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TESIS

STUDY OF THE EFFECT OF IMMUNEPOTENT-CRP ON NK AND T LYMPHOCYTES AND T-ACUTE LYMPHOBLASTIC LEUKEMIA CELL LINES

POR

HELEN YARIMET LORENZO ANOTA

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THESIS

BY

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STUDY OF THE EFFECT OF IMMUNEPOTENT-CRP ON NK AND T LYMPHOCYTES AND T-ACUTE LYMPHOBLASTIC LEUKEMIA CELL LINES

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ABBREVIATIONS

Δψm Mitochondrial membrane potential

ACD Accidental cell death

ADCC Antibody-dependent cellular cytotoxicity

AMP Antimicrobial peptides

ADCD Autophagy-dependent cell death

AnnV Annexin-V

APC Antigen presenting Cells

bDLE bovine dialyzable leukocyte extract

BM Bone marrow

CFSE Carboxyfluorescein succinimidyl ester

ConA Concanavalin A

DCFDA Dichlorodihy drofluorescein diacetate

DHE Dihydroethidium

ER Endoplasmic Reticulum

ETPs Early thymocyte progenitors

FACS Fluorescence-Activated Cell Sorting

IL-C Innate lymphoid cells

I-CRP IMMUNEPOTENT-CRP

ICD Immunogenic cell death

IP_3R Inositol-1,4,5-trisphophate receptors

KLR Killer cell lectin-like receptors

LDCD Lysosome-dependent cell death

MHC Major histocompatibility complex

MPT Mitochondrial permeability transition

NAC N-acetylcysteine

NCCD Nomenclature Committee on Cell Death

NCR Natural cytotoxicity receptors

NK Natural killer cells

PBMC Peripheral blood mononuclear cells

PCD Programmed cell death

RCD Regulated cell death

ROS Reactive oxygen species

RyR Ryanodine receptors

T-ALL T-cell acute lymphoblastic leukemia

TCR T cell receptor

TMRE Tetramethylrhodamine ethyl ester

TNF-alpha Tumor necrosis factor

TRAIL TNF-related apoptosis-inducing ligand

ABSTRACT

Immunotherapies have been used in different diseases and recently, in various types of cancer. IMMUNEPOTENT-CRP (I-CRP) has been demonstrated antitumor activity against human and murine cancer cell lines leading to ROS-dependent regulated cell death and recently, has been classified as an immunogenic cell death inductor. It has also demonstrated to prevent chemotherapy induced cytotoxicity in murine bone marrow cells and to improve the life quality of lung and breast cancer patients, increasing the number of total leukocytes as well as the $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ subpopulations. For these reason in this thesis, it was evaluated the effect of I-CRP on different human lymphocytes subsets and the cytotoxic effect on T-cell acute lymphoblastic leukemia (T-ALL) cell lines were evaluated. The activation, proliferation, cytokines release and major lymphocytes subsets were tested in peripheral blood mononuclear cells (PBMC) from healthy donors. We analyzed activation, repertoire receptor modifications and effector activity of NK lymphocytes. In addition, we tested principal subsets of T lymphocytes and their correlation with activation, senesce and exhaustion. The cell death mechanism was evaluated on T-ALL cells. I-CRP did not affect cell viability, however, induced activation without proliferation of PBMC, increasing of pro- and anti-inflammatory cytokines release and modulating of $CD56^{dim}/CD16^{-}$NK subset. Particularly, increased CD69, NKp30, NKp44, NKp46, NKG2D, NKG2C and KIR receptors expression on NK lymphocytes, whereas no significant differences on CD160, CD85j and CD226 were detected and improved their effector activity against target cell. We did not observe changes in CD3 expression on T cells, however, CD4 and CD8 markers diminish, in addition enhance CD69 expression without PD-1 and CD160 expression. On T-ALL cell, ICRP induced in cytoplasmic Ca^{2+} augmentation, provoking to endoplasmic reticulum Ca^{2+} release through IP_3R and RyR Ca^{2+} channels, which lead to a massive ROS production that orchestrated nuclear and mitochondrial damage, effector caspases activation and pro-survival autophagy, interestingly pro-death signals generate apoptosis pathway. In conclusion, I-CRP is a potent immunomodulator of NK and T Lymphocytes derived from healthy donors and a cytotoxic apoptosis inductor on T-ALL cell lines.

RESUMEN

Las inmunoterapias han sido utilizadas en diferentes enfermedades y recientemente, en varios tipos de cáncer. El IMMUNEPOTENT-CRP (I-CRP) ha demostrado actividad antitumoral en líneas celulares humanas y murinas, induciendo muerte celular regulada ROS-dependiente, y ha sido recientemente clasificado como inductor de muerte celular inmunogénica. Sobre el sistema inmune ha demostrado prevenir la citotoxicidad de quimioterapias en células de medula ósea y mejorar la calidad de vida de pacientes de cáncer de mama y pulmón, aumentando el número total de leucocitos así como las poblaciones $CD4^+$, $CD8^+$, $CD16^+$ y $CD56^+$. Por lo tanto, se evaluó el efecto del I-CRP en diferentes subtipos de linfocitos humanos y en líneas celulares de leucemia linfoblástica aguda-T (LLA-T). Se analizó la activación, proliferación, liberación de citocinas y los subtipos de linfocitos más comunes en células mononucleares del sangre periférica (PBMC) de donantes sanos. Analizamos activación, modificaciones en el repertorio de receptores y actividad efectora de linfocitos NK, evaluamos las principales poblaciones de linfocitos T y lo correlacionamos con marcadores de activación, senescencia y agotamiento. El mecanismo de muerte celular se evaluó en células de LLA-T. El I-CRP no afectó la viabilidad, sin embargo indujo activación sin proliferación en PBMC, aumentando la liberación de citocinas pro- y anti-inflamatorias y modulado el fenotipo NK $CD56^{dim}/CD16^{-}$. Particularmente, en linfocitos NK aumentó la expresión de CD69, y de los receptores NKp30, NKp44, NKp46, NKG2D, NKG2C y KIR, no se mostró diferencia en los receptores CD160, CD85j y CD226, lo que mejoró la actividad efectora contra una célula blanco. No se observó cambios en la expresión de CD3 en linfocitos T, sin embargo los marcadores CD4 y CD8 disminuyeron, aumentando la expresión de CD69 sin expresión de PD-1 y CD160. En células LLA-T el ICRP indujo un aumento de Ca^{2+} citoplasmático, provocando la liberación de Ca^{2+} del retículo endoplásmico mediante los canales de Ca^{2+} (IP_3R y RyR), lo que generó la producción masiva de ROS que orquestaron el daño nuclear y mitocondrial activando caspasas y autofagia pro-supervivencia, particularmente las señalas de muerte generan apoptosis. En conclusión, el I-CRP es un potente inmunomodulador de linfocitos NK y T derivados de donantes sanos y un agente citotóxico inductor de apoptosis en células de LLA-T.

1 INTRODUCTION

1.1 IMMUNE SYSTEM

The immune system is a sophisticated system composed by different types of cells subsets, interacting cells, soluble factors, tissues, and organs, all of which are interconnected (Varadé, Magadán, & González-Fernández, 2021). The major function is to provide protection the host from environmental agents such as pathogens (viruses, bacteria, parasites) or chemicals, thereby preserving the integrity of the body. The diversity of components (soluble factors as complement proteins and diverse cellular components) provides a success in the host protection (Cohen & Efroni, 2019).

The immune system can be envisaged as having 2 arms of defense, nonspecific and specific, defined as innate and adaptative immune responses. Particularly, the non-specific arm is the first line of defense against infection and is the first obstacle for a pathogen. The components of the innate immune system cannot differentiate the own from the other in a precise way; additionally, they are not so specialized since they lack the capacity to distinguish minimal differences in the foreign molecules. In contrast, the specific arm, is a second broad line of defense that eliminates pathogens that evade or persist in the first line. The most important characteristic is the immune memory. Both arms involve several distinct components, however they work in a systematic way (De & Tomar, 2014; Kennedy, 2010; Netea et al., 2020). Several cell subsets orchestrated the innate and adaptative immune response. Being, phagocytes, neutrophils, macrophages, natural killer lymphocytes, mast cells, basophils, dendritic cells, and eosinophils, the cellular components in innate immune system, and T and B lymphocytes on adaptative immune system (figure 1). Interestingly, Natural killer T cells and γδ T cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity (De & Tomar, 2014; Dranoff, 2004). Interestingly, Natural killer lymphocytes and T lymphocytes are the major cellular effectors on innate and adaptive immune response, respectively. The development of new specialized equipment and immunological techniques have allowed identification of a large variety of cell subpopulations and the functional analysis (Varadé et al., 2021).

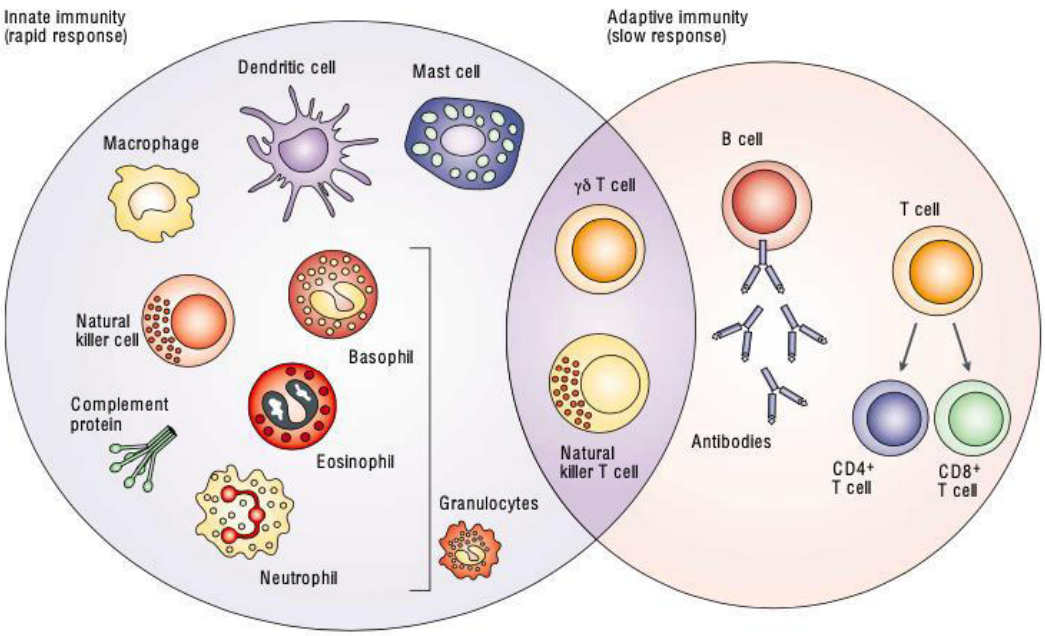

Figure1. Cellular component in the innate and adaptive immune response. The innate immune response is formed by soluble factors, such as complement proteins, and diverse cellular components including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells. The adaptive immune response consists of antibodies, B cells, and CD4+ and CD8+ T lymphocytes. Natural killer T cells and γδ T cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity. Adopted from (Dranoff, 2004).

1.2 NATURAL KILLER CELLS

The natural killer cells (NK) were described in early 1970 as lymphoid cells that possess the ability to lysed tumor cells without prior immunization, determining that they possess a cytotoxic "natural" reactivity. (Herberman et al., 1975; Kiessling, Klein, and Wigzell, 1975). Subsequently, they were classified as a different lineage of lymphocytes, with cytotoxic and cytokine-producing capacity; and phenotypically defined as CD56+ CD3- in humans. They represent 10% of the total population of peripheral blood mononuclear cells (PBMC), and comprise the third population in percentage of lymphocytes, after B and T cells (Mandal & Viswanathan, 2015). Currently, it is known that they also possess the ability to control microbial infections by limiting their spread and subsequent tissue damage and recent research has shown

that NK cells are also regulatory cells involved in interactions with dendritic cells, macrophages, including with T cells and endothelial cells. In this sense, NK lymphocytes can regulate the immune response, limiting or exacerbating cell populations.

1.2.1 NK cell receptor repertoire

NK cells possess a wide range of receptor that are implicated in their natural reactivity expressed on cell surface which can be grouped into different families, such as: natural cytotoxicity receptors (NKp46, NKp44, NKp30), CD244 (2B4), C-type lectin receptors (NKG2D, NKG2A, NKG2C), leukocyte inhibitor receptors (LILRB1) and KIR receptors (Killer Immunoglobulin-like Receptor). In table 1, it is shown the activator receptors and in table 2 the inhibitors receptors of NK cells, as well as the ligands with which they interact.

Table 1. Human NK cell inhibitor receptors and their ligands.

Inhibitor Receptor	Ligand
KIR2DL1	HLA-C2 group (Lys^{80})
KIR2DL2/3	HLA-C1 Group(Asn^{80}), HLA-C2 Grup (Lys80)
KIR2DL5A/B	Undeterminated
KIR3DL3	Undeterminated
KIR3DL1	HLA-A, Bw4
KIR3DL2	HLA-Aw3, Aw11
CD94/NKG2A/B	Signal peptide presented in HLA-E
LILRB1	HLA class I
KLRG1	Cadherin
SIGLECS	Sialic Acid
NKRP1A	LLT1(*Lectine-like transcript-1*)

Table 2. Human NK cell activator receptors and their ligands.

Activator Receptor	Ligand
KIR2DS1	HLA-C2 Group (Lys^{80})
KIR2DS2	HLA-C2 gruop with Viral Peptide
KIR2DS3	Undeterminated
KIR2DS5	HLA-C2
KIR3DS1	HLA-F
KIR2DL4	HLA-G
CD16	Fc fraction antibody
CD94/NKG2C	HLA-E
NKG2D	MIC-A, MIC-B, ULBP1-6
CD94/NKG2E/H	HLA-E
DNAM-1	CD155, CD112
CD58	CD2 (LFA-2)
NKp30	B7-H6, HCMV-pp65, BAG6, heparan sulfate
NKp44	MLL5, viral hemaglutinin, PCNA, PDGF-DD
NKp46	Complement factor P, viral hemagglutinin, heparan sulfate
NKp65	KACL
NKp80	AICL

NK cell we noticed the existence of a population

1.2.2 Subsets

Several subsets of NK cells have been described based on relative expression of the adhesion molecule CD56 and the activating receptor CD16. In table 3, we showed human natural killer cell subsets and their function(Kaur, Trowsdale, & Fugger, 2013). Whereas blood $CD56^{bright}CD16^{dim/-}$ NK cells are classically viewed as immature precursors and cytokine producers, the larger $CD56^{dim}CD16^{bright}$ subset is considered as the most cytotoxic. In peripheral blood of healthy donors, it has been noted that there

is a population of $CD56^{dim}CD16^{dim}$ NK cells that was frequently higher in number than the $CD56^{bright}$ subsets (Amand et al., 2017; Kobyzeva et al., 2020; Poli et al., 2009; Vujanovic et al., 2019). Among other functions, NK cells also have an immunoregulatory role, as cytokines release, such as interferon (IFN-γ), tumor necrotic factor (TNF), macrophage inflammatory protein-1α (MIP-1α), interleukin (IL)-6, granulocyte, and macrophage colony stimulator factor (GM-CSF) and CCL5 (RANTES). Human NK cells can be classified into two subsets, depending on their phenotype and function: CD56dim and CD56bright. CD56dim makes up 90% of the total population of NK cells in peripheral blood, these have high cytotoxic activity and express a low-affinity receptor for the constant region of Immunoglobulin G, Fc γ RIIIa (CD16). The remaining 10% belong to the CD56bright subset and are involved in cytokine production and are mainly found in secondary lymphoid tissues, such as tonsils, lymph nodes, and the spleen (table 3) (Mandal & Viswanathan, 2015). Within the $CD56^{dim}$ NK cell population, further distinctions of individual subsets based on phenotypic and functional characteristics can be made (Figure 2) (Pfefferle et al., 2020).

Table 3. Human Natural Killer Cell Subsets

	CD56dim CD16+ Natural Killer cells	CD56bright CD16- Natural Killer cells
Relative abudance	Constitute 90% of Natural Killer cells in blood	Constitute 10% of Natural Killer cells in blood; predominant in lymphoid organs
KIR expression	High	Low
Cytotoxic activity	High	Low
Cytokine production upon stimulation	Low	High

Adopted from (Kaur et al., 2013).

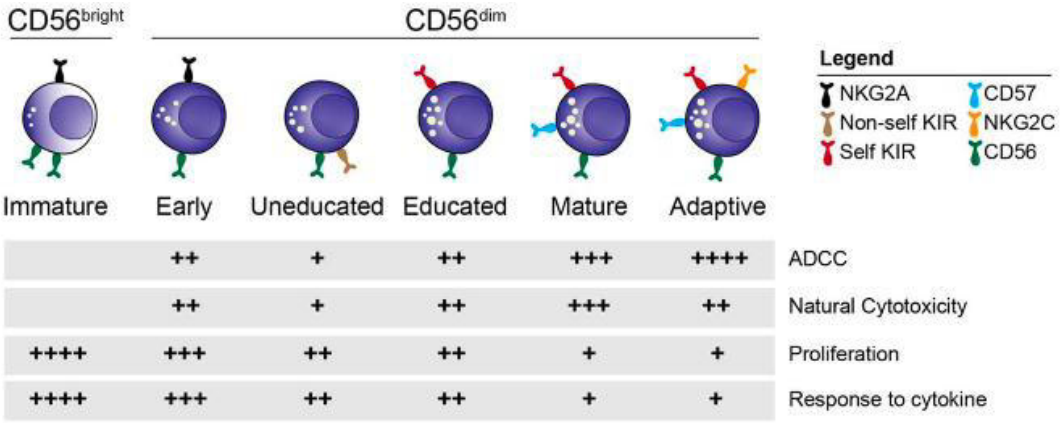

Figure 2. NK cell subsets. Overview of the distinct stages of NK cell differentiation based on phenotypic and functional properties. Adopted from (Pfefferle et al., 2020).

1.2.3 NK Cell Activation

The cytotoxic effect of NK cells could be due to direct or indirect target recognition (Figure 3). In the direct pathway, NK cells directly recognize activation signals in their membrane that induces to activating signals. The principal molecules that could be identified by NK cells are surface glycoproteins present on all nucleated cells, including major histocompatibility complex I (MHC I) or viral antigens. The expression of ligands for activating NK cell receptors must exceed the expression of molecules binding to inhibitory receptors to accomplish target cell lysis. In other hand, an indirect recognition is called antibody-dependent cellular cytotoxicity (ADCC) utilizes the ability to express the FcγRIIIa receptor (CD16) by NK cells, which in turn enables antibody-coated target cell detection. The effect of correct target recognition is the activation of killing mechanisms in NK cells: exocytosis of cytotoxic granules or death receptor-mediated cytotoxicity (Grudzien & Rapak, 2018).

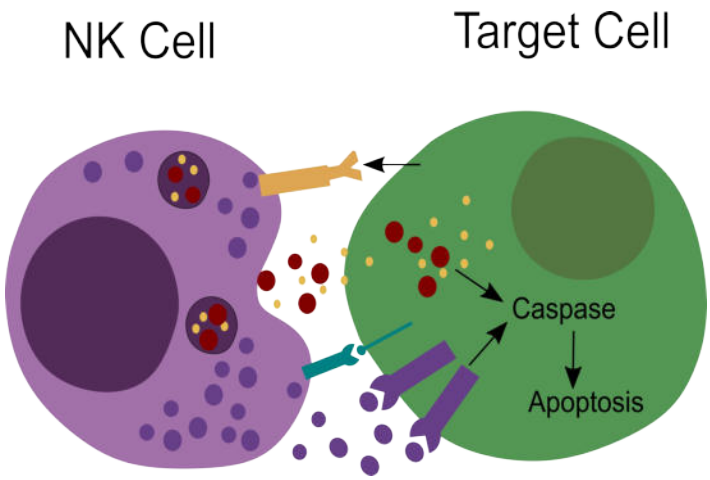

Figure 3. Target recognition and cytotoxicity mechanism in NK cells. Cytotoxicity mechanisms are colored in red and yellow; target recognition pathways are colored in purple. The death receptor recognizes their ligand and apoptosis pathway. The exocytosis of cytotoxic granules leading to apoptosis pathway on the target cell. The ligand recognition by activating receptor leading to apoptosis pathway. The antibody-dependent cellular cytotoxicity (ADCC).

