peptide synthesis have been developed. In the case of the TSP-1 interaction with CD47, the 4N1K peptide (KRFYVVMWKK) has been the most largely used, despite one more peptide, the 7N3 (FIRVVMYEGKK), was simultaneously described with it (Gao & Frazier 1994). Of note is that the 7N3 has only been used as a function-blocking peptide, or to study the role of CD47 as an adhesion molecule (Csányi et al., 2012; Kaur et al., 2010, 2014; Kaur & Roberts, 2011; Sipes, Krutzsch, Lawler, & Roberts, 1999), which is only possible thanks to its interaction with other membrane receptors such as integrins, VEGFR-2 or CD36 (Soto-Pantoja, Kaur & Roberts, 2015). Direct induction of CD47 signaling (including RCD) through 7N3, though, has not been reported yet. This might be explained by the recent discovery that the VVM motif also requires the intact "FYV" overlapping sequence in order to bind and induce CD47-mediated RCD (Denèfle, et al. 2016), and perhaps a bouquet of signals that the 7N3 fails to activate.

In accordance with this, and the thesis claiming that 4N1K-derived peptides could be a novel approach to treat CLL (Martínez-Torres, et al. 2015), the main goal of the present work was to demonstrate that PKHB1 was able to directly induce caspase-independent RCD on different types of leukemia cells as well.

Among fine additional details, the present notion considers the following:

- TSP-1 binds CD47 through its C-terminal domain containing the "FYVVM" motif
- Diverse peptides containing FYVVM motif triggers CD47 signaling (these are CD47 agonist peptides) in a variety of cell types, similarly to TSP-1 itself
- CD47 agonist peptides selectively induce RCD on different types of cancer cells

It is worth mentioning that it was our group of investigation who recently proved that soluble 4N1K kills primary CLL cells (Martínez-Torres, et al. 2015), and a variety of soluble cancer cell lines (Jurkat and CEM: T-ALL; RAJI and RAMOS: Burkitt's Lymphoma; RPMI-8226 and RPMI-8866: multiple myeloma) (Denèfle 2016). Consistently, in the present study MEC-1 (CLL), CEM (T cell ALL) and L5178Y-R (murine T cell leukemia) cells were observed to be sensitive to killing through 4N1K at similar concentrations (Fig. 2A). In addition, the upgraded version of the 4N1K, PKHB1, which had formerly shown to be more efficient to induce RCD in CLL (Martínez-Torres, et al. 2015; Denèfle, et al. 2016), also showed greater cytotoxicity in CEM and L5178Y-R cells, evidencing its improved stability and affinity with CD47.

Having considered the previous, results showing that PKHB1 induces cell death of different types of leukemia at similar concentrations are interesting to discuss.

9.3. Uniformity and selectivity: clues to a homogeneous machinery in leukemia

The median lethal dose of PKHB1 for virtually all types of leukemia cells tested in the present study was around 200 μM. The only exception was the murine cell line, L5178Y-R, which median lethal dose was 150 μM (Fig. 6). This slight difference can be attributed to two different options: whether 1) PKHB1 has more affinity with murine CD47, or 2) L5178Y-R has a phenotype that slightly favors CD47-induced death signaling. The first option should not be discarded as a differential peptide affinity with murine CD47 has not been demonstrated experimentally. However, human TSP1 controls CD47 signaling equally in human, bovine, porcine, rat and murine cells (Soto-Pantoja, Kaur & Roberts, 2015); thus, this option seems unlikely. Moreover, death signaling triggered by 4N1K varies even within specific cell types (e.g., T-cells in different phases of the immune response [Van V. Q. et al., 2012]), suggesting that is the singularity in each cell what determines the strength of a signal in every case.

Maybe the strongest argument supporting this hypothesis is this peptide's selectivity for leukemic but not healthy cells. Previous reports disclose no cytotoxicity of PKHB1 in healthy B or T cells (Martínez-Torres, et al., 2015; Denèfle, et al., 2016). In the

present study it was shown that, likewise, PKHB1 spares total human PBMCs (Fig. 4A). In addition, as freshly isolated mouse lymphoid cells were extremely fragile (having cell death rates of over 20%, data not shown), a control-normalizing cell viability test was used for a fitter measure of PKHB1-induced damage. And, although this test does not provide cell death-specific characteristics, the analysis indicate that the overall viability of healthy mouse lymphoid cells is not affected by exposure to PKHB1 either (left in occasions for as long as 6 h, data not shown); differently to the ETO which was clearly cytotoxic (Fig. 4B-E).