1.3 T CELLS

The lymphocytes T (T Cells) are a type of leukocyte essential on cellular immune response. T cells are defined as $CD45^+$ and $CD3^+$ cells. In human peripheral blood around of 15 to 30% of all $CD45^+$ leucocytes are T Cells. Particularly, on isolated PBMC, T cells represents 45 to 70% of total. T Cells originate in bone marrow (BM) and mature in thymus, where differentiated in to major populations helper $CD4^+$, cytotoxic $CD8^+$ or another recognize antigens through T cell receptor (TCR) (Kumar, Connors, & Farber, 2018) (Velardi, Tsai, & van den Brink, 2021).

Development of T cells, defined as hematopoietic cells expressing either αβ or γδ TCR complexes, requires a primary lymphoid organ, the thymus, which provides a specific microenvironment essential for T cells differentiation (Taniuchi, 2018). Once homing of hematopoietic progenitors, referred to as early thymocyte progenitors (ETPs). ETPs initiate genetic programs directing their development into T cells through exposure to the thymic microenvironment. Typically, T cell develop process in the thymus has been

divided into four stages according to the CD4 and CD8 surface markers expression (Figure 4) (Taniuchi, 2018).

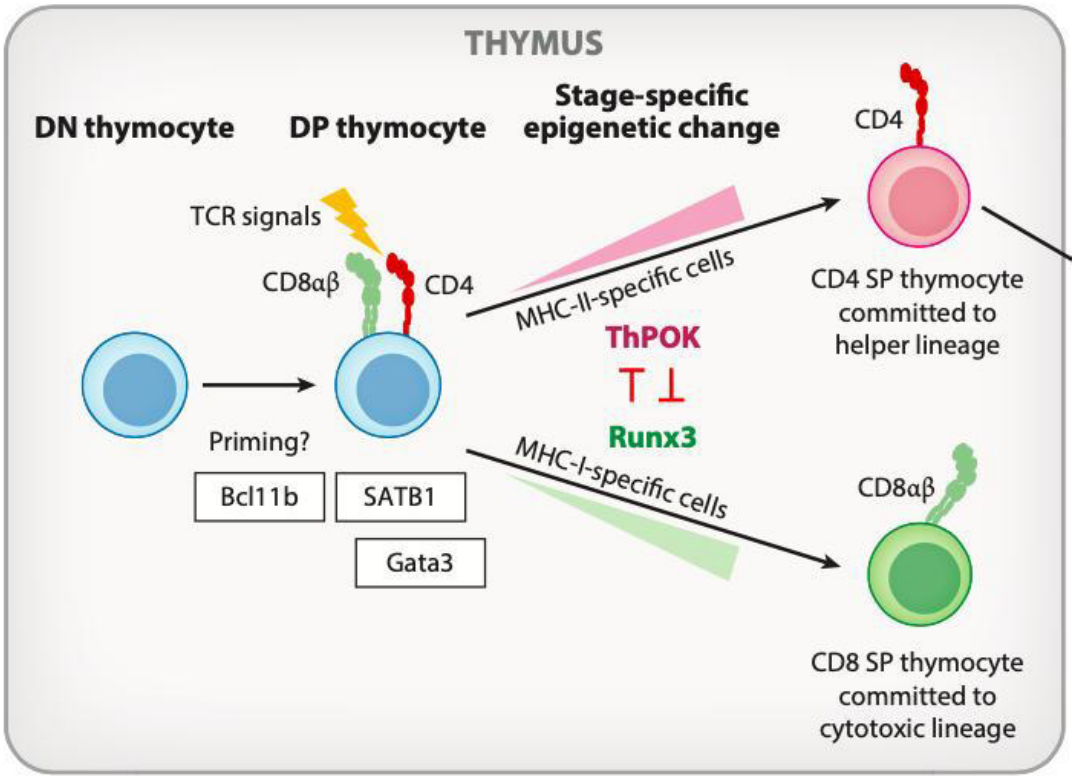

Figure 4. Schematic model of CD4 helper and CD8 cytotoxic T cell differentiation. Adopted from (Taniuchi, 2018).

1.3.1 T cell activation

Within the immune synapse formed between APCs and T cells, three signals are required for antigen-specific T cell activation. T cell activation involves complex interactions, TCR signaling, CD28 co-stimulation and cytokines release by antigen-presenting cells (APCs) are essential required. TCR interacts with foreign antigen in the context of self-MHC, which provided "signal 1". However, signal 1 by itself is insufficient to enable T cell activation. "Signal 2" is provided when CD28 receptor (expressed on T Cell), binds to B7-1 (CD80) and B7-2 (CD86) molecules that are expressed on APCs but not on tumor cells. "Signal 3" is produced by the secretion of cytokines by APCs, which signal via cytokine receptors on T cell to polarize them

toward an effector phenotype. (Figure 5). Therefore, tumor cells alone are unable activated T cell. T cell requires tumor cell fragment to be phagocytosed by APCs such as dendritic cells, with eventual antigen processing and presentation by APCs. T cells then interact with APC to receive three signals for an appropriate T cell activation, which leads to cysteine production and proliferation as well as active killing of tumor cells (Sharma, Campbell, Yee, & Goswami, 2015) (Hwang, Byeon, Kim, & Park, 2020) (Gutcher & Becher., 2007).

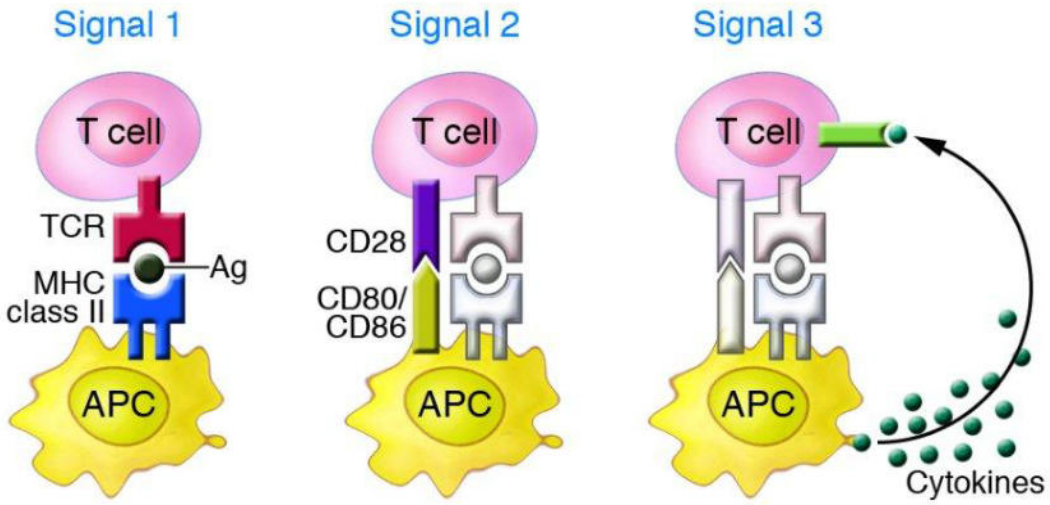

Figure 5. T Cell activation. The signal 1 for T cell activation, occurs following the recognition of MHC-peptide complex on an APC by the TCR on a T cell. The signal 2 for T cell activation is provided by binding of B7 molecules (CD80/ 86) on the APC to CD28 on the T cells. Following this interaction, T cells are activated and perform various effector functions. The signal 3 is produced by the secretion of cytokines by APCs, which signal via cytokine receptors on T cells to polarize them toward an effector phenotype. Adopted from Gutcher & Becher., 2007.

1.3.2 Helper $CD4^+$ T cell

$CD4^+$ T cells, represent around 65% of peripheral T cells. The major function is recognized peptides presented by MHC class II molecules on APCs. They play a major role in instigating and shaping adaptive immune responses. After activation signaling, $CD4^+$ T cells differentiated into distinct effector subtypes playing a major role in mediating immune response through the secretion of specific cytokines. The CD4+T

cells carry out multiple functions, ranging from activation of the cells of the innate immune system, B-lymphocytes, cytotoxic T cells, as well as nonimmune cells, as well as a critical role in the suppression of immune reaction. The principal T $CD4^+$ cells are, T-helper 1 (Th1), T-helper 2 (Th2) cells, T-helper 17 (Th17), follicular helper T cell (Tfh), induced T-regulatory cells (iTreg), T-helper 22 (Th22), regulatory type 1 cells (Tr1) and T-helper 9 (Th9) (Figure 6). The environment, complex network of specific cytokine signaling, and transcription factors followed by epigenetic modifications could induce different subsets of $CD4^+$ cells (Golubovskaya & Wu, 2016; Luckheeram, Zhou, Verma, & Xia, 2012; Tubo & Jenkins, 2014).

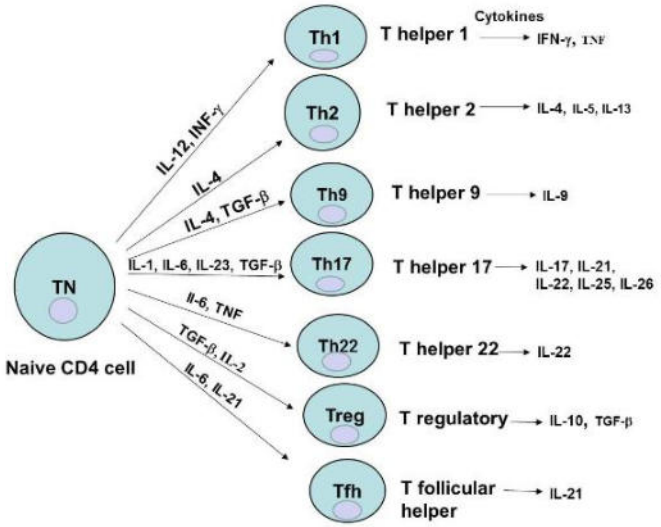

Figure 6. Different $CD4^+$ T cell subsets. The different $CD4^+$ subsets are generated from the naive T cells by the different cytokines. Each $CD4^+$ subset produces a different type of interleukins. Adopted from (Golubovskaya & Wu, 2016).

1.3.3 Cytotoxic $CD8^+$ T cells

$CD8^+$ T cells, also known as cytotoxic T cells, are characterized by the lack of $CD4^+$ in contrast, are characterized by the expression of $CD8^+$ in the plasma membrane. These recognize peptides bound to class I MHC molecules, which are found in all nucleated cells. Once your antigen has been recognized and activated, it can respond in three

different ways. First, through the secretion of cytokines such as TNF-α and IFN-ϒ, with antitumor and antimicrobial effects. The second, through the production and release of cytotoxic granules with perforins and granzymes. The third, by destruction via interaction with FAS ligand, due to FasL expression, inducing an intracellular signaling cascade of its target cell (Iwasaki & Medzhitov, 2015).

1.4 T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic malignancy characterized by aberrant proliferation of immature thymocytes. According to INEGI in México is the most common childhood cancer (INEGI, 2016). Currently, T-ALL represents approximately 12% to 15% of all newly diagnosed ALL cases in pediatric patients and up to 25% of adult (Raetz & Teachey, 2018) (Cordo', van der Zwet, Canté-Barrett, Pieters, & Meijerink, 2021; Roberts, 2018). Several genetic defects are correlated with the progression of T-ALL, such as 9p deletions resulting in inactivation of CDKN2A (p16) and CDKN2B (p15) and translocations affecting the T-cell receptor genes (Roberts, 2018). The gene expression incorporation has provided an additional view on the subgroups present in T-ALL (Fattizzo, Rosa, Giannotta, Baldini, & Fracchiolla, 2020; Girardi, Vicente, Cools, & Keersmaecker, 2017). Currently, has been identifies around 100 genes that could be mutated in pediatric and adults T-ALL patients. A large variety of genes is mutated in adults and pediatric patients are showed in table 4. Based on all genomic data has been correlated with the T-ALL treatments.

Table 4. Mutations frequencies in adults and pediatric patients T-ALL.

Gene	Type of genetic aberration	Frequency (%)	
		Pediatric	Adult
NOTCH1 signaling pathway			
FBXW7	Inactivating mutations	14	14
NOTCH1	Chromosomal rearrangements/Activating mutations	50	57
Cell Cycle			
CDKN2A	9p21 deletion	61	55
CDKN2B	9p21 deletion	54	46
RB1	Deletions	12	
Transcription factors			
BCL11B	Inactivating mutations/deletions	10	9
LMO2	Chromosomal rearrangements/deletions/expression	13	21
MYB	Chromosomal rearrangements/duplications	7	17
TAL1	Chromosomal rearrangements/5´super-enhancer mutations/delections/expression	30	34
TLX1	Chromosomal rearrangements/deletions/expression	8	20
TLX3	Chromosomal rearrangements/ expression	19	9
WT1	Inactivating mutation/deletion	19	11
Signaling			
AKT	Activating mutations	2	2
DNM2	Inactivating mutation	13	13
JAK1	Activating mutations	5	7
JAK3	Activating mutations	8	12
IL7R	Activating mutations	10	12
NRAS	Activating mutations	14	9
PTEN	Inactivating mutations/deletion	19	11
Epigenetic factors			
DNMT3A	Inactivating mutations	1	14
EZH2	Inactivating mutations/deletion	12	12
PHF6	Inactivating mutations/deletion	19	30
SUZ12	Inactivating mutations/deletion	11	5

Adopted from (Girardi et al., 2017).

1.5 REGULATED CELL DEATH

A strict balance between cell survival and cell death is fundamental to development and homeostasis. Normally, every day cells die in our body, it is estimated that per second around one million cells die during normal tissue turnover, hence, cell death is one of the principal homeostatic processes and an essential part of life.

From a conceptual point of view, cell death is defined as the permanent degeneration of vital cellular functions (L. Galluzzi et al., 2015). In other hand, pragmatically speaking it is difficult clarify and identify a difference between a reversible alteration in homeostasis and an irreversible loss of cellular activities appears. To circumvent this issue, the Nomenclature Committee on Cell Death (NCCD) they suggest that a cell should be considered dead when any of the following criteria are met: (1) the permanent loss of the barrier function of the plasma membrane; (2) the breakdown of cells into discrete fragments, which are commonly referred to as apoptotic bodies; or (3) the engulfment of cells by professional phagocytes or other cells endowed with phagocytic activity (Lorenzo Galluzzi et al., 2018)(L. Galluzzi et al., 2015) (Kroemer et al., 2009).

Under these criteria, cell death can be classified as programed, regulated, and accidental cell death (Figure. 7). Accidental cell death (ACD), or also defined as necrosis, could be triggered by physical (abrupted changes in temperature (high/low) or high pressures), chemical (extreme variations in pH or potent detergents) and mechanical (shearing) stimuli. Also, is virtually immediate and is insensitive to pharmacologic or genetic interventions of any kind. Programmed cell death (PCD) stands for those physiological instances of cell death that occur in embryonic or post-embryonic development and tissue homeostasis. In contrast to ACD, the regulated cell death (RCD) involves a genetically encoded molecular machinery and indicates cases of cell death (programmed or not), consequently, can be inhibited or modulated by

pharmacological and/or genetic interventions targeting the key components of such a machinery (L Galluzzi et al., 2014; Lorenzo Galluzzi et al., 2018; Tang, Kang, Berghe, & Vandenabeele, 2019).

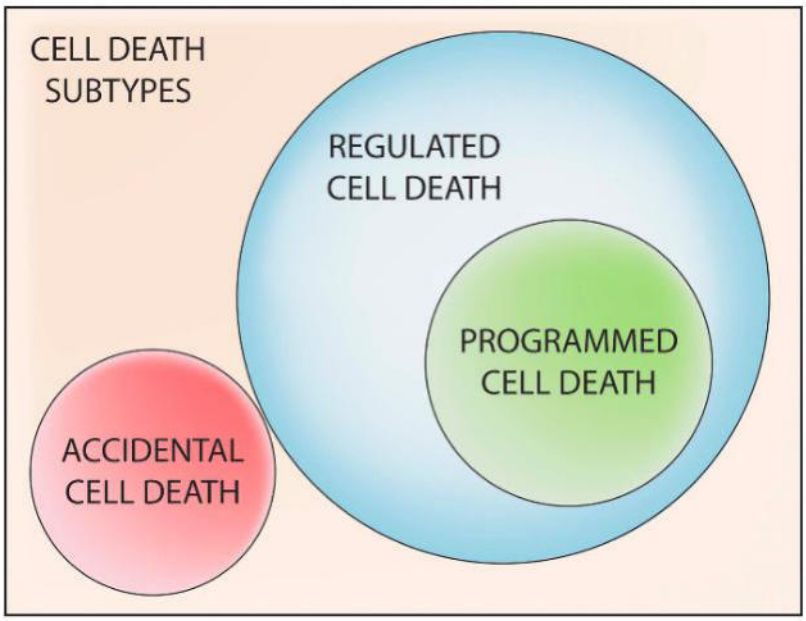

Figure 7. Types of cell death. Regulated cell death can be initiated by a genetically encoded machinery and can be modulated by specific pharmacologic or genetic interventions. Programmed cell death is used to indicate instances that occur as part of a developmental program or to preserve physiologic adult tissue homeostasis Accidental cell death refers a type of cell death that occurs when cells are exposed to an extreme physical, chemical, or mechanical stimulus succumbing in a completely uncontrollable manner, reflecting the immediate loss of structural integrity. Adopted from (L. Galluzzi et al., 2015).

Modulation of intracellular signaling is crucial for cell survival or death. Signaling for cell death can be activated after stimulation of death receptors, damage to cellular structures, deregulation of the system that controls ion movements across cell membranes or other stimuli (X. M. Hu et al., 2021). The major actors involved in the signaling pathway define the type of cell death that would be triggered (Del Re, Amgalan, Linkermann, Liu, & Kitsis, 2019). The NCCD formulated guidelines for the definition and interpretation of cell death from morphological, biochemical, and functional perspectives (Figure 8) (Lorenzo Galluzzi et al., 2018). However, it is

important to mention that an interconnection can exist between different cell death modalities (Lorenzo Galluzzi et al., 2018).

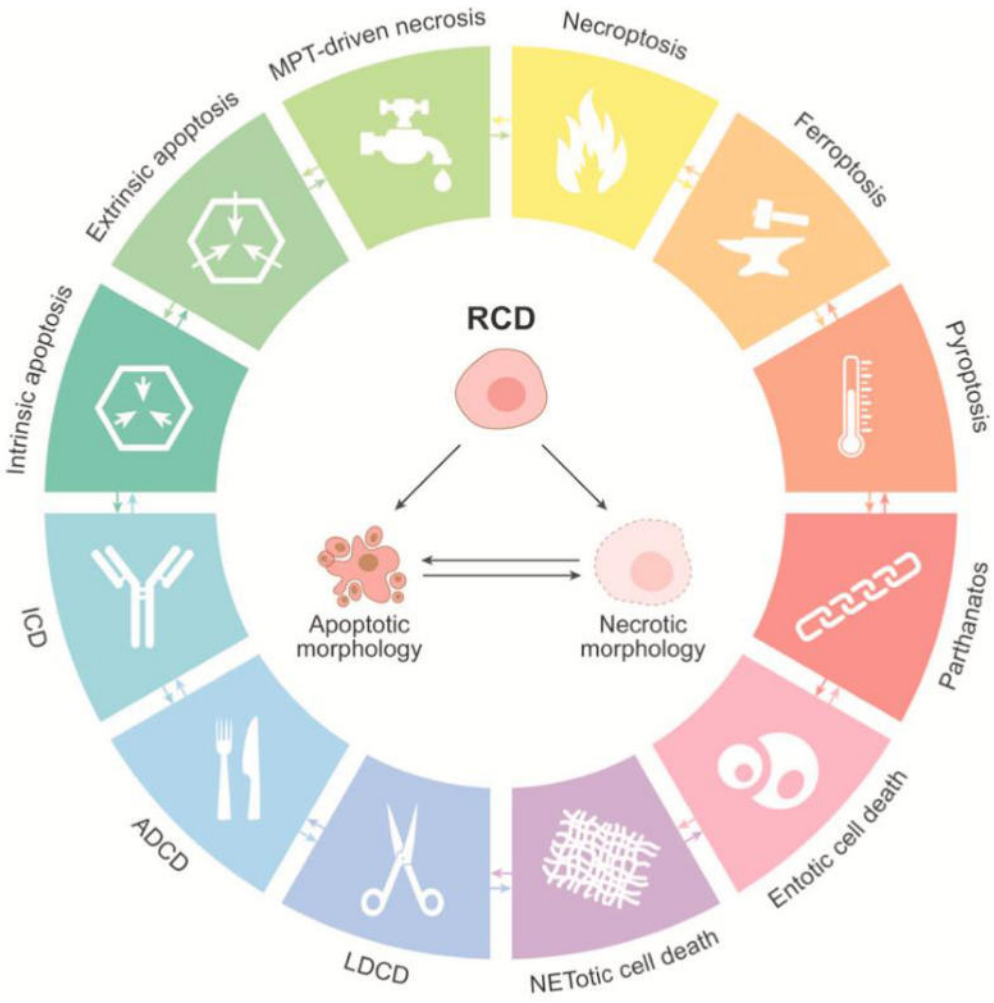

Figure 8. Major cell death subroutines. The cells exposed to perturbation of the intracellular or extracellular microenvironment can activate one of many signal transduction cascades ultimately leading to their demise. Each of such regulated cell death modes is initiated and propagated by molecular mechanisms that exhibit a considerable degree of interconnectivity. Moreover, each type of regulated cell death can manifest with an entire spectrum of morphological features ranging from fully necrotic to fully apoptotic, and an immunomodulatory profile ranging from anti-inflammatory and tolerogenic to pro-inflammatory and immunogenic. ADCD: autophagy-dependent cell death, ICD: immunogenic cell death, LDCD: lysosome-dependent cell death, MPT: mitochondrial permeability transition. Adopted from (Lorenzo Galluzzi et al., 2018).

Table 5. Principal subroutines or Regulated Cell death.

Cell death subroutines	Definition
Extrinsic apoptosis	Specific variant of RCD initiated by perturbations of the extracellular microenvironment detected by plasma membrane receptors, propagated by caspase 8 and precipitated by executioner caspases, mainly caspase 3.
Intrinsic apoptosis	Type of RCD initiated by perturbations of the extracellular or intracellular microenvironment, demarcated by MOMP, and precipitated by executioner caspases, mainly caspase 3.
Autophagy-dependent cell death	A form of RCD that mechanistically depends on the autophagic machinery (or components thereof).
Entotic cell death	A type of RCD that originates from actomyosin-dependent cell-in-cell internalization (entosis) and is executed by lysosomes.
Ferroptosis	A form of RCD initiated by oxidative perturbations of the intracellular microenvironment that is under constitutive control by GPX4 and can be inhibited by iron chelators and lipophilic antioxidants.
Immunogenic cell death	A form of RCD that is sufficient to activate an adaptive immune response in immunocompetent hosts.
Lysosome-dependent cell death	A type of RCD demarcated by primary LMP and precipitated by cathepsins, with optional involvement of MOMP and caspases.
Mitotic death.	Specific variant of RCD (most often, intrinsic apoptosis) driven by mitotic catastrophe.
Necroptosis	A modality of RCD triggered by perturbations of extracellular or intracellular homeostasis that critically depends on MLKL, RIPK3, and (at least in some settings) on the kinase activity of RIPK1.
NETotic cell death	A ROS-dependent modality of RCD restricted to cells of hematopoietic derivation and associated with NET extrusion.

Parthanatos	A modality of RCD initiated by PARP1 hyperactivation and precipitated by the consequent bioenergetic catastrophe coupled to AIF-dependent and MIF-dependent DNA degradation.
Pyroptosis	A type of RCD that critically depends on the formation of plasma membrane pores by members of the gasdermin protein family, often (but not always) as a consequence of inflammatory caspase activation.

Adopted from (Lorenzo Galluzzi et al., 2018).

1.5.1 APOPTOSIS A REGULATED CELL DEATH MECHANISM

Apoptosis is a type of RCD characterized by membrane blebbing, cell shrinkage, condensation of chromatin, and DNA fragmentation events orchestrated by caspases (cysteine-dependent aspartate-directed proteases) and can be initiated by extrinsic or intrinsic pathway (L. Galluzzi et al., 2015; Lorenzo Galluzzi et al., 2018) (Tang et al., 2019).

Extrinsic apoptosis strictly can be initiated by extracellular microenvironment perturbations. Extrinsic apoptosis is mostly driven by either of two types of plasma membrane receptors: (1) death receptors, whose activation depends on the binding of the cognate ligand(s), and (2) dependence receptors, whose activation occurs when the levels of their specific ligand drop below a specific threshold (Lorenzo Galluzzi et al., 2018; Lestari & Rifa, 2018). Death receptors include (but are not limited to): Fas cell surface death receptor (FAS; also known as CD95), and TNF receptor superfamily member 1A (TNFRSF1A; best known as TNFR1), 10a (TNFRSF10A; best known as TRAILR1 or DR4), and 10b (TNFRSF10B; best known as TRAILR2 or DR5) (Lorenzo Galluzzi et al., 2018). In general, death receptor ligation allows for the assembly of a dynamic multiprotein complex at the intracellular tail of the receptor, such as so-called “death-inducing signaling complex” (DISC), which operate as molecular platforms to regulate the activation and functions of caspase 8 (or caspase 10, in a limited number of settings). The molecular mechanisms regulating caspase 8 activity upon death receptor stimulation have been extensively investigated. Caspase 8 maturation involves a cascade of events initiated by the binding of caspase 8 to FADD at the DISC. This interaction enables the assembly of a linear filament of caspase 8

molecules that facilitates homodimerization and consequent activation by autoproteolytic cleavage (Figure 9) (Lorenzo Galluzzi et al., 2018).

Intrinsic apoptosis is a form of RCD initiated by a variety of microenvironmental perturbations including (but not limited to) growth factor withdrawal, DNA damage, endoplasmic reticulum (ER) stress, reactive oxygen species (ROS) overload, replication stress, microtubular alterations or mitotic defects. Apoptotic cells retain plasma membrane integrity and metabolic activity (to some degree) (Lorenzo Galluzzi et al., 2018). The critical step for intrinsic apoptosis is irreversible and widespread mitochondrial outer membrane permeabilization (MOMP), which is controlled by pro-apoptotic and anti-apoptotic members of the BCL2, apoptosis regulator (BCL2) protein family, a group of proteins sharing one to four BCL2 homology (BH) domains (Mariño & Kroemer, 2013). In response to apoptotic stimuli, MOMP is mediated by BCL2 associated X, apoptosis regulator (BAX), and/or BCL2 antagonist/killer 1 (BAK1; best known as BAK), both of which contain four BH domains and a conserved transmembrane domain. These pro-apoptotic members of the BCL2 protein family (which contain a single BH3 domain) are activated transcriptionally or post-translationally as specific organelles or cellular compartments experience perturbations of homeostasis, de facto operating as cellular transducers of stress signaling (Olsson et al., 2016). In addition, some anti-apoptotic BCL2 family members have been proposed to promote cellular survival by: (1) regulation in the homeostasis of ion calcium (Ca^{2+}) on endoplasmic reticulum (ER); (2) promoting bioenergetic metabolism upon interaction with the F1FO ATP synthase; and (3) favoring to the redox homeostasis regulation. Moreover, MOMP directly induces the cytosolic release of apoptogenic factors that commonly are in intermembrane space on mitochondria. These mitochondrial proteins include (but are not limited to) cytochrome c, somatic (CYCS), which usually operates as an electron shuttle in the mitochondrial respiratory chain, and diablo IAP-binding mitochondrial protein (DIABLO; also known as second mitochondrial activator of caspases, SMAC) (Lorenzo Galluzzi et al., 2018). The release of CYCS and SMAC to the cytosol is favored by mitochondrial cristae remodeling, which relies on the oligomerization and activation of OPA1, mitochondrial dynamin like GTPase (OPA1). The cytosolic pool of CYCS binds to

apoptotic peptidase activating factor 1 (APAF1) and pro-caspase 9 (caspase 9) in a deoxyATP- dependent manner to form the supramolecular complex known as apoptosome, which is responsible for caspase 9 activation. The activated caspase 9 can catalyze the proteolytic activation of effector caspases as caspase 3 and caspase 7, which are widely perceived as the enzymes responsible for cell demolition during intrinsic (and extrinsic) apoptosis in mammalian cells (and hence are commonly known as executioner caspases) (Figure 9).

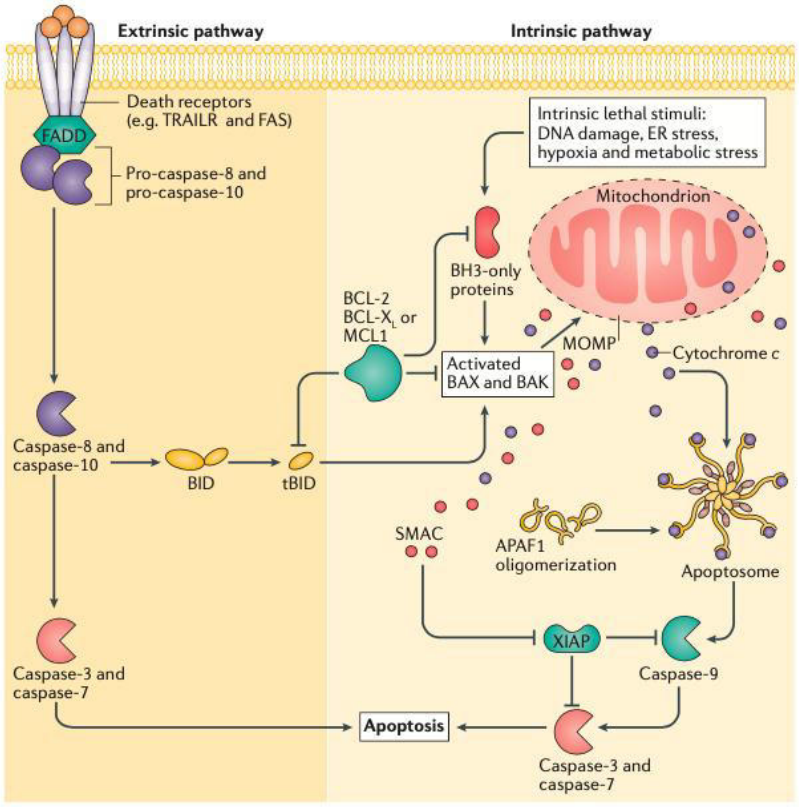

Figure 9. Extrinsic and intrinsic apoptotic signaling pathways. In the extrinsic apoptotic pathway, upon binding to their cognate ligand, death receptors such (TRAILR of FAS) that can activate initiator caspases (caspase-8 and caspase-10) through dimerization mediated by adaptor proteins such as FAS-associated death domain protein (FADD). Active caspase-8 and caspase-10 then cleave and activate the effector caspase-3 and caspase-7, leading to apoptosis. The intrinsic (or mitochondrial) pathway of apoptosis requires mitochondrial outer membrane permeabilization (MOMP). Following MOMP, mitochondrial intermembrane space proteins such as second mitochondria-derived activator of caspases (SMAC) and cytochrome c are released into the cytosol. Cytochrome c interacts with apoptotic protease activating factor 1 (APAF1), triggering apoptosome assembly, which activates caspase-9. Active

caspase-9, in turn, activates caspase-3 and caspase-7, leading to apoptosis. Mitochondrial release of SMAC facilitates apoptosis by blocking the caspase inhibitor X-linked inhibitor of apoptosis protein (XIAP). Caspase-8 cleavage of the BH3-only protein BH3-interacting death domain agonist (BID) enables crosstalk between the extrinsic and intrinsic apoptotic pathways. Adopted from (Ichim & Tait, 2016).

1.6 IMMUNOTHERAPIES

Immunotherapies, also called biologic therapy by the National Cancer Institute (NCI), are therapies that use patient's immune system to fight different diseases, such as tuberculosis (Zielinski et al. 1984), rheumatoid arthritis, infections, and cancer (Baxevanis, Perez, and Papamichail 2009). With this type of therapeutic strategies in cancer, it is expected to release immune control points and in turn inhibit signals or molecules derived from tumors that impede effective tumor immunity (Baxevanis, Perez, and Papamichail 2009).

Immunotherapy may be specific or nonspecific (Oiseth and Aziz 2017). Specific immunotherapy seeks the activation of the antigen-specific immune response through T-cell-dependent cell immunity or through antibody-producing B-lymphocyte-dependent humoral immunity. On the other hand, non-specific immunotherapy, through non-specific immune responses; for example, when the overall immune response is reinforced. In both cases, the response can be conferred by passive immunization, an example of this type of therapy are cancer vaccines, or active, using serum and/or immune system cells or their products (Oiseth and Aziz 2017).

Current cancer therapies have not been entirely successful, due to the molecular characteristics of cancer cell and all its mechanisms to evade the immune system, however, several research has been investigated in the deployment of immunotherapy as a cancer treatment. These therapies found eliminate cancerous cells, while providing signals and tumor antigens to promote the innate and adaptive immune system to simultaneously support tumor elimination.

1.6.1 DIALYSABLE LEUKOCYTE EXTRACTS

Dialysable leukocyte extracts (DLE) are a type of immunotherapy that consists of a heterogeneous mixture of low molecular weight substances obtained from blood or lymphoid tissue, that has immunomodulatory properties. Several reports demonstrated that DLE derived from human blood or lymphoid tissue from pig, or crocodile blood, or bovine leukocytes from spleen or colostrum could modulate multiple molecular targets promoting to immunomodulatory activities in conditions such as immunodeficiencies, autoimmune, bacterial diseases, asthma, allergies, and some types of cancer (Ramírez-Ramírez et al., 2016; Zajícová et al., 2014). The bioactive peptides obtained from DLE, regardless of the species from which they were obtained, have demonstrated similar effects on leukocytes from mice and humans, which include the activation of similar signaling pathways related to their immunomodulatory effects.

1.6.2 IMMUNEPOTENT-CRP

IMMUNEPOTENT-CRP (I-CRP) a kind of immunotherapy, is a dialysable extract obtained from bovine spleen (bDLE), which provides several applications in human. Several studies have been demonstrated that could modulates human and murine immune cells, and that induces cytotoxicity against human and murine tumoral cell lines.

Particularly, their cytotoxic effect has been shown in breast cancer cell lines (MCF-7, BT-474, MDA-MB231, and 4T1) (M. A. Franco-Molina et al., 2008; Martínez-Torres et al., 2020; Reyes-Ruiz, Calvillo-Rodriguez, Martínez-Torres, & Rodríguez-Padilla, 2021a) of lung cancer (A-427, CALU, U937) (Martinez-Torres et al., 2019), in murine lymphoma (L5178Y-R) (M. A. Franco-Molina et al., 2006) and in cervical cancer cells (HeLa) (Martinez-Torres et al., 2019; Martinez-Torres, Reyes-Ruiz, Benítez-Londoño, Franco-Molina, & Rodríguez-Padilla, 2018; Martínez-Torres et al., 2020); without affection on human peripheral blood mononuclear cells (PBMC).

Additionally, several reports demonstrated that I-CRP promotes an exacerbated ROS production, lead to mitochondrial damage, DNA degradation and cell cycle arrest, triggering to ROS-dependent regulated cell death on tumoral cell lines (Martinez-Torres et al., 2019; Martínez-Torres et al., 2020; Martínez-Torres, Reyes-Ruiz, Benítez -Londoño, Franco-Molina, & Rodríguez-Padilla, 2018; Reyes-Ruiz, Calvillo-Rodriguez, Martínez-Torres, & Rodríguez-Padilla, 2021b). Interestingly, I-CRP revealed in a murine breast cancer model to induce immunogenic cell death (ICD) involving DC maturation in lymph nodes, and augmentation of CD8+ T-cell in lymph nodes, peripheral blood and tumor site leading to long-term memory (Reyes-Ruiz et al., 2021b), and similar in murine melanoma model I-CRP combined with oxaliplatin increased DAMPs release and the rate of ICD (Rodríguez-Salazar et al., 2017). Recently, I-CRP plus doxorubicin/cyclophosphamide chemotherapy remodel the tumor microenvironment in an air pouch triple-negative breast cancer murine model decreasing PD-L1, IDO and Gal-3 expression, IL-6, IL-10, and MCP-1 levels, and increasing IFN-γ, and IL-12 levels (Santana-Krímskaya et al., 2020).

In other hand, I-CRP demonstrated an immunomodulator effect on immune system. Particularly, I-CRP decreased pro-inflammatory cytokines such as TNF-α, IL-6, and NO in human and murine macrophages stimulated with lipopolysaccharide (LPS) (M. A. Franco-Molina et al., 2007; Moisés A. Franco-Molina, Mendoza-Gamboa, Castillo-León, Tamez-Guerra, & Rodríguez-Padilla, 2004), increasing the antioxidant activity of CAT, GPx and SOD, decreasing the production activities of NO, and TNF-α, COX-2 and PGD2, phosphorylation of IkB, DNA binding activity of the subunit NF-kB p50 and p65 in human macrophages stimulated by LPS(M. A. Franco-Molina et al., 2006, 2007). Additionally, I-CRP demonstrated a chemoprotective activity, increasing the percentages of granulocytes, improving hematological levels, and recovering the weight gain of treated mice (Evangelina & Moisés Armides Franco-Molina, 1 EdgarMendoza-Gamboa, 1 Heriberto Prado-García, 2 Lydia Guadalupe Rivera-Morales, 1 Pablo Zapata-Benavides, 1 María del Carmen Rodríguez-Salazar, 1 Diana Caballero-Hernandez, 1 Reyes Silvestre Tamez-Guerra, 2016). In clinical trials, I-CRP was shown to increase total leukocytes and platelets in neonates with septic shock in the first weeks of life (Rodríguez Balderrama, 1999). Moreover, in other

breast and lung cancer clinical trials, I-CRP also demonstrated enhances the total number of leukocytes in the $CD4^+$, $CD8^+$, $CD16^+$, and $CD56^+$ subpopulations, improving to life´s quality (Moises A. Franco-Molina et al., 2017; Lara, Turrent, Garza -Treviño, Tamez-Guerra, & Rodriguez-Padilla, 2010).

Therefore, this work was focused to analyze the effect of I-CRP in the immunophenotype and effector activity in non-cancerous NK and T lymphocytes and on the other hand in the cytotoxic effect on T-ALL cell lines. For, to propose new alternatives for its use, in pathologies associated with the immune system, extrapolate it to other types of cancer and to be able to be coupled with other treatments.