9.3.1. The potential mechanism of RCD in leukemia does not depend on caspases…

Another aspect that provides evidence that PKHB1-induced killing requires a special phenotype in order to activate RCD is the fact that it does it in a caspase-independent manner regardless of the cell line. This is a recurrent characteristic of CD47-mediated RCD in a variety of cell types (Johansson, Higginbottom, & Londei, 2004; Manna & Frazier, 2004; Mateo et al., 1999; Pettersen, Hestdal, Olafsen, Lie, & Lindberg, 1999; Saumet et al., 2005; Uno et al., 2007). In the present study it was shown that in contrast to the ETO, PKHB1-induced RCD still occurs after pre-incubation with the broad-spectrum caspase-inhibitor, Q-VD-OPH, and even in MEC-1 and K562 cells that are not sensitive to ETO-induced RCD (Fig. 5).

This is relevant because MEC-1 has a mutant *p53*, and consequently it is resistant to most chemotherapies. Similarly, K562, a Philadelphia chromosome positive cell line, is resistant to apoptosis through mechanisms mediated by the BCR-ABL translocation gene (see *2.4.4. Chronic myeloid leukemia*). These mechanisms include a microenvironment-dependent activation of the Jak/STAT signaling pathway and consequent up-regulation of anti-apoptotic Bcl-2 family members (Bewry, et al. 2008). Therefore, the fact that death occurs on both cell lines supports the idea that a non-apoptotic RCD pathway is activated after CD47 engagement with PKHB1 in different types of leukemia.

Studies regarding PKHB1 triggering of RCD on imatinib-resistant CML cells, ATRA-resistant AML cells, K562 or HL-60 cells in presence of anti-apoptotic microenvironmental factors (such as IL-4 or co-culture with endothelial cells) would supply a closer look for a prospective therapeutic use in these types of leukemia, as microenvironment is a source of chemoresistance and disease relapse (see *2.2. Microenvironment and chemoprotection*). Nonetheless, together with the awareness that PKHB1-induced killing in CLL is independent of cells' interaction with microenvironment factors (Martínez-Torres et al. 2015), our results showing that co-culture of CEM or L5178Y-R cells with mouse BMSC does not affect PKHB1-induced cytotoxicity of leukemic cells leads us to believe that this could be a possibility.

To this regard, interesting findings might surge from the study of the effects of CD47 agonist peptides on cells exposed to the strong cell–cell interactions present in the leukemia–microenvironment crosstalk (such as that between T-cells and endothelial cells), as CD47 roles in homotypic and heterotypic cell–cell adhesion have been widely described (Brown & Frazier, 2001; Soto-Pantoja, Kaur & Roberts, 2015). First-step screenings on this have already been realized in the present study by co-culturing CEM cells along with human umbilical vein endothelial cells (HUVEC) (data not shown), however interaction among treated cells is too strong for them to be fully recovered and accurately quantify cell death by flow cytometry. Modifications in the methodology should permit to overcome this issue, though. Still, CD47 implication in the CEM–HUVEC interaction is suggested, and therefore the implication of other co-stimulatory molecules that could be present in the microenvironment might be proposed in the future.

9.3.2. ... but on calcium

Indeed, CD47 can interact with several proteins in the inside and the outside of the cell, and these interactions, as well as their consequent activities, are cell-specific. Still, these are limited, and the fact that cytoplasmic calcium signaling is constantly implicated in CD47 signaling transduction remains. For example, CD47 is part of the complex

of the Rh group, only found in erythrocytes. CD47 is needed for the correct functioning of this complex that is linked to the cytoskeleton via ankyrin (Van Kim et al. 2006), which regulates calcium signaling (Hayashi & Su, 2001). Moreover, CD47 selectively regulates integrin-dependent calcium influx in endothelial cells. It was demonstrated that CD47-function blocking antibody, B6H12, inhibited Ca^{2+} influx stimulated by an integrin ligand, but not that stimulated by histamine, implying that TSP1-CD47 signaling positively regulates cytoplasmic calcium (Schwartz et al., 1993). Consistently, recombinant C-terminal TSP1 containing the CD47 binding domain elevated cytoplasmic Ca^{2+} in Jurkat cells, which in turn inhibited the activation of soluble guanylyl cyclase by nitric oxide (Ramanathan et al., 2011). Furthermore, cross-linking CD47 of rat brain micro-vascular endothelial cells increased cytoplasmic Ca^{2+} mobilization, which was related to an increase in Src kinase activity (Martinelli et al., 2013). In cardiac myocytes, treatment with 7N3 increased cytoplasmic calcium (Sharifi-Sanjani et al., 2014). Notably, inhibition of the Na^{+}/Ca^{+} exchanger using amiloride prevented the 7N3-induced Ca^{2+} mobilization. Conversely, TSP1-CD47 signaling had previously been shown to inhibit an ionomycin-stimulated calcium flux in endothelial cells (Bauer et al., 2010), It is still unclear whether these disparate Ca^{2+} mobilization mechanisms are a result of cell-specific effects of CD47 signaling on cytoplasmic calcium flux or depend on the agonist.