2 JUSTIFICATION

T-cell acute lymphoblastic leukemia (T-ALL) is a type of cancer derived from the bone marrow, affects T lymphocytes, and occurs mainly in pediatric patients. Currently, the main treatments for T-ALL are combinations of chemotherapies and corticosteroids, and stem cell transplantation, being the immunotherapies the last line of therapies. Immunotherapies strengthen the immune system to fight multiple diseases such as infections, immunodeficiencies, and autoimmune diseases, and recently, they are being used as an adjuvant in cancer treatment. On the other hand, current antitumor therapies lack selectivity and directly affect the immune cells of the cancer patients. So today it is difficult to find a therapy that can eliminate tumor cells and positively modulate the immune system.

IMMUNEPOTENT-CRP (I-CRP) is an immunotherapy that consists in a dialysable leukocyte extract obtained from bovine spleen (bDLE). Previously, it has been demonstrated that it increases leucocytes when used as an adjuvant in breast and lung cancer treatment. Additionally, it decreases cytotoxic chemotherapy in murine bone marrow cells and decreases pro-inflammatory cytokines in human and murine macrophages during LPS stimulation. In other hand, I-CRP has demonstrated cytotoxic activity on tumor murine and human cell lines, leading to ROS-dependent cell death, and was recently classified as an immunogenic cell death inductor.

Thus, on the one hand I-CRP has an antitumor capacity on tumor cell lines, and on the other hand it modulates immune cells. These characteristics led to the following

questions: how does I-CRP modulate lymphoid cells? and how does I-CRP kill T-ALL cells? Thus, the aim of this thesis project was to understand the immunomodulator activity of I-CRP on lymphoïd cells and the cytotoxic effect on T-ALL cell lines. This work provides new knowledge about the effect of I-CRP in leukemic lymphocytes and in non-cancerous lymphocytes, in order to improve I-CRP application, and to target its use in hematological malignancies and to be able to be coupled with other treatments.

3 HYPOTHESIS

IMMUNEPOTENT-CRP induces cell death in T-cell acute lymphoblastic leukemia cells, and improves the proliferation, viability, and activation of different subpopulations of non-cancerous lymphocytes.

4 OBJECTIVES

4.1 Main objective

To analyze the effect of IMMUNEPOTENT-CRP on the viability of T-cell acute lymphoblastic leukemia cells and non-cancerous lymphocytes, and on the proliferation and activation of different subpopulations of non-cancer lymphocytes.

4.2 Specific objective

1. To analyze the immunomodulator effect of IMMUNEPOTENT-CRP on peripheral mononuclear cells derived from healthy donors.
2. To analyze the effect of IMMUNEPOTENT CRP on immunophenotype and effector activity of NK lymphocytes derived from healthy donors.
3. To investigate the effect of IMMUNEPOTENT CRP on T lymphocytes derived from healthy donors.
4. To evidence the cell death mechanism of IMMUNEPOTENT CRP-induced T acute lymphoblastic leukemia cell lines.

5 CHAPTERS

5.1 CHAPTER I

IMMUNEPOTENT-CRP (I-CRP): induces Changes in the Natural Killer cell repertoire and function

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Keywords: Natural Killer Cells, Dialyzable Leukocyte Extract, Cell Activation, NK Cell Receptors Repertoire, Effector Activity, Immunotherapy

5.1.1.1 Abstract

Natural killer (NK) cells are $CD3^{-}$, $CD16^{+}$, $CD56^{+}$ large granular lymphocytes that play a crucial role in the immune response by recognizing and eliminating a variety of virus-infected, malignant, and antibody-coated target cells. In this study, we examined activation, changes in the NK cell repertoire, and effector functions of normal donors' NK cells treated with IMMUNEPOTENT-CRP (I-CRP), a bovine dialyzable leukocyte extract (DLE) containing bioactive peptides. We found that I-CRP induces the activation of NK cells and a positive modulation on $CD56^{Dim}$ $CD16^{-}$ subset, without inducing proliferation. NK cell receptor analyses indicated an increase on NKp30, NKp44, NKp46, NKG2D, NKG2C and KIR, however no significant differences on CD160, CD85j and CD226 where observed. I-CRP-treated NK cells exhibited an increased degranulation activity against K562 target cells, as shown by the CD107a assay, and correlate to the cytotoxicity against K562 cells observed in the calcein release assay. These results indicate that I-CRP can modify human NK cells receptor repertoire leading to an increased cytotoxic activity, providing strong evidence for its use to stimulate NK cells against viruses, bacteria, or cancer cells.

5.1.1.2 Introduction

Natural killer (NK) cells are innate lymphoid cells (ILC) commonly defined as $CD56^{+}$ (neural cell adhesion molecule) and/or $CD16^{+}$ (low-affinity antibody-binding receptor

Fcγ- receptor IIIa) and absence of CD3 molecule on the surface (Cichocki, Grzywacz, & Miller, 2019; Grudzien & Rapak, 2018). CD56 and CD16 markers are frequently used to define the NK cell functions as immunomodulatory or cytotoxic (Miller, 2001)(Vivier, Tomasello, Baratin, Walzer, & Ugolini, 2008). Biological functions of NK cells play an important role in host defense, as they are involved in recognizing and killing of tumor or pathogen infected cells. They also play a key immunoregulatory role, as NK cells can secrete various set of cytokines, such as interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha, among others (Fauriat, Long, Ljunggren, & Bryceson, 2010)(Zwirner & Ziblat, 2017)(Fuqua, Parsek, & Greenberg, n.d.).

NK cells constantly interact with other cells in the organism. While normal cells, with adequate amounts of major histocompatibility complex (MHC) class I molecules on their surface, cannot be eliminated by NK cells (except Dendritic cells); transformed cells, with a lowered or lost expression of MHC class I molecules and increased expression of stress molecules, can be recognized and killed (Orr & Lanier, 2010). Thus, the altered expression of class I antigens or increased expression of stress molecules, a common event in tumor transformation or following a pathogen infection, leads to NK cell-mediated target cell lysis (Abel, Yang, Thakar, & Malarkannan, 2018; Iwasaki & Medzhitov, 2015). This "natural" cytotoxicity is controlled by a wide range of receptors expressed on the cell surface, which can be classified on different families, such as: natural cytotoxicity receptors (NCR; NKp46, NKp44, NKp30), killer cell lectin-like receptors (NKG2D, NKG2A, CD161), leukocyte inhibitory receptors (LILRB1), and killer immunoglobulin-like receptor (KIR; CD158a/h, CD158b/j, CD158d) (Fuqua et al., n.d.; Pende et al., 2019; Sternberg-Simon et al., 2013). Depending on the subset, NK cells express different combinations of inhibitory or activating receptors, resulting in a diversity within the NK cell populations with the potential to respond to a variety of stimuli, under different pathological conditions (Held, Coudert, & Zimmer, 2003; Pende et al., 2019). A fine regulation between opposite signals delivered by inhibitory and/or activating receptors dictates “final” NK cells effectors functions (Chaouat, 2008; Held et al., 2003).

Tumor progression and chronic infections generally lead to the depletion or exhaustion of NK cells, limiting their anti-tumor/infection potential (Abel et al., 2018)(Vidal,

Khakoo, & Biron, 2011)(Sungur & Murphy, 2014)(Höglund & Klein, 2006). Multiple mechanisms contribute to these effects, such as dysregulations in receptor signaling, as well as suppressive effects of regulatory cells and/or soluble factors in the microenvironment (Sungur & Murphy, 2014). It has been shown that this condition could be surpassed by cytokine-mediated activation as well as the use of other NK-cell target immunotherapies, increasing immunity against the tumor or improving the clinical response to the conventional treatment being carried out (Cantoni et al., 2016)(Mandal & Viswanathan, 2015)(W. Hu, Wang, Huang, Sui, & Xu, 2019).

Immuno-directed therapies have taken an important place in the treatment of several disease. Among them, Dialyzable leukocyte extracts (DLE), which are a mixture of low molecular weight substances, including bioactive peptides, obtained from blood or lymphoid tissues, could have immune-modulation action (Arnaudov & Kostova, 2015). They have been shown to transfer cell-mediated immunity from an immune donor to a non-immune recipient, and beneficial effects were observed when implemented in conditions such as immunodeficiencies, autoimmune, bacterial diseases, asthma, allergies, and some types of cancer (Arnaudov & Kostova, 2015; Salazar-Ramiro, Hernández-Pedro, Rangel-Lopez, Cruz, & Estrada-Parra, 2018; Zajícová et al., 2014). Phase I clinical studies performed shown that IMMUNEPOTENT-CRP (I-CRP) treatment improve life´s quality in lung and breast cancer patients , increasing the number of total leukocytes as well as the $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ subpopulations (M. A. Franco-Molina et al., 2008; Lara et al., 2010). Also, I-CRP is selectively cytotoxic against cancer cell lines *in-vitro*, while it possesses an immunomodulatory effect on immune system cells (Lara et al., 2010; Lorenzo-Anota, Martínez-Torres, Scott-Algara, Tamez-Guerra, & Rodríguez-Padilla, 2020a; Martinez-Torres et al., 2019, 2020, 2018). However, the direct effect on lymphocytes' functionality has not been clearly defined, and its effect in NK cells has not been assessed. Thus, the aim of this study was to analyze the *in-vitro* effect of I-CRP in human NK cells, focusing on its potential activation, repertoire modifications and effector activity of NK cells. By testing their activation, repertoire modifications and effector activity.

5.1.1.3 Material and Methods

5.1.1.3.1 IMMUNEPOTENT-CRP

IMMUNEPOTENT-CRP (I-CRP), bovine dialyzable leukocyte extract (bDLE), was produced by the Laboratorio de Inmunología y Virología of Facultad Ciencias Biológicas of Universidad Autónoma de Nuevo León (UANL, México), as previously described (Moisés A. Franco-Molina et al., 2004). One unit (U) of I-CRP is defined as the product obtained from 1×10^{8} leukocytes. I-CRP was resuspended in RPMI 1640 medium, (GIBCO Thermofisher, Waltham, Massachusetts, USA) supplemented with 1 µg/mL amphotericin B, 1 µg/mL penicillin and 2.5×10^{-3} µg/mL streptomycin (GIBCO® by Life technologies) and 10% of FBS (GIBCO® by Life technologies), referred as culture medium. The stock solution was stored at 4°C.

5.1.1.3.2 Cell isolation

Peripheral blood mononuclear cells (PBMC) were obtained after centrifugation on Ficoll-Paque™ PLUS (GE Healthcare, Chicago, Ilinois, USA). PBMC were maintained at 1×10^{5} cells/mL at 37ºC in 5% CO_2 atmosphere, using culture medium. Human myelogenous leukemia cell line, K562, (ATCC® CCL-243™) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and was maintained under suggested conditions.

5.1.1.3.3 NK cells purification

NK cells ($CD56^{+}$, $CD16^{+}$ and $CD3^{-}$) were isolated from PBMC culture of six healthy individuals (n=6) by negative selection using magnetic-activated cell sorting (MACS) microbead technology (Miltenyi Biotec, Bergisch Gladbach, Germany; >90% purity and >90% viability), with anti-CD20 microbeads (cat:130-091-104, Miltenyi Biotec), anti-CD3 microbeads (cat: 130-050-101, Miltenyi Biotec), and anti-CD14 microbeads (cat:130-050-201; Miltenyi Biotec) cocktail, as stated by manufacturer's instructions. NK cells culture was maintained at same conditions than PBMC culture.

5.1.1.3.4 Analysis of lymphocyte populations

The principal lymphocytes subsets ($CD3^+$, $CD20^+$ and $CD56^+$ $CD16^+$) of PBMC were evaluated as follows: 1×10^5 PBMC per well were seeded in conic 96-well plates (Corning Inc. Costar®, NY, USA) and treated with different concentration of I-CRP (0.3, 0.7 and 1.5 U/mL) or with the mitogen concanavalin A as control (ConA; 5g/mL; Sigma-Aldrich, Germany) for 24h and 48h. After treatment, cells were recollected, washed and stained with anti-CD56 (CD56-PE; cat:555516, BD Biosciences), anti-CD16 (CD16-PerCP-Cy; cat:560717, BD Biosciences), anti-CD3 (CD3-FITC; cat:555916, BD Biosciences) and anti-CD20 (CD20-APC; cat:559776, BD Biosciences) in 100 L of FACS buffer at 37ºC in darkness. After 30 min of incubation, cells were washed, assessed by flow cytometry (Fluorescence-Activated Cell Sorting [FACS]; BDAccury6; Becton Dickinson, San Jose, CA, USA), and analyzed using FlowJo Software (Tree Star Inc., Ashland, OR, USA).

5.1.1.3.5 NK-cells receptors repertoire

All phenotypic studies were implemented on PBMC, by seeding 1×10^5 per well (96-well round bottom plate) and treated with I-CRP (1.5 U/mL), or IL-2 (200 U/mL) as positive control, or left unstimulated cells as negative control, during 24h and 48h. Thereafter, supernatant was discarded, and cells were stained for 30 min with the following Abs CD45 (clone J33), CD3 (UCHT1), CD16 (3G8), CD160 (191B8), NKp44 (BAB281), NKp30 (Z25), CD85j (HP-F1), CD158e1/2 (Z27.3.7), CD158i (FES172), NKG2D (ON72), NKp46 (BAB281) from Beckman Coulter, CD56 (B159), CD69 (L78), CD226 (DX11), CD161 (DX12) from BD Biosciences, NKG2C (134591), CD158d (181703) from R&D Systems, and CD158b/j (DX27) CD158a/h (11PB6) from Miltenyi Biotec. Analyses were performed by nine-color staining flow cytometry using a CytoFLEX S cytometer and analyzed using Kaluza Analysis 2.1 software (Beckman Coulter). Lymphocytes were gated in FSC/SSC dot plot and at least 30,000 events were collected, then $CD45^+$ were selected and $CD3^+$ lymphocytes were eliminated from the analysis by plotting lymphocytes in a FSC/CD3 expression dot plot. Negative CD3 cells were evaluated by the expression of CD56 and/or CD16 and

NK cell receptor expression analysis was performed in $CD3^{-}CD56^{+}CD16^{+/-}$ lymphocyte population.

5.1.1.3.6 Cell viability and early activation assay

To determine effects on viability and the activation induced by I-CRP on PBMC and NK cells, we used Calcein-AM (Thermofisher, Waltham, Massachusetts, USA) staining to test viability and the assessment of the early activation marker, CD69. First, PBMC or NK cells were seeded at 1×10^5 cells per well in 96-well conic bottom plates (Corning Inc. Costar ®, NY, USA) and treated during 24h and 48h with different concentrations of I-CRP (0.3, 0.7 and 1.5 U/mL) and the mitogen concanavalin A (ConA; 5 μg/mL; Sigma-Aldrich, Germany), IL-2 (200 U/mL; R&D Systems®, US) or IL-15 (ng/mL; Gibco®, US) as positive control of activation. After treatment exposure, cells were recollected in conical microtubes and washed with phosphate-buffered saline (PBS). Then, cells were resuspended in FACS buffer (PBS and 2% FBS) and Calcein-AM (0.25 μM) for 30 min at 37ºC in darkness, after incubation cells were washed twice with PBS. Finally, cells were incubated with an anti-CD69-allophycocyanin antibody (CD69-APC, cat:555533, BD Biosciences) at 37ºC in darkness. Cells were then washed and resuspended in FACS buffer to be assessed by flow cytometry (Fluorescence-Activated Cell Sorting [FACS]; BDAccury6; Becton Dickinson, San Jose, CA, USA) and analyzed using FlowJo Software (Tree Star Inc., Ashland, OR, USA).

5.1.1.3.7 Proliferation assay

To test proliferation on PBMC, we used Carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Carlsbad, USA) staining (1 μM) as indicated by the manufacturer's instructions. In brief, the dye was added to 1×10^9 PBMC suspension, samples were vortexed and incubated at 37°C in the dark for 10 min. PBMC were then washed twice with warm PBS. Stained PBMC were treated with I-CRP (1.5 U/mL) or ConA (5 μg/mL) for 96h. Cells were then seeded in 96-well conic bottom plates at the same conditions, previously described. After stimuli, cells were recollected, washed twice with PBS, stained with Calcein-AM (as previously described), assessed by flow

cytometry (Fluorescence-Activated Cell Sorting [FACS]; BDAccury6; Becton Dickinson, San Jose, CA, USA), and analyzed using FlowJo Software (Tree Star Inc., Ashland, OR, USA).

5.1.1.3.8 Cytokine release assay

The supernatants from the indicated cultures and co-cultures were collected for IL-2, IL -4, IL-6, IL-10, IL-17a, and TNFα assessment (BD CBA Human Th1/Th2/Th17 Cytokine Kit, San Jose, CA, USA) by flow cytometry following manufacturer's instructions.

5.1.1.3.9 Degranulation assay (CD107a)

After I-CRP treatment (1.5 U/mL) PBMC or NK cells were recovered, washed twice with PBS to eliminate treatment, and co-cultivated with K562 cells (PBMC 10:1 K562, NK 5:1 K562). After 1h, the anti-CD107a (H4A3, CD107a-FITC; cat:555800, BD Biosciences) was added, and co-cultures were incubated for 3h. Afterwards, cells were recovered, washed (PBS) and assessed by flow cytometry (BDAccury6) and analyzed using FlowJo Software (Tree Star Inc., Ashland, OR, USA). Lymphocytes were gated in FSC/SSC dot plot and 10,000 events were collected, then CD107a was evaluated.

5.1.1.3.10 NK cell cytotoxicity (Calcein-AM release assay)

After PBMC or NK cells were treated with I-CRP for 24h, cells were then washed twice and subsequently, cells were co-incubated with K562 target cells labeled with Calcein-AM (2μM) at an E:T ratio of 10:1 for 4h at 37 C in 5% CO_2. NK cell–mediated target cell apoptosis was assessed by flow cytometry and determined by the loss of Calcein-AM stain–expressing K562 target cells. K562 cells were gated in FSC/SSC dot plot and 10,000 events were collected, then calcein-AM was evaluated. Using K562 cells alone as a control of size and granularity.

5.1.1.3.11 Statistical analyses

Statistical analyses were conducted using the nonparametric Mann-Whitney U test (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$). The level of significance in figures

4 and 5 was adjusted for multiple testing using the Bonferroni correction. The results given in this study represent the median of at least three independent or more experiments done in triplicate (median±SD). The data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

5.1.1.4 Results

5.1.1.4.1 I-CRP induces activation but not proliferation of PBMC

First, cell viability and activation were evaluated on peripheral blood mononuclear cells (PBMC) derived from healthy human donors after I-CRP incubation. In figure 10A, it shows the gating strategy used to determine cell viability and activation of PBMC. Viability of PBMC treated with different concentrations of I-CRP or Concanavalin A is shown (Fig. 10B). Loss of cell viability is not observed after 24 and 48h of I-CRP treatment, even at 0.7 U/mL, a CC_{100} (lethal dose 100) in leukemic T-cells (Lorenzo-Anota et al., 2020a) compared to the control, or 1.5 U/mL (more than CC_{50} in solid tumor cells) (Martinez-Torres et al., 2020) (Martinez-Torres et al., 2018) (Martinez-Torres et al., 2019); suggesting that I-CRP is non-toxic on PBMC from normal donors. Next, we tested PBMC activation by assessing CD69 expression on viable cells after PBMC stimulation, as shown in figure 10B. We observed that I-CRP increases the CD69 expression on viable PBMC at 0.7 U/mL and 1.5 U/mL compared to the control, and to similar values are observed after ConA stimulation (figure 10C). This effect was observed since 24h of treatment and were more pronounced after 48h of incubation. Thus, I-CRP seems to be an early activator of PBMC in a dose-dependent manner. The next step was to assess if I-CRP could induce proliferation. PBMC were stained with CFSE before I-CRP or ConA incubation and cultured during 96h. Figure 10D shows several peaks with lower CFSE fluorescence in PBMC after stimulation with ConA as expected. In contrast, in PBMC stimulated with I-CRP, we did not observe any proliferative peaks (Fig. 10D), revealing that I-CRP promotes signals to induce early activation but not proliferation in PBMC, at the studied points.

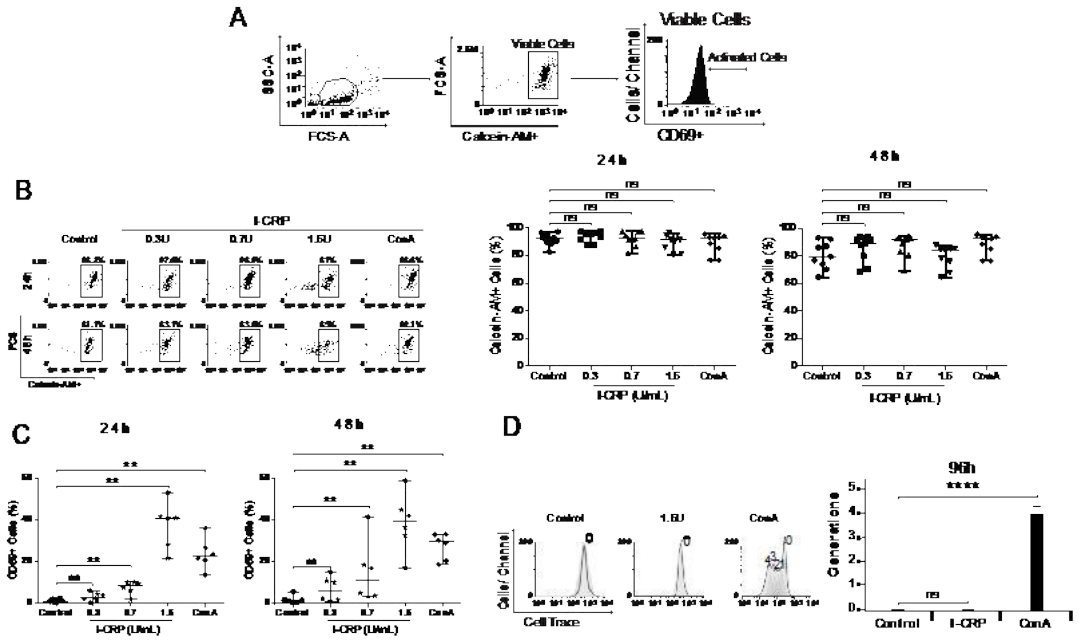

Figure 10. I-CRP increases CD69 expression without inducing proliferation on PBMC. A) Gating strategies for flow cytometry analysis of Calcein-AM and CD69 on PBMC. **B)** Representative dot plots and quantification of cell viability analysis by flow cytometry using calcein-AM staining in PBMC from 9 healthy subjects treated with different concentrations of I-CRP (0.3 U/mL, 0.7 U/mL and 1.5 U/mL) for 24 and 48h or Concanavalin-A (ConA, 5 μg/mL). **C)** Quantification of CD69 expression levels by flow cytometry of PBMC isolated 6 multiple healthy individuals were treated with different concentrations of I-CRP (0.3 U/mL, 0.7 U/mL and 1.5 U/mL) for 24 and 48h, or Concanavalin-A (ConA, 5 μg/mL). **D)** Representative histograms (right) and quantification (left) of proliferation pics by flow cytometry through CFSE staining in PBMC 4 healthy individuals treated with I-CRP (1.5 U/mL) or Concanavalin A (Con A, 5 μg/mL) for 96h. All data shown is median and range. Statistics were performed by non-parametric Mann–Whitney U-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

5.1.1.4.2 I-CRP induces cytokine release in human PBMC

To determine if the activation induced by I-CRP generates cytokine release, we tested cytokine secretion on supernatant of I-CRP stimulated PBMC and IL-15 stimulation was used as a positive control. The cytokine analysis is shown in figure 11. I-CRP promotes TNF-alpha secretion on PBMC, at a higher level than those observed after IL-15 stimulation (Fig.11A). Concerning IL-2 secretion, we observed lower concentrations after the stimulation with I-CRP, when compared to IL-15 stimulation (Fig. 11B). In contrast, we did not detect any differences in IL-4 production between

both stimulations (Fig. 11C). For the other cytokines, the secretion of IL-6 (Fig. 11D), IL-10 (Fig. 11E) and IL-17A (Fig. 11F) increased in I-CRP-stimulated cells, when compared to those stimulated with IL-15. These results indicate that, stimulation of PBMC with I-CRP could induce a different profile of cytokine secretion compared to IL-15 stimulated PBMC.

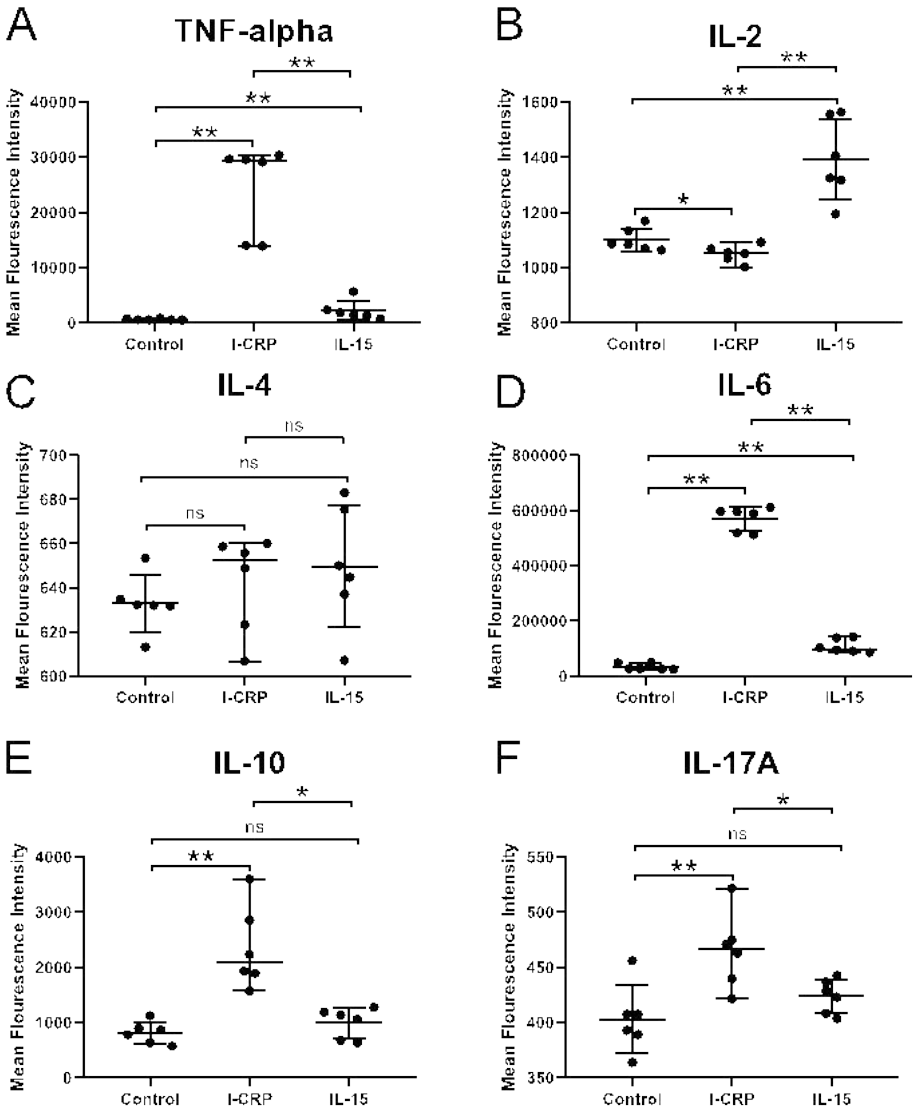

Figure 11. I-CRP induces cytokines' release. Quantification on median fluoresce intensity of supernatant of PBMC from 6 healthy subjects treated with I-CRP (1.5 U/mL) for 24h and IL-15 (15 ng/mL), of **A)** TNF-alpha, **B)** IL-2, **C)** IL-4, **D)** IL-6**, E)** IL-10, and **F)** IL-17A release. All data shown is

median and range. Statistics were performed by non-parametric Mann–Whitney U-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

5.1.1.4.3 I-CRP modulates $CD56^{Dim}$ $CD16^{+/-}$ subset in PBMC

As I-CRP can activate PBMC and induce the release of cytokines, we decided to determine which cell subsets could be modified, using a flow cytometry strategy. In figure 12A, we show the gating strategy from a representative healthy donor. We firstly selected lymphocytes using forward and side scatter analysis, and then, T cells were defined as $CD3^{+}$, B cells as $CD20^{+}$ and NK as CD3 and CD20 negative population. CD56 and CD16 were used to classify NK cells in I:$CD56^{Bright}$ $CD16^{-}$, II:$CD56^{Bright}$ $CD16^{+}$, III:$CD56^{Dim}$ $CD16^{-}$, IV:$CD56^{Dim}$ $CD16^{+}$ and V:$CD56^{-}$ $CD16^{+}$ subsets. After 24h of culture, we did not observe significant differences on T cells (Fig. 12B) and B cells (Fig. 12C) in PBMC from normal donors, stimulated with different concentrations of I-CRP or untreated controls. Concerning NK cells, an increase in $CD56^{Dim}$ $CD16^{-}$ NK cells with a decrease in $CD56^{Dim}$ $CD16^{+}$ population was detected in I-CRP (1.5 U/mL) treated cells compared to control cells after 24h of treatment. After 48h of culture, we observed just a slight decrease in B cells after 48h of treatment, at the highest concentration tested (Fig. 12C). In contrast, on NK cells subsets analysis we observed at 1.5 U/mL of I-CRP during 24h that $CD56^{Dim}$ $CD16^{-}$ subset increased compared to controls, and $CD56^{Dim}$ $CD16^{+}$ subset diminished after 48h of treatment (Fig. 12D). This result shows that I-CRP modulates NK cell subsets, by increasing the $CD56^{Dim}$ $CD16^{-}$ subset.

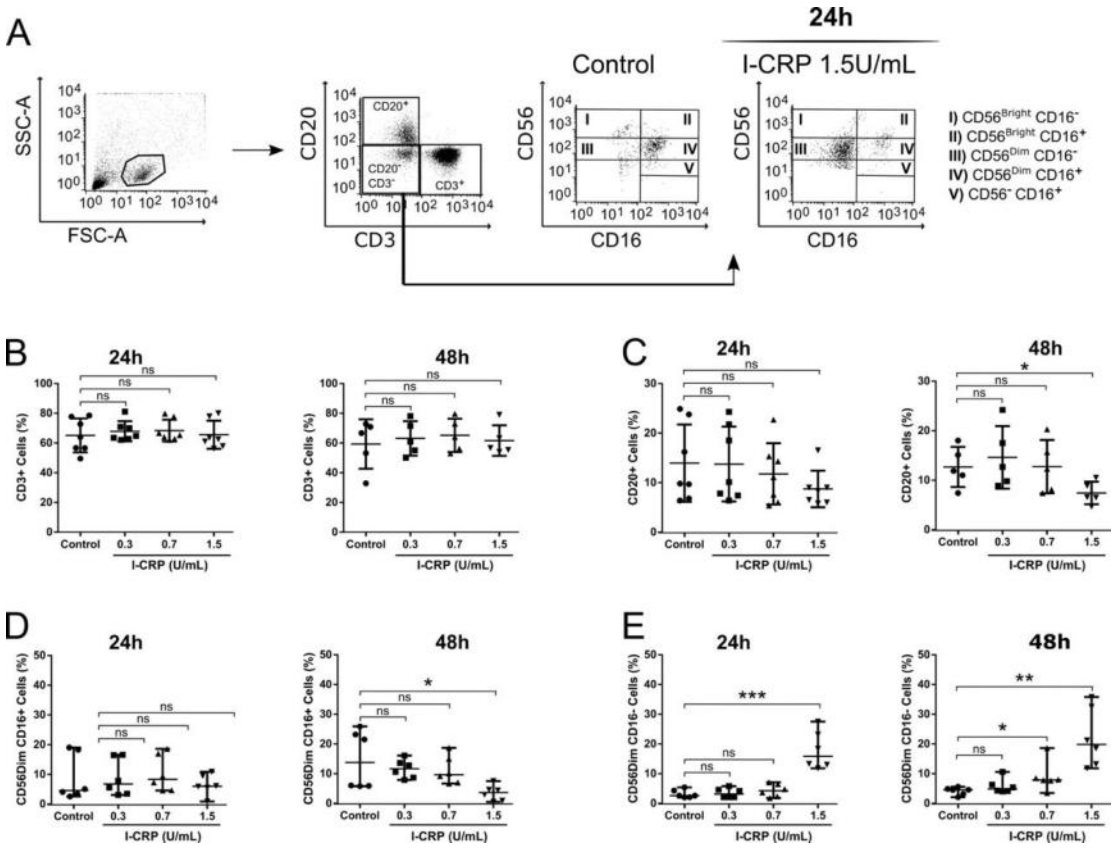

Figure 12. I-CRP increases CD56^{Dim} CD16D^{im} NK subset on PBMC. A) Gating strategy for the analysis of lymphocytes CD3 (T cells), CD20 (B cells) and CD56/CD16 (NK cells) on PBMC by flow cytometry. **B)** Quantification of CD3^{+} T Cells on PBMC treated by I-CRP or Concanavalin A, with different concentrations as indicated for 24 (left) and 48 (right) h. **C)** Quantification of CD20^{+} B Cells on PBMC treated with different concentrations of I-CRP or Concanavalin A, as indicated during 24 (left) and 48 (right) h. **D)** Quantification of CD56^{Dim} CD16^{+} subset of NK cells on PBMC treated with different concentrations of I-CRP or Concanavalin A, as indicated for 24 (left) and 48 (right) h. **E)** Quantification of CD56^{Dim} CD16^{-}NK cell subset on PBMC treated by I-CRP or Concanavalin A, with different concentrations as indicated for 24h (left) and 48h (right). All data shown is median and range. Statistics were performed by non-parametric Mann–Whitney U-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

5.1.1.4.4 NK cells modulate accessory and killer cell lectin-like receptors upon I-CRP stimulation

To get more information about the changes in the major NK cell subsets of CD56^{Dim} CD16^{-} subsets in NK cells with the activation in PBMC, we analyzed in deep the principal repertoire of NK cells. (Results are shown in figure 13). We confirmed that I-

CRP induces early activation marker (CD69) expression at 24 and 48h on NK cells, as well as observed in IL-2 stimulated cells (Fig. 13A). When the NK cell repertoire was analyzed, we detected an increased expression of CD161 (Fig. 13B), NKG2D and NKG2C receptors after I-CRP treatment, when compared to controls at 24 and 48h (Fig. 13C). These results suggest that I-CRP can modulate NK cell receptor repertoire of human NK cells from normal donors.

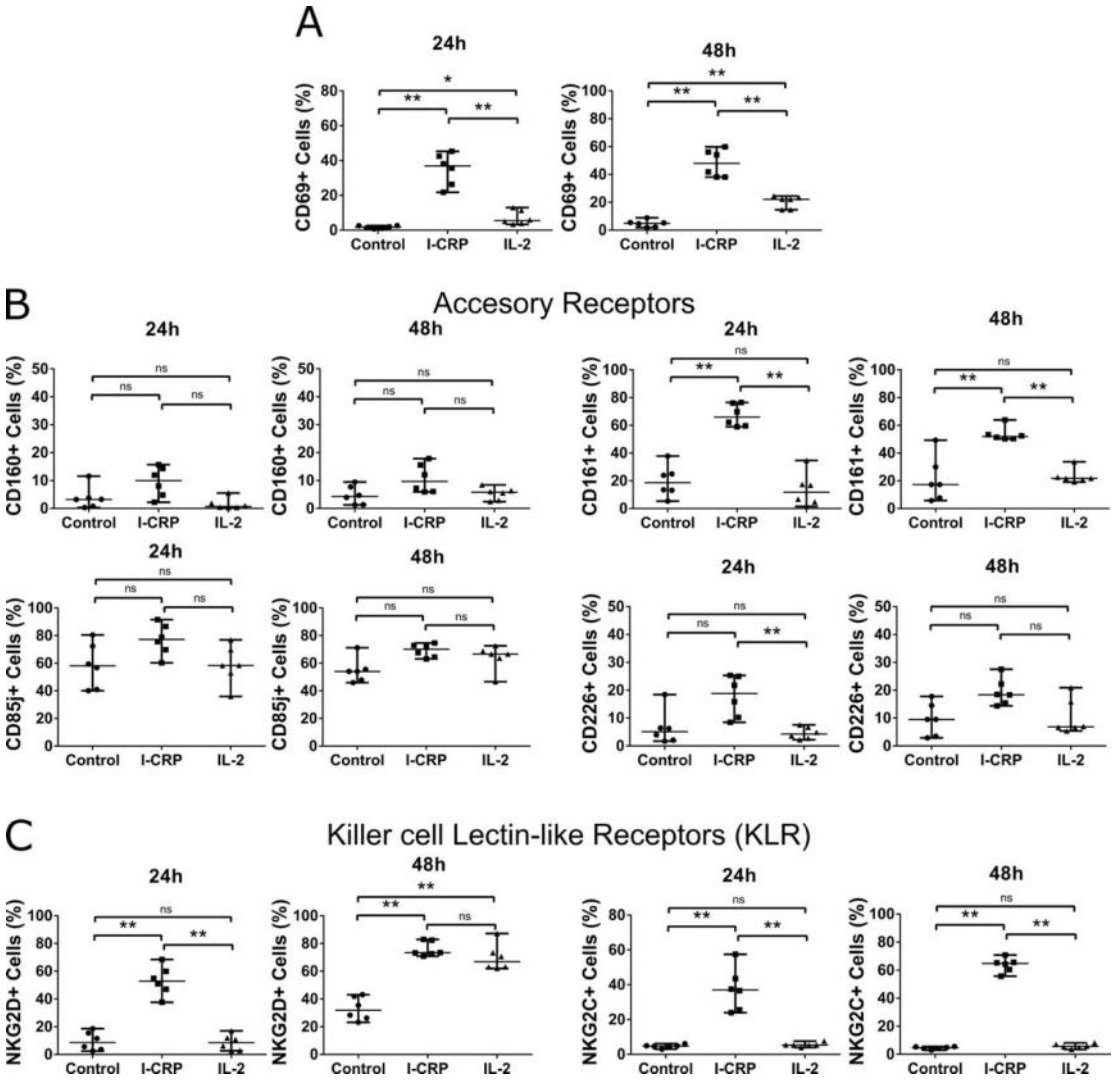

Figure 13. I-CRP promotes early activation and enhances NKG2C expression on NK cells. A) Quantification of CD69 expression on NK cells after PBMC incubation with I-CRP (1.5 U/mL) or IL-2 (200 U/mL) during 24 and 48h. **B)** Quantification of accessory receptors (CD161, CD161, CD85j and CD226) on NK cells after PBMC stimulation with I-CRP (1.5 U/mL) or IL-2 (200 U/mL) during 24 and 48h. **C)** Quantification of Killer cell lectin-like receptors (NKG2D and NKG2C) on NK cells after PBMC incubation with I-CRP (1.5 U/mL) or IL-2 (200 U/mL) during 24 and 48h. All data shown is

median and range from healthy donors n=6. Statistics were performed by non-parametric Mann–Whitney U-test with Bonferroni correction (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

5.1.1.4.5 NK cells modulate killer immunoglobulin-like receptors and natural cytotoxicity receptors after I-CRP treatment

In view of the modifications observed in the NK cell repertoire, we explored if I-CRP-activated NK cells could also modulate KIR and NCR repertoire (Fig. 14). The KIR analysis shows an increase in their expression compared to controls, after 24 and 48h of I-CRP stimulation (Fig. 14A). Moreover, increased NCR expression is detected after 24h and 48h of I-CRP stimulation when compared to controls. It is important to note that NKp44 and NKp30 upregulation is earlier than those observed after IL-2 stimulation, suggesting a different pathway of activation.