Finally, although Ca^{2+} release is not directly demonstrated in several reports studying the cellular functions of the TSP1-CD47 signaling, known Ca^{2+}-dependent cellular mechanisms (such as those involving cytoskeleton remodeling and actin rearrangement) are commonly described. One example is that, similarly to endothelial cells, CD47 interaction with VEGFR2 in Jurkat T-cells is necessary for VEGFR2-Y^{1175} phosphorylation, and its downstream signaling controlling trafficking, cell proliferation, cell-cell adhesion, and migration. (Kaur et al. 2014) Altogether, the evidence shows that CD47 indirectly controls Ca^{2+} signaling.

It is also clear that Ca^{2+} signaling is altered in cancer cells, although its nature as a cause and/or a consequence of carcinogenesis is not completely understood (Stewart, et

al. 2015). Altered Ca^{2+} transporter protein expression is associated with some cancers, and specific aspects of the Ca^{2+} signal appear to be altered in some cancer cells. (Stuart, et al. 2015) In present work, the role of calcium signaling via CD47 in leukemic T cells of mouse (L5178Y-R) and human (CEM) was demonstrated. This highlights the possibility that, as in CLL, the CD47-mediated cell death pathway in other types of leukemia takes advantage of the altered calcium signaling.

X. CONCLUSIONS

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

- PKHB1 kills different types of leukemia cells, but not healthy human peripheral-blood mononuclear cells or cells from mouse lymphoid organs.
- PKHB1 induces caspase-independent cell death of different types of leukemia.
- PKHB1-triggered cell death overcomes the common bone marrow stromal cells-conferred chemoprotection of T-cell leukemia.
- Ca^{2+} influx is needed for leukemic T-cells to die after ligation of PKHB1.

XI. PERSPECTIVES

The perspectives of the present work mainly comprehend three aspects:

1. Unravel the mechanisms controlling Ca^{2+} influx after peptide engagement

Although it was shown that Ca^{2+} influx was necessary for leukemic T cells to die, the downstream components regulating this well-known executioner mechanism of RCD remain undiscovered. Multiphoton confocal microscopy approaches, and intracellular Ca^{2+} chelators will permit to determine whether intracellular Ca^{2+} mobilization is required to trigger Ca^{2+} influx mechanisms. Moreover, since the PLCγ1, which regulates Ca^{2+} mobilization mechanisms, was demonstrated to control PKHB1-induced cell death in CLL cells, the participation of this and other phospholipases should be tested. Finally, based on the similarities between the mechanisms of RCD triggered by PKHB1 in CLL and ALL, and the caspase-independence on cell death, the study of this mechanism could be extended to myeloid leukemia.

2. Testing the in vivo potential of CD47 agonist peptides in a model using L5178Y-R

The present thesis work widened the potential therapeutic use of PKHB1 from CLL to other types of leukemia, including the murine leukemia/lymphoma cell line L5178Y-R. This opens the possibility to assess the impact of the treatment with PKHB1 in tumor growth of L5178Y-R–allotransplanted mice. With this, the role that the immune system plays *in vivo*, as a result of both potential immunogenicity of CD47-triggered cell death, and activation of CD47 in immune cells, could be studied as well.

3. Improvement in the CD47 agonist peptides efficacy

The concentration of PKHB1 used for cell death induction is still on the micro-molar range, being that the generally accepted *in vitro* potency of a drug is governed by the so called “nanomolar rule” which theorizes that drugs performing at nanomolar scale *in vitro* should be potent enough to be efficient *in vivo*. Being aware of this, improvement of this kind of peptides might be focused on improving peptide stability, CD47 specificity, and facilitating RCD through co-stimulatory signals, molecules or CD47 composition.

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XIII. BIBLIOGRAPHICAL ABSTRACT

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