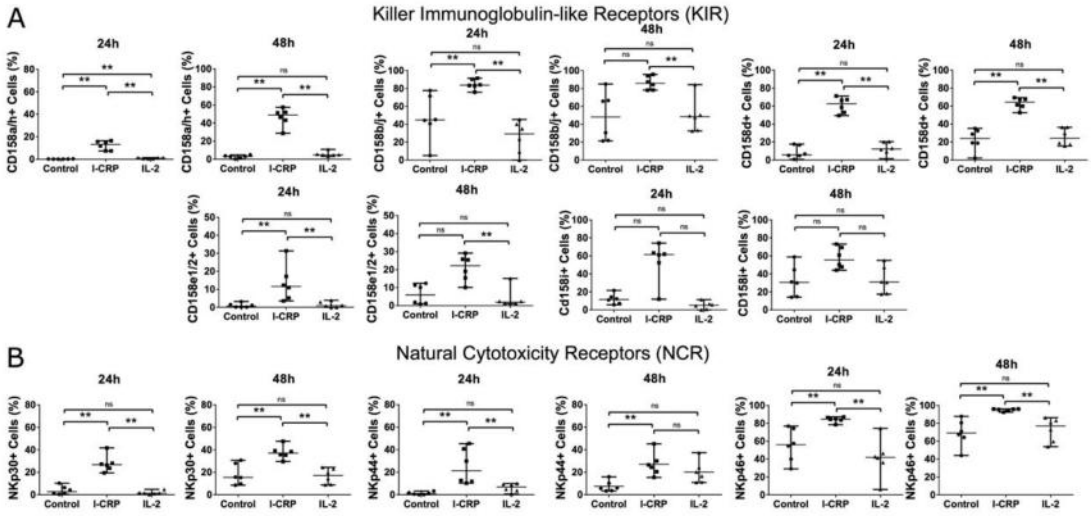

Figure 14. I-CRP modulates Killer Immunoglobulin-like receptors and Natural cytotoxicity receptors expression on NK cells. A) Quantification of Killer Immunoglobulin-like receptors (KIR, CD158a/h, CD158b/j, CD158d, CD158e1/2 and CD158i) on NK cells after PBMC incubation with I-CRP (1.5 U/mL) or IL-2 (200 U/mL) during 24 or 48h. **B)** Quantification of Cytotoxicity receptors (NKp30, NKp44 and NKp46) on NK cells after PBMC stimulation with I-CRP (1.5 U/mL) or IL-2 (200 U/mL) at 24 and 48h. All data shown is median and range from healthy donors n=6. Statistics were performed by non-parametric Mann–Whitney U-test with Bonferroni correction (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

5.1.1.4.6 I-CRP enhances natural cytotoxicity activity of PBMC from normal donors

We next tested the natural cytotoxic capacity of I-CRP stimulated PBMC. To determine the natural activity of PMBC against NK target cells, we tested activation, the degranulation marker expression (CD107a), and cell viability of target cells after co -culture with I-CRP pre-incubated PBMC with K562 target cell line and compared to unstimulated or IL-15 stimulated PBMC (Fig. 15A). We can observe that CD69 expression increases in PBMC pre-incubated with I-CRP after K562 stimulation and was similar to those observed in IL-15 stimulated cells. We next measured the expression of the degranulation marker CD107a as a marker of the natural cytotoxic activity. As seen in Figure 15C, CD107a expression is increased in PBMC incubated with I-CRP or IL-15 compared to unstimulated cells. To correlate CD107a expression with cytotoxicity, we assessed the lysis of the target cell using cell viability test. As observed on figure 15D, PBMC stimulated with I-CRP induce more cytotoxicity on K562 cell line than untreated or IL-15-treated control cells. These results suggest that I-CRP increases the effector activity of NK cells against target cells, through their activation, release of cytotoxic granules (CD107a expression), and killing of target cells.

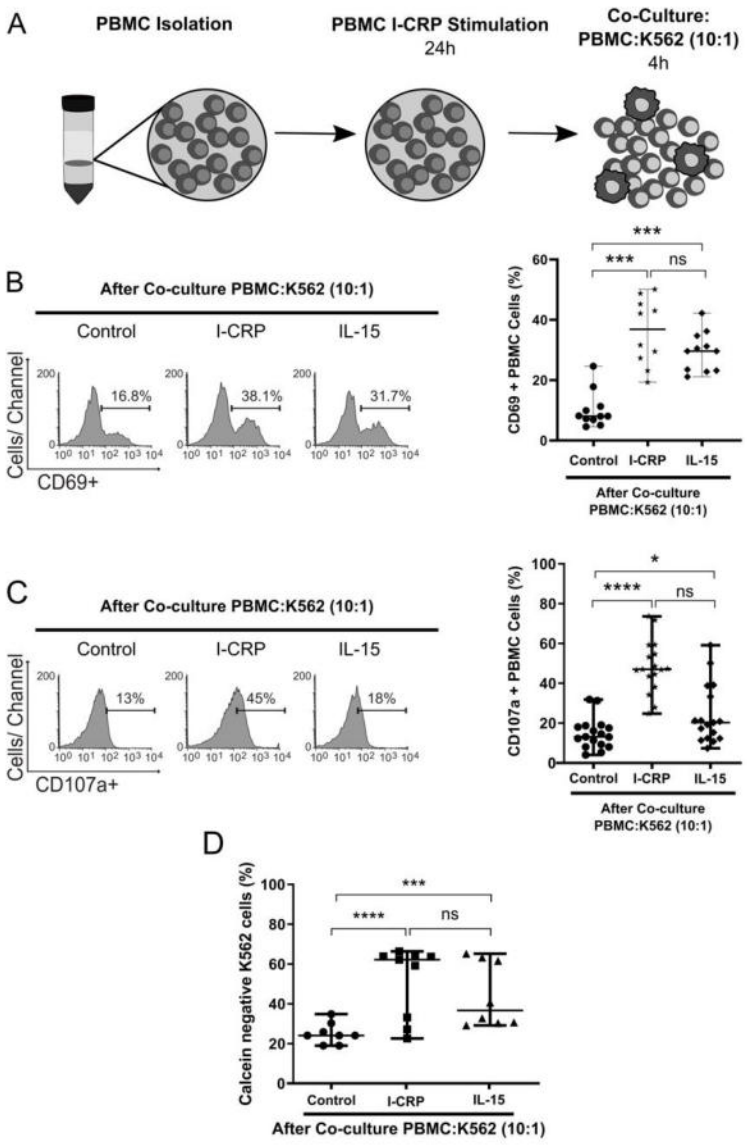

Figure 15. I-CRP promotes effector activity of PBMC from healthy donors. A) Schematic representation of experiment design. **B)** Representative histograms (left) and quantification (right) of CD69 expression by flow cytometry on PBMC isolated from 11 healthy individuals, pre-stimulated with I-CRP (1.5 U/ml) or IL-15 (15 ng/mL) for 24h and then co-cultured with K562 cells in ratio 1:10 (K562:NK) for 4h. **C)** Representative histograms (left) and quantification (right) of CD107a expression by flow cytometry on PBMC isolated from 17 healthy individuals, pre-incubated with I-CRP (1.5 U/ml) or IL-15 (15 ng/mL) for 24h and then co-cultured with K562 cells in ratio 1:10 (K562:NK) for 4h. **D)** Percentage quantification of calcein-AM negative K562 cells co-cultured during 4h with PBMC from healthy individuals pre-stimulated with I-CRP (1.5 U/ml) and IL-15 (15 ng/mL) for 24h (K562:NK ratio 1:10). All data shown is median and range. Statistics were performed by non-parametric Mann–Whitney U-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

5.1.1.4.7 Cytotoxic activity of purified NK cells is higher after I-CRP-treatment

To know if the changes observed in PBMC incubated with I-CRP is mediated by a direct effect on NK cells or indirect action provided by other PBMC, we isolated NK cells from PBMC by negative selection (to avoid their activation). In figure 16A, we show the median (95%) of purity of NK cells after negative selection. First, we tested cell viability of isolated NK cells stimulated with I-CRP or IL-15 compared to no stimulation. We found that I-CRP incubation did not affect significatively the viability of NK cells when compared to their control and with IL-15 treated cells (Fig. 16B), confirming results observed in total PBMC (Fig 10B). Concerning CD69 activation marker, we found that I-CRP induces early activation of NK cells (43%) compared to unstimulated control (13%), and similar to IL-15 stimulation (45%) (Fig. 16C). To verify the modulation of NK cell markers, we tested NK subsets according to CD56 and CD16 expression. In figure 16C, we can observe the dot plots from a representative healthy donor indicating the percentages of NK cells subsets (I:$CD56^{Bright}$ $CD16^{-}$, II:$CD56^{Bright}$ $CD16^{+}$, III:$CD56^{Dim}$ $CD16^{-}$, IV:$CD56^{Dim}$ $CD16^{+}$ and V:$CD56^{-}$ $CD16^{+}$) in untreated cells, and I-CRP and IL-15 stimulated NK cells for 24 and 48h. We found that I-CRP stimulation decreased the $CD56^{Dim}$ $CD16^{+}$ subset and increased $CD56^{Dim}$ $CD16^{-}$cells when compared to control or IL-15 treatment (Fig. 16D). To determine the effect of the preincubation of NK cells with I-CRP in the effector activity against K562, we measured CD107a expression. I-CRP increases the expression of CD107a to 45% on NK cells, compared to control (15%) and IL-15 stimulation (18%) (Fig. 16E), which could be correlated to enhanced CD69 expression (Fig. 16F). These results indicate that preincubation of NK cells with I-CRP enhances their degranulation capacity and confirm those observed in PBMC (Fig.15).

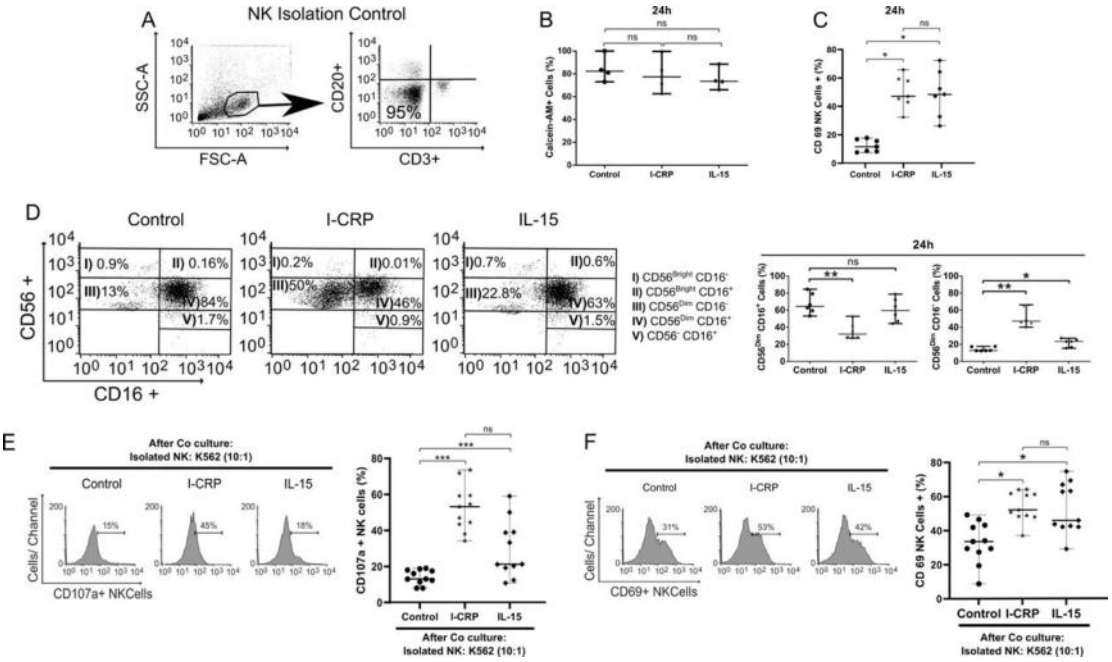

Figure 16. I-CRP increases NK cells effector activity. A) Representative gating strategy and dot plot (CD20 vs CD3) obtained from one healthy donor after negative NK cell isolation from PBMC. The median of purity efficacy in NK cell population was 95%. **B)** Quantification of cell viability by flow cytometry using calcein-AM staining in NK cells treated with I-CRP (1.5 U/mL) or IL-15 (15 ng/mL) during 24h (data from 4 healthy donors). **C)** Quantification of CD69 expression by flow cytometry on NK cells from 7 healthy individuals. NK cells were incubated with I-CRP (1.5 U/ml) or IL-15 (15 ng/mL) during 24 h. **D)** Representative dot plots (left panels) of NK cells subsets using CD56 and CD16 markers (I:$CD56^{Bright}CD16^{-}$, II:$CD56^{Bright}CD16^{+}$, III:$CD56^{Dim}CD16^{-}$, IV:$CD56^{Dim}CD16^{+}$ and V:$CD56^{-}CD16^{+}$) untreated or treated with I-CRP (1.5 U/mL) or IL-15 (15 ng/mL) for 24h. Right panel, median values of $CD56^{Dim}CD16^{+}$ and $CD56^{Dim}CD16^{-}$ subsets after I-CRP or IL-15 stimulation for 24h (data from 6 healthy donors). **E)** CD107a expression on NK cells non-incubated or pre-incubated with I-CRP or IL-15 after co-cultured with K562 cells for 4h (K562:NK ratio 1:10). Left panel, a representative analysis from one individual. Right panel, median values of CD107a expression in the same culture conditions (data from 11 healthy individuals). **F)** Representative histograms (left panel) and median values (right) of CD69 expression on NK cells cultured as in (**E**), data from 7 healthy individuals. All data shown is median and range. Statistics were performed by non-parametric Mann–Whitney U-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

5.1.1.5 DISCUSSION

In this study, we examined changes in the NK cell repertoire and functions occurring in response to I-CRP treatment. We show that I-CRP does not affect PBMC viability and induces NK cells activation, modifications on the repertoire of NK markers, and an augmented recognition against K562 cell line. These processes are closely linked to the changes on the expression of the activation and inhibitory NK cell receptors, which control activation and provide NK cell diversity and functionality (Horowitz et al., 2013), indicating that I-CRP improves NK cells functional activity.

Several markers of early NK cell activation have been described, including the C-type lectin receptor, CD69 (Lopez-Cabrera et al., 1993). CD69 is not expressed on resting NK cells but is highly upregulated on NK cells following activation (Fogel, Sun, Geurs, Carayannopoulos, & French, 2013). In this study CD69 was consistently higher in I-CRP treated NK cells, even when NK cells were previously isolated. The increase of the NK cells $CD56^{Dim}$ $CD16^{-}$ subset was observed after I-CRP treatment, this can be related to an observation reported by Lajoie et al. in 2014, who indicated that CD16 shedding is restricted to activated cells and this process is partially ADAM-17 dependent (Lajoie et al., 2014). Despite ADAM-17 inhibitors were not implemented in our study, CD16 shredding is strongly correlated with degranulation (Lajoie et al., 2014), which was observed with a high constitutive potential on I-CRP-treated NK cells, using the CD107a functional assay. Fogel et al. (Fogel et al., 2013) observed that CD16 shedding increased NK cell motility and facilitate detachment of NK cells from target cells (Fogel et al., 2013; Srpan et al., 2018). We observed an increase in target cell recognition and cytotoxic activity in I-CRP stimulated NK cells, suggesting that CD16 shedding could be implicated in these processes.

The phenotypic analysis of the NK cell repertoire identified patterns of NK cell receptors that may define NK cell activation by DLE, such as enhanced CD161, NKG2D, NCR and KIR expression. CD161 engagement appears to have a complex role in the modulation of immune NK functions with both activating and inhibitory effects (Ljutic et al., 2005). CD161 is involved in triggering the NK cell cytotoxicity against human melanoma (Montaldo et al., 2012) as well as in the ability to respond to IL-12 and IL-18, upregulating NKp30, CD160 and CD25 (Kurioka et al., 2018). Similarly, I-CRP induces an increase in the expression of NKp30 and CD69, but not in

CD160, suggesting a different kind of activation. Increased NKG2D expression is related to NK cell activation, as it can have functions as an activating and a co-stimulatory receptor, playing a fundamental role in the surveillance of microbial infections and cancer (Wensveen, Jelenčić, & Polić, 2018). NKG2D was increased after stimulation with I-CRP, supporting the activation of NK cells. Moreover, NKp44, a NCR which is only up regulated in activated NK cells, was also increased. Taken together, these results indicate that I-CRP can activate isolated NK cells or NK cells in PBMC. This activation does not involve proliferation, but involves NKG2C and general KIR expression, characteristics mostly observed in adaptive-like NK cells, most well studied in HCMV-seropositive patients, which are defined as $CD57^{+}NKG2C^{+}$ NK cells (Gumá et al., 2004; Hendricks et al., 2014). In this study CD57 was not analyzed, but the I-CRP-activation pattern we report here has important similarities with the adaptive-like NK cells, like CD16 decrement, which is observed after acquisition of NKG2C in $CD56^{dim}$ cells (Kobyzeva et al., 2020), and a better degranulation activity, also reported in $NKG2C^{+}$ NK cells, compared to $NKG2C^{-}$ NK cells (Kobyzeva et al., 2020). Another similarity is the expression of inhibitory and activating KIR in NK cells, which has been related to the adaptation to HCMV-infection in addition to NKG2C (Béziat et al., 2013). It has been determined that adaptive NK cells possess unique KIR repertoire, and display strong cytotoxicity against tumor cells (Béziat et al., 2013; Liu et al., 2017; Manser et al., 2019), and, on the other hand, the KIR expression have been shown to be clinically relevant in allogeneic transplantations to treat acute leukemia (Sivori et al., 2020). It remains unclear if these cells can surpass NK cell-resistant tumor cells, but it suggests a better cytotoxic activity as seen in matured adaptive-like NK cells.

In this context, other reports on DLE study revealed *in vitro* differentiation of functional $CD56^{+}CD16^{+}CD11c^{+}$ NK-like cells from $CD34^{+}$ cells (Ramírez-Ramírez et al., 2016), this supports the hypothesis that I-CRP activation of NK cells might involve a maturation mechanism. We have mentioned the CD161 and NCR expression of I-CRP-treated NK cells, these receptors are not usually observed in HCMV-adaptive-like NK cells but recently were related to a $BMPRIA^{+}$ (type IA BMP receptor) immature thymocytes, progenitors able to generate higher numbers of NK cells (Robson et al.,

2014) and promoting maturation of a resulting $CD94^{+}CD161^{+}CD56^{+}$NKp46+ NK cells (Hidalgo et al., 2012). The increased expression of NKG2C and CD161, and CD16 loss could be the result of the heterogeneous peptide mixture composing I-CRP, which could include peptides derived from MHC class I molecules (Rölle et al., 2014), as well as peptides encoding by UL40 in HCMV (Hammer et al., 2018), which can activate adaptive-like NKG2C NK cells, furthermore antimicrobial peptides (AMP), like LL-37, can mediate ADAM activation (Reiss & Bhakdi, 2012) and BMP pathway engagement (Wu et al., 2010). However, further studies about the exact peptides contained in DLEs and inhibition studies must be done to better describe these mechanisms of action.

Overall, our results show that I-CRP induces NK cell activation (CD69 expression), increasing the $CD56^{Dim}$ $CD16^{-}$ subset and modulating the expression of the main NK receptors repertoire (Fig.17A), resulting in a NKG2C+CD161+NK cells with improvements in their cytotoxic response against target cells (Fig.17B). This work helps to understand the mechanism of I-CRP as immunostimulant and its possible implications on NK cell differentiation/activation. In addition, these results pointed-out the possible utilization of I-CRP in diseases having a mayor dysfunction of NK cells like viral, bacterial and cancer.

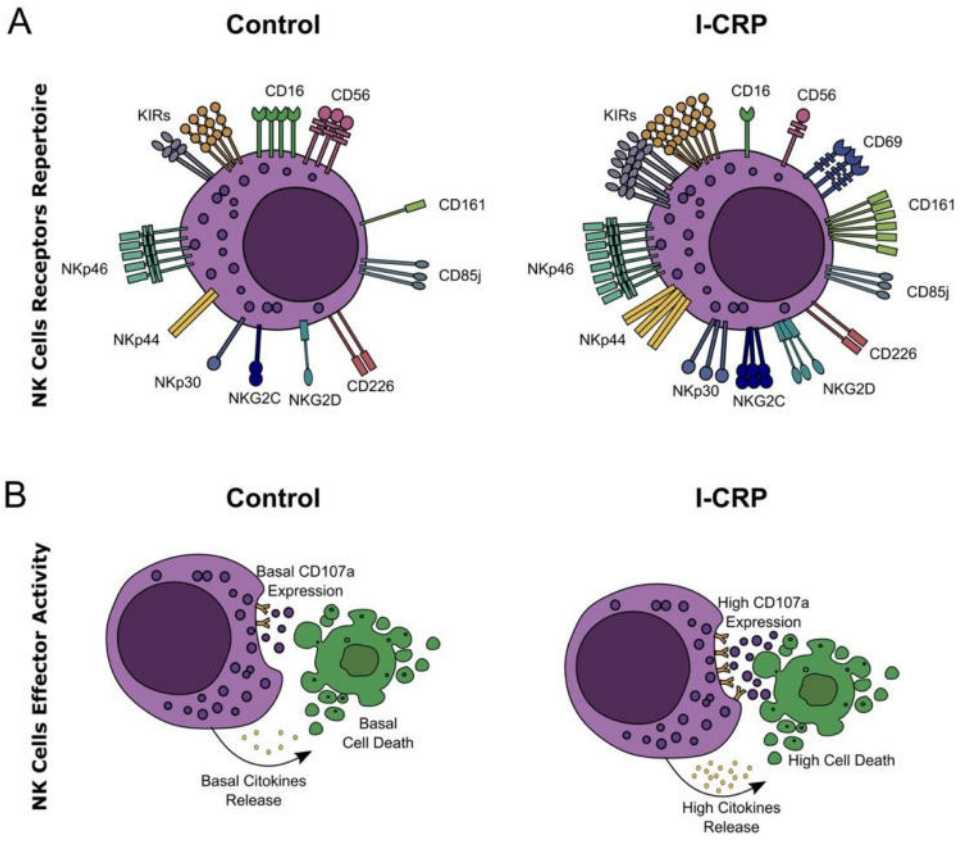

Figure 17. Schematic representation of I-CRP effect on NK cells. A) I-CRP increase early activation marker CD69, promotes over expression of CD161 an accessory/activator receptor, killer cell lectin-like receptors (KLR) NKG2D and NKG2C, Killer Immunoglobulin-like receptors (KIR) and enhances natural cytotoxicity receptors (NCR, NKp30, NKp44 and NKp46) on NK cells. B) I-CRP improves the effector activity of NK cells by enhancing degranulation marker (CD107a), promoting cytokines release and improving cytotoxicity on K562 target cells.

5.1.1.6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5.1.1.7 Author Contributions

HYLA, ABML, RTG, ACMT, DSA and CRP analyzed and interpreted data. HYLA, and ABML performed all the experimental work. DSA and ACMT conceived and

supervised the project. HYLA, ABML and ACMT wrote the manuscript. RTG, DSA, and CRP revised the manuscript. All authors approved the final manuscript.

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5.2 CHAPTER II

Bovine dialyzable leukocyte extract IMMUNEPOTENT-CRP induces selective ROS-dependent apoptosis in T-Acute Lymphoblastic Leukemia cell lines

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5.2.1.1 ABSTRACT

Immunotherapies strengthen the immune system to fight multiple diseases such as infections, immunodeficiencies and autoimmune diseases and, recently, they are being used as an adjuvant in cancer treatment. IMMUNEPOTENT-CRP (I-CRP) is an immunotherapy made of bovine Dialyzable Leukocyte Extract (bDLE) that has chemoprotective and immunomodulatory effects in different cellular populations of the immune system, and antitumor activity in different cancer cell lines. Our recent results suggest that the antineoplastic effect of I-CRP is due to the characteristics of cancer cells. To confirm, we evaluated whether that selectivity is due to cell lineage or a characteristics of cancer cells, testing cytotoxicity in T-acute lymphoblastic leukemia cells and their cell death mechanism.

Here we assessed the effect of I-CRP in cell viability and cell death. To determine the mechanism of cell death, we tested cell cycle, mitochondrial and nuclear alterations as well as caspases and reactive oxygen species (ROS), and their role in cell death mechanism. Our results show that I-CRP does not affect cell viability in non-cancer cells and induces selective-cytotoxicity in a dose-dependent manner in leukemic cell lines. I-CRP also induces mitochondrial damage through pro- and anti-apoptotic protein modulation (Bax and Bcl-2) and ROS production, nuclear alterations including DNA damage (γ-H2Ax), over expression of p53, cell cycle arrest and DNA degradation. I-CRP induced ROS-dependent apoptosis in leukemic cells. Overall, here

we show that I-CRP cytotoxicity is selective to leukemic cells, inducing ROS-dependent apoptosis. This research opens the door to further exploration of their role in the immune system and the cell death mechanism that could potentially work in conjunction with other therapies including hematological malignances.

KEYWORDS: Leukemia, Immunotherapy, Dialyzable Leukocyte Extract, Apoptosis, ROS, caspases.

5.2.1.2 INTRODUCTION

Regulated cell death (RCD) is a mechanism by which the cell activates its own machinery for self-destruction; it involves structured signaling cascades and molecularly defined effector mechanisms, and is important for the maintenance of tissues (Galluzzi et al. 2018; Kroemer et al. 2009; Tang et al. 2019; Ashkenazi and Salvesen 2014). Apoptosis is the most widely described RCD mechanism; it is characterized by cell shrinkage (pyknosis), membrane blebbing, apoptotic body formation, DNA fragmentation (karyorrhexis), and chromatin condensation (Nagata 2018; Manley 2013; Singh, Letai, and Sarosiek 2019). In cancer, there is a break in the circuits that regulate division and regulated cell death, leading to deregulation of apoptosis and generating uncontrolled cell growth (Galluzzi et al. 2018).

T-Acute Lymphoblastic Leukemia (T-ALL) is a type of cancer derived from the bone marrow that affects T-lymphocytes and is the most common cause of cancer in children worldwide (Karrm and Johansson 2016; Terwilliger and Abdul-Hay 2017). Malignant progression is promoted by the myeloprotection provided by the bone marrow and evasion of the host's immune system, evidencing the resistance capacity to treatments of the leukemic cells (Karrm and Johansson 2016; Rutering et al. 2016). At present, the main therapies for leukemia include combinations of chemotherapies and corticosteroids, kinase inhibitors and stem cells transplant (Karrm and Johansson 2016; Jacobs and Gale 2010); however, they cause important side effects in healthy immune system cells. Therefore, it is important to develop therapies that eliminate cancer cells without affecting healthy immune system cells, as well as stimulate the immune system to fight cancer.

Immunotherapy consists of treatments that use the immune system to fight multiple diseases such as infections, autoimmune diseases, immunodeficiencies and, lately, in cancer treatment as an adjuvant (Oiseth and Aziz 2017; Anusha et al. 2017). IMMUNEPOTENT-CRP (I-CRP) is an immunotherapeutic agent composed of bovine dialyzable leukocyte extract (bDLE) obtained from disintegrated spleen. In previous reports, I-CRP showed a potential immunomodulatory effect in preclinical trials of breast cancer treatments, (Lara et al. 2010; M. Franco-Molina et al., 2008.) and in in vivo assays, it showed an anti-tumor effect (Moises A. Franco-Molina et al. 2018; Coronado-Cerda et al. 2016). Several in vitro studies reveal its immunomodulatory properties in human and mouse monocytes and macrophages (M. A. Franco-Molina et al. 2007; Moisés A. Franco-Molina et al. 2005) and their cytotoxic effect in different cancer cell lines (M. A. Franco-Molina et al. 2006; Martínez-Torres, Reyes-Ruiz, et al. 2018). In the breast cancer cell line MCF-7, I-CRP inhibits cell growth, suppresses DNA-binding activity of AP-1, decreases c-Jun protein expression and modulates the mRNA expression of cell death proteins such as NFκB, p53, c-myc, bax and bcl-2 (Mendoza-Gamboa et al. 2008). Also, in the cervical cancer cell line HeLa, I-CRP decreases cell viability through cell cycle arrest in the G2/M phase with caspase-3 activation and ROS production, inducing caspase-independent but ROS-dependent RCD (Martínez-Torres, Reyes-Ruiz, et al. 2018). In lung cancer cells it induces cell cycle arrest, cell death, and caspase-independent but ROS-dependent RCD (Martinez-Torres et al. 2019).

At present, all results suggest a dual role of I-CRP: in healthy immune system cells as an immunomodulator, and in cancer cells as a cytotoxic agent. However, the effect of I-CRP on cancers derived from the immune system is unknown. The limited information of its molecular action mechanisms has hindered its widespread use in different pathologies. The aim of this research was to analyze the effect of I-CRP in T-ALL cells, (human cancer cells derived from immune system) and their mechanisms of cytotoxicity. We used Molt-4 and CEM cells as T-ALL model cancer cells, assessed the mechanism of cell death by evaluating mitochondrial (ratio Bax-Bcl-2, ROS and TMRE) and nuclear (ϒ-H2Ax, cell cycle and DNA degradation) alterations and the role of ROS and caspases in a cell death mechanism.

5.2.1.3 MATERIALS AND METHODS

5.2.1.3.1 Cell culture

Molt-4 (ATCC® CRL-1582™) and CEM (ATCC® CCL-119™) cell lines (T-acute lymphoblastic leukemia cells), were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained under suggested conditions. Cells were cultured in plastic sterile flasks (Corning Inc. Costar ®, USA) at 37 ºC in 5% CO2 atmosphere, using RPMI 1640 medium (GIBCO Thermofisher, Waltham, Massachusetts, USA) supplemented with 1µg/mL amphotericin B, 1µg/mL penicillin and 2.5X10-3 µg/mL streptomycin (GIBCO Thermofisher, Waltham, Massachusetts, USA) and 10% of FBS (GIBCO Thermofisher, Waltham, Massachusetts, USA).

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors after obtaining written informed consent by density gradient centrifugation with Ficoll-PaqueTM PLUS (GE Healthcare, Chicago, Ilinois, USA) and maintained at 4X106 cells/mL in cell culture plates at 37 ºC in 5% CO2 atmosphere, using RPMI 1640 medium (GIBCO Thermofisher, Waltham, Massachusetts, USA) supplemented with 1µg/mL amphotericin B, 1µg/mL penicillin and 2.5X10-3 µg/mL streptomycin (GIBCO Thermofisher, Waltham, Massachusetts, USA) and 10% of FBS (GIBCO Thermofisher, Waltham, Massachusetts, USA). This study was approved by the Institutional Ethics Committee at the Universidad Autónoma de Nuevo León, College of Biological Sciences. To analyze T-lymphocytes in PBMC culture we used the antiCD3 antibodies CD3-APC (BD Biosciences, San Jose, CA; cat:555335, clone:UCHT 1) and CD-FITC (BD Biosciences; cat:555916, clone:UCHT 1).

T-lymphocyte purification

T-lymphocytes (CD3+) were isolated from PBMC culture by negative selection using magnetic-activated cell sorting (MACS) microbead technology (Miltenyi Biotec, Bergisch Gladbach, Germany; >90% purity and >90% viability), with anti-CD20 microbeads (cat:130-091-104, Miltenyi Biotec), anti-CD16 microbeads (cat:130-045-701, Miltenyi Biotec), and anti-CD14 microbeads (cat:130-050-20; Miltenyi Biotec)

cocktail, as stated by manufacturer's instructions. T-lymphocytes culture were maintained at same conditions of PBMC culture.

5.2.1.3.2 Immunepotent-CRP

The bovine dialyzable leukocyte extract, IMMUNEPOTENT CRP (I-CRP) was produced by the Immunology and Virology Laboratory at the College of Biological Sciences, Universidad Autónoma de Nuevo León (San Nicolás de los Garza, Nuevo León, México) and was dissolved in RPMI 1640 medium (GIBCO Thermofisher, Waltham, Massachusetts, USA) supplemented with 1µg/mL amphotericin B, 1µg/mL penicillin and 2.5X10-3 µg/mL streptomycin (GIBCO Thermofisher, Waltham, Massachusetts, USA) and 10% of FBS (GIBCO Thermofisher, Waltham, Massachusetts, USA). One unit of I-CRP is defined as the product obtained from 1×108 leukocytes.

5.2.1.3.3 Cell viability assessment

Cell viability was determined with Calcein-AM (Thermofisher, Waltham, Massachusetts, USA). Briefly, cells were seeded at 5X104 cells per well in 96-well plates (Corning Inc. Costar ®, NY, USA) and exposed to different concentrations of I-CRP (0.4, 0.6, 0.8, 1.0U/mL; after the 24- and 48- hours of treatment, the cells were washed with phosphate-buffered saline (PBS) then resuspended in 100µL of FACS buffer and Calcein-AM (2M) for 30 minutes at room temperature, finally the cells were washed with PBS. Cells were then assessed by flow cytometry (Fluorescence-Activated Cell Sorting [FACS]; BDAccury6; Becton Dickinson, San Jose, CA, USA) and analyzed using FlowJo Software (Tree Star Inc., Ashland, OR, USA).

5.2.1.3.4 Cell death analysis

Cell death was determined after 24 and 48 hours of I-CRP exposure analyzing phosphatidylserine exposure using Annexin V-allophycocyanin (APC) (AnnV, 0.25 µg/mL; BD Biosciences Pharmingen, San Jose, CA, USA) and cell membrane permeability with Propidium Iodide (PI; 0.5 µg/mL; MilliporeSigma, Eugene, OR, USA) stain. Cells were seeded at 5X104 cells per well in 96-well plates (Corning Inc.

Costar ®, NY, USA) and exposed to different concentrations of I-CRP (0.4, 0.6, 0.8, 1.0U/mL) in subsequent assays; this allowed to define the median cytotoxic concentration of I-CRP required to induce cell death by 50% (CC50). After treatment, cells were recollected and washed with phosphate-buffered saline (PBS) then resuspended in binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl2), and stained during 30 minutes at 4 ºC.

5.2.1.3.5 Mitochondrial membrane potential assessment

To determine mitochondrial damage, we tested loss of mitochondrial membrane potential by tetramethylrhodamine ethyl ester stain (TMRE, 50nM; Sigma, Aldrich, Darmstadt, Germany) (Martínez-Torres, Zarate-Triviño, et al. 2018). Cells were seeded at 5X104 cells per well in 96-well plates (Corning Inc. Costar ®, NY, USA) and treated with I-CRP (CC50) and incubated 24 hours. After treatment, cells were recollected and washed with PBS, finally stained for 30 minutes at 37 ºC.

Western-blot analysis

Cells treated with CC50 of I-CRP for 24 hours and were lysed in lysis buffer (50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Samples containing 100 μg of whole cell protein were separated on 15% SDS-PAGE gels and blotted onto nitrocelulose transfer membranes. After blocking with 5% non-fat milk, each membrane was incubated overnight with a primary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) against p53 (1:1,000), Bax (1:1,000), Bcl-2 (1:1,000) and β-actin (1:1,000), and then with a secondary anti-mouse antibody (1:10000) (Santa Cruz Biotechnology). Protein expression was visualized with an enhanced chemoluminescence (ECL) detection kit (Thermo Scientific, Waltham, MA, USA).

5.2.1.3.6 Reactive Oxygen Species production analysis

Reactive oxygen species (ROS) production levels were measured with two different stains Dihydroethidium (DHE; Invitrogen, St Louis, MO, USA) and dichlorodihy drofluorescein diacetate (DCFDA; Invitrogen, St Louis, MO, USA). After treatment

for 24 hours with CC50 of I-CRP, cells were washed with PB, stained with DHE (10μM) or DCFDA (1 μM) and incubated for 30 minutes at 37 ºC.

5.2.1.3.7 Nuclear alteration analysis

To measured DNA damage we tested γ-H2AX histone phosphorylated. Cells were seeded at 5X105 cells/mL in 6-well sterile plates (Corning Inc. Costar®, NY, USA) and treated for 24 hours with CC50 of I-CRP. Cells were washed with PBS and fixed by bubbling with methanol (100%) and conserved overnight at -20 ºC. After fixed, cells were blocked (FACS buffer 10%) for 30 minutes at 4 ºC and washed (FACS buffer 2%). Then, cells were incubated with a specific primary antibody to the variant phosphorylated histone (γ-H2AX; ABCAM, Cambridge, UK) in a 1:100 dilution (Kiner; et al., 2008) during one hour at room temperature in constant agitation. Negative controls (isotype) were incubated only with FACS buffer 2%. After, we incubated for 30 minutes at room temperature the secondary antibody (Anti-Mouse FITC; ABCAM, Cambridge, UK) in a 1:100 dilution, and conserved overnight at 4 ºC. The p53 expression was analyzed using specific primary antibody to p53 (p53; ABCAM, Cambridge, UK) in a 1:100 dilution by flow cytometry as described previously.

5.2.1.3.8 Cell cycle analysis

For cell cycle analysis we quantified intracellular DNA using Propidium Iodide (PI) staining by Flow Cytometry (Martínez-Torres, Zarate-Triviño, et al. 2018). After treatment for 24 hours with CC50 of I-CRP, cells were recollected and washed with PBS, then we fixed in 70% ethanol by bubbling and conserved at -20 ºC overnight. Additionally, cells were washed again with PBS and incubated with RNase (MilliporeSigma, Eugene, OR, USA) and PI (10 μg/mL; MilliporeSigma, Eugene, OR, USA) at 37 °C for 30 minutes. For DNA degradation, we quantified the SubG1 population.

5.2.1.3.9 Cleaved Caspase-3 analysis

We used a specific detection kit, FITC-DEV-FMK (ABCAM; Cambridge, UK) (Martínez-Torres, Reyes-Ruiz, et al. 2018) to assess caspase-3 activation. In brief, the cells were seeded at 1X105 cells per well in 96-well plates (Corning Inc. Costar ®, NY, USA) and after 24 hours of treatment with I-CRP (CC50), were recovered and stained following the manufacturer's instructions.

5.2.1.3.10 Pharmacological inhibition

We used different pharmacological inhibitors to determine the cell death mechanism of I-CRP in leukemic cells. QVD-OPh (QVD, 10 μM) as a general caspase inhibitor; Z-DEVD (1μM) as caspase-3 inhibitor; Z-IETD (1μM) as caspase-8 inhibitor; Z-LEHD (1μM) as caspase-9 inhibitor; were acquired from Bio Vision (CA, USA). N-acetyl-cysteine (NAC, 5mM) was used as a ROS inhibitor. The inhibitors were added 30 minutes before CC50 I-CRP treatment. All stock solutions were wrapped in foil and stored at −20 °C.

5.2.1.3.11 Statistical Analysis

Western blot analysis was measured using ImageJ software. Statistical analyses were done using paired Student's t-test. The statistical significance was defined as $p<0.005$. The data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). The results given in this study represent the mean of at least three independent experiments done in triplicate (mean±SD).

5.2.1.4 RESULTS

5.2.1.4.1 Immunepotent-CRP decreases selective cell viability in leukemic cells

We assessed whether I-CRP induces selective cytotoxicity in leukemic cells. For this, we analyzed cell viability in the T-acute lymphoblastic leukemia (T-ALL) cell lines Molt-4 and CEM; and in the healthy counterpart peripheral blood mononuclear cells (PBMC) and T-lymphocytes (Fig. 18). In figure 18, we show histograms of cell viability analysis in Molt-4 (Fig. 18A), CEM (Fig. 18B), PBMC (Fig. 18C), T-

lymphocytes in total PBMC (CD3+) (Fig.18D) and in isolated T-lymphocytes (Fig. 18E) at different concentrations of I-CRP (0.4, 0.6, 0.8, and 1.0U/mL) at 24 and 48 hours of treatment. In figure 18F, we observed that I-CRP decreases cell viability in a time and concentration-dependent manner in T-ALL cell lines, however we observed that cell viability of healthy counterpart was not affected; including T-lymphocytes (CD3+). These results showed that I-CRP decreases selectively the viability in malignant cells only.

Figure 18. Cell viability of T-ALL cell lines and healthy counterpart after I-CRP treatment. A) Representative histograms of cell viability analysis by Flow cytometry using calcein-AM staining in Molt-4, B) CEM, C) PBMC D) CD3+ cells in PBMC, E) Isolated CD3+ treated with different concentrations (0.4, 0.6, 0.8, 1.0U/mL) of I-CRP for 24 and 48 hours. F) Quantification of cell viability. The results are presented as mean ± standard deviation of three different experiments.

5.2.1.4.2 Immunepotent-CRP induces selective cell death in a leukemic cell lines

To confirm that the loss of cell viability is due to the cytotoxic effect of I-CRP and not to a metabolic effect, we used a cell death assay analyzing phosphatidylserine (PS) exposure (Annexin-V) and membrane permeabilization (propidium iodide, PI) at different concentrations of I-CRP (0.4, 0.6, 0.8, 1.0U/mL); after 24 and 48 hours of treatment (Fig. 19) in T-ALL cells and healthy counterpart. As shown in Figure 19A

and B, I-CRP at 0.8U/mL increases cell death with double positive population for Annexin-V and PI staining and enhances concentration and time-dependent cell death at 24- and 48- hours in Molt-4 (Fig. 19A) and CEM (Fig. 19B) cells, and the mean cytotoxic concentration that killed 50% of the cells (CC50) was 0.6U/mL at 24 hours and the CC100 (killed 100% of cells) was 1.0U/mL. We did not observe affectations in the cell integrity of the healthy counterpart (Fig. 19C, D and E) even after 48 hours of treatment. Cell death induction by I-CRP in T-ALL cells was confirmed by microscopy assessment, where we observed morphological alterations, including apoptotic bodies and cell shrinkage (Supp.1). These results indicated that I-CRP is a selective cell death inductor in T-ALL cells, does not affect healthy cells at same doses and times of treatment, thus confirming the selective cytotoxic effect in cancer cells.

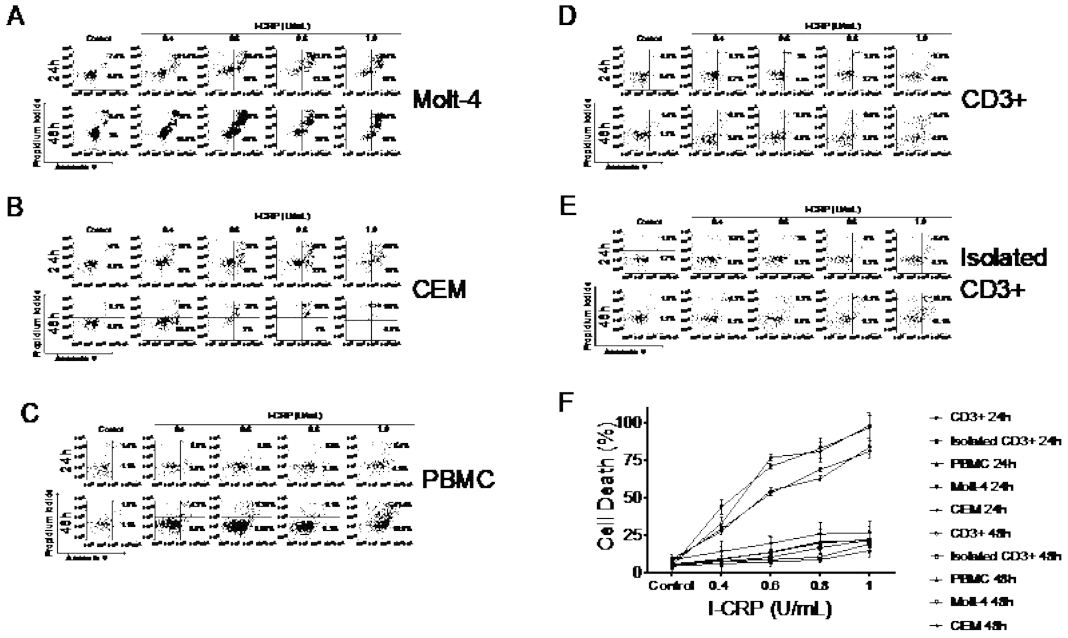

Figure 19. Phosphatidylserine exposure and membrane permeability of T-ALL cell lines and healthy counterpart after I-CRP treatment. A) Representative dot plots of cell death analysis by Flow cytometry using Annexin-V and propidium iodide (PI) staining in Molt-4, B) CEM, C) PBMC, D) CD3+ cells in PBMC, E) Isolated CD3+ treated at different concentrations (0.4, 0.6, 0.8, 1.0U/mL) of I-CRP for 24 and 48 hours. F) Quantification of cell death. The results are presented as mean ± standard deviation of three different experiments.

5.2.1.4.3 Immunepotent-CRP induces mitochondrial alterations in leukemic cells

Mitochondria are the bioenergetic center of the cell and a reservoir of pro-death factors and have an essential role in the intrinsic pathway of apoptosis, activating caspase proteases (Ashkenazi and Salvesen 2014; Tait, S.W., and Green 2013). To elucidate the cell death mechanism induced by I-CRP in T-ALL cells, we analyzed mitochondrial alterations Molt-4 (Fig. 10) and CEM (Supp. 2A and B). We tested loss of mitochondrial membrane potential (LMMP) with TMRE marker, Bax-Bcl-2 ratio (pro- and anti-apoptotic proteins) by western blot, and reactive oxygen species (ROS) production, using DHE and DCFDA stainings. We found that I-CRP enhances LMMP in Molt-4 from 6% to 58% (Fig. 20A), indicating that I-CRP induces mitochondrial impairment. In ratio Bax-Bcl-2 analysis (Fig. 20B), we showed that treatment increases Bax expression and reduces Bcl-2 expression. We also found that I-CRP increases ROS production from in Molt-4 cells (Fig. 20C and D). We did not observe mitochondrial alterations in PBMC (Supp. 2C and D). These mitochondrial alterations suggest cell death by apoptosis in T-ALL cell lines.

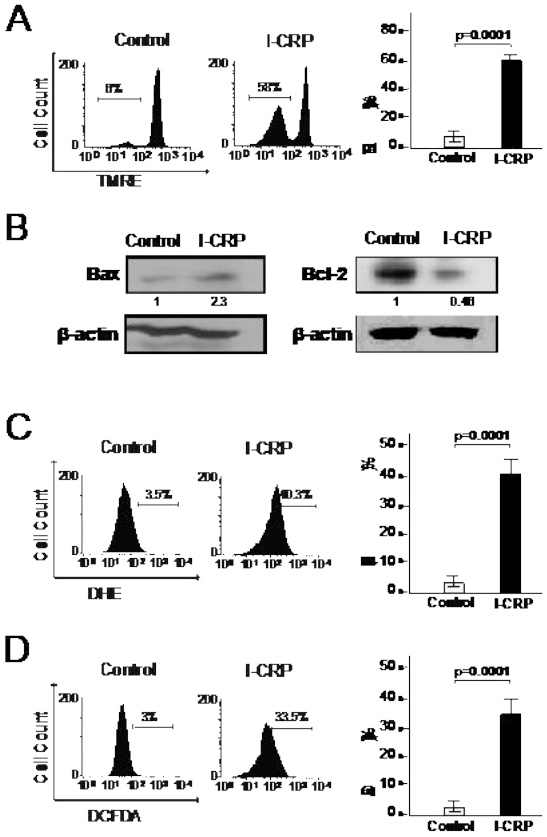

Figure 20. Mitochondrial alterations in Molt-4 cell line upon I-CRP. A) Representative histogram of mitochondrial membrane potential loss analysis and quantification, using TMRE by Flow cytometry. B) Western blot analysis showed the expression of Bax and Bcl-2 proteins. The same blot was reproved with β-actin to confirm equal loading of each lane. C) Representative histogram of ROS analysis and quantification by Flow cytometry using DHE stain. D) Representative histogram of ROS analysis and quantification by Flow cytometry using DCFDA stain. The results are presented as mean ± standard deviation of three different experiments.

5.2.1.4.4 Immunepotent-CRP induce nuclear alterations

Usually, RCD is associated with DNA damage. During DNA damage, H2AX, a variant of the H2A protein family, is phosphorylated in serine 139 (γ-H2Ax) to recruit repair proteins that can induce p53 activation, leading to cell cycle arrest followed by cell death characterized by DNA degradation (Galluzzi et al. 2018; Ashkenazi and Salvesen 2014). We tested phosphorylation of H2Ax (γ-H2Ax) in Molt-4 cells; we found that the percentage of γ-H2Ax increases from 3% to 32% in trated cells (Fig. 21A), indicating that I-CRP induces DNA damage. Then, we analyzed p53 expression and found it increased from 18% to 38% in treated cells (Fig. 21B). The western blot analysis (Fig. 21C) confirmed overexpression of p53 in treated cells with I-CRP. These results suggest that I-CRP has a role cell cycle arrest and cell death. Finally, to determine cell cycle alterations, we quantified DNA using PI staining and RNase. The results show an increased percentage of cells in G2 phase in comparison to the control in Molt-4 (Fig. 21D) and CEM (Supp. 3A) treated cells. Further, low percentage of DNA degradation of 17% in Mol-4 (Fig. 21E) and CEM (Supp. 3B) cells treated with I-CRP at 24 hours of treatment. These results demonstrate that I-CRP induces DNA damage (γ-H2AX), leading to p53 augmentation, and cell cycle arrest in the G2 phase, suggesting a classical apoptosis cell death in T-ALL cell lines.

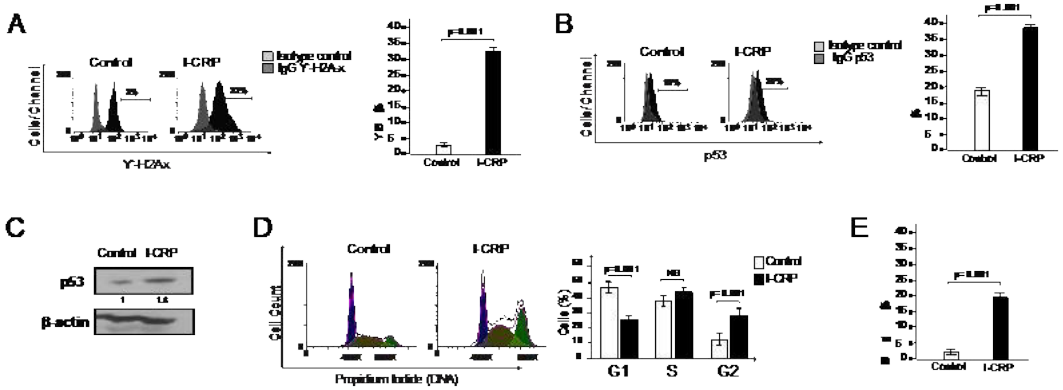

Figure 21. Nuclear alterations in Molt-4 cell line after I-CRP treatment. A) Representative histogram of nuclear damage analysis and quantification measure γ-H2AX by Flow cytometry. B) Representative histogram of p53 analysis and quantification by Flow cytometry in Molt-4 cells. C) Western blot analysis showed the expression p53 protein and β-actin to confirm equal loading of each lane. D) Representative histogram of cell cycle analysis and quantification, using RNAse and propidium iodide (PI) stain, by Flow cytometry. E) DNA degradation analysis by Flow cytometry. The results are presented as mean ± standard deviation of three different experiments.

5.2.1.4.5 Immunepotent-CRP induces caspase-dependent cell death in T-ALL cells

Caspase-3 is the principal caspase effector in apoptosis (Manley 2013; Xu and Shi 2007), previous studies described that I-CRP induces cleavage of capase-3, but triggers caspase-independent cell death mechanism in tumoral cells (Martínez-Torres, Reyes-Ruiz, et al. 2018). To determine the cell death mechanism induced by I-CRP in Molt-4 cells, we tested caspase-3 activation induced by I-CRP and etoposide (ETO) as an apoptotic inductor (positive apoptosis inductor control) in presence or absence of QVD -Oph (QVD; as a pan-caspase inhibitor). In Molt-4 cells, I-CRP induced slight caspase-3 activation from 5% to 25% (Fig. 22) at 24 hours of treatment. Further, QVD prevented caspase-3 activation induced by I-CRP and ETO (Fig. 22B). To reveal whether I-CRP induced apoptosis, we used the pan-caspase inhibitor (QVD) and specific caspases inhibitors Z-DEVD (caspase-3 inhibitor), Z-IETD (caspase-8 inhibitor), and Z-LEHD (caspase-9 inhibitor). In figure 22C, we can observe that the percentage of cell death diminishes in comparison to the CC50 of I-CRP when using caspases inhibitors. Caspase-dependence for cell death was confirmed in CEM cells

(Supp. 4A), where we also observed cell death inhibition when inhibiting caspases. This indicates that the mechanism of cell death induced by I-CRP is caspase-dependent in T-ALL cells.

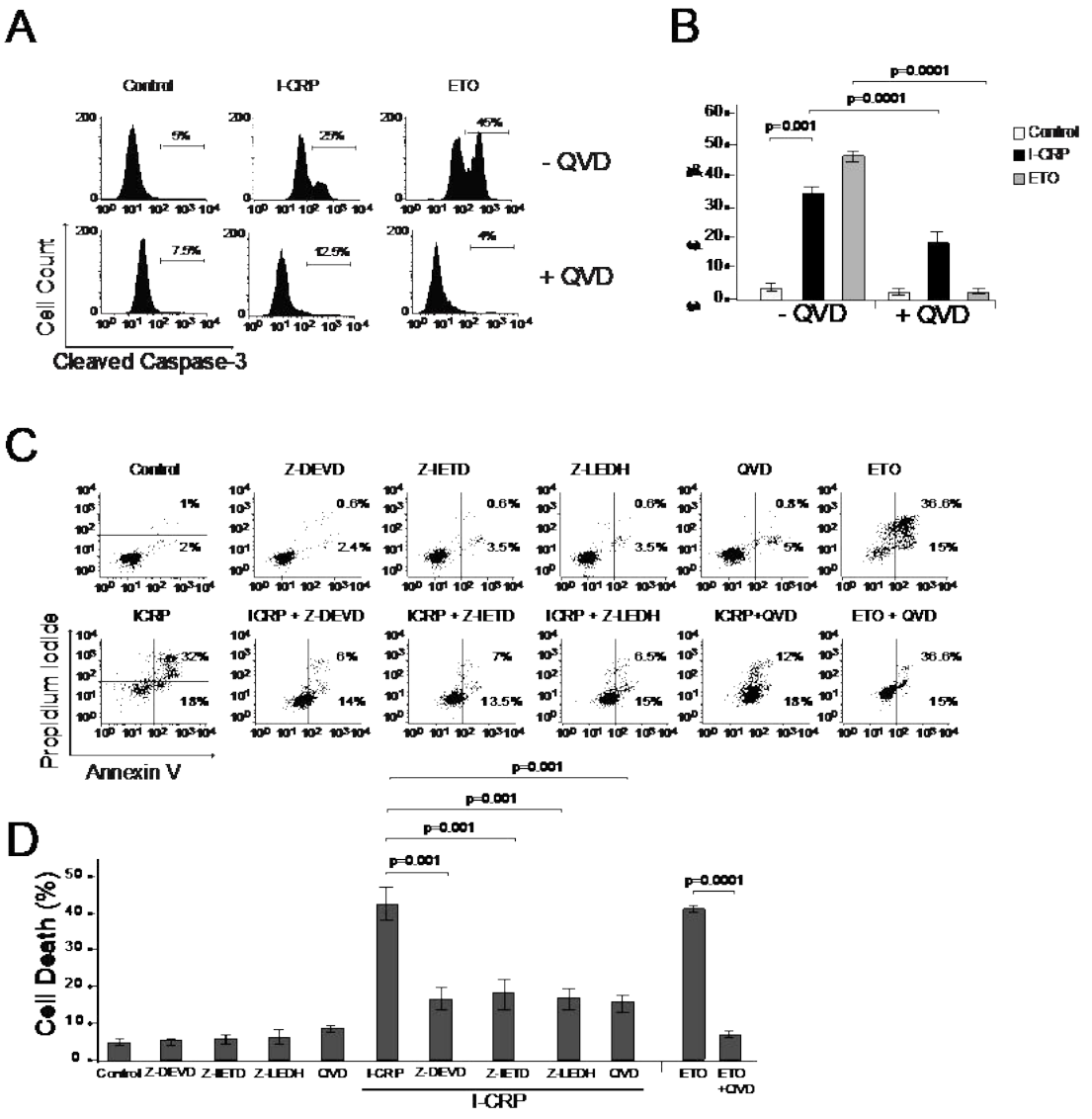

Figure 22. Caspase-3 activity and effects of caspases inhibition on I-CRP-treated Molt-4 cells. A) Representative histogram of caspase-3 activity analysis and B) quantification by flow cytometry with FITC-DEVD-FMK staining in Molt-4 cells treatment with I-CRP and etoposide (ETO), using QVD as a pan caspase inhibitor. C) Representative dot plots of cell death analysis and D) quantification by Flow cytometry with Annexin-V and propidium iodide (PI) staining in Molt-4 cells after I-CRP and etoposide (ETO) treatments, using Z-DEVD (Caspase-3 inhibitor), Z-IETD (Caspase-8 inhibitor), Z-LEHD

(Caspase-9 inhibitor) and QVD (pan-caspase inhibitor). The results are presented as mean ± standard deviation of three different experiments.

5.2.1.4.6 Role of caspases in the mitochondrial alterations induced by I-CRP in T-ALL cells

The critical step for intrinsic apoptosis is the irreversible and widespread permeabilization of the mitochondrial outer membrane (MOMP), which directly promotes the cytosolic release of apoptogenic factors which induce caspases activation. To analyze the role of caspases in mitochondrial damage, we tested LMMP with TMRE marker and ROS production using DHE stain during caspases inhibition with QVD. We confirmed that I-CRP increased LMMP from 5% to 58%, and that during caspase inhibition with QVD the LMMP is not changed (51%) (Fig. 23A), these results were also observed in CEM cells (Supp. 4B). We also observed that during caspase inhibition with QVD the ROS production is not changed in Molt-4 (34%) (Fig. 23B) and CEM cells (Supp. 4C). All results suggest that I-CRP induces LMMP and enhances ROS production, which leads to caspases activation and intrinsic apoptosis in T-ALL cells.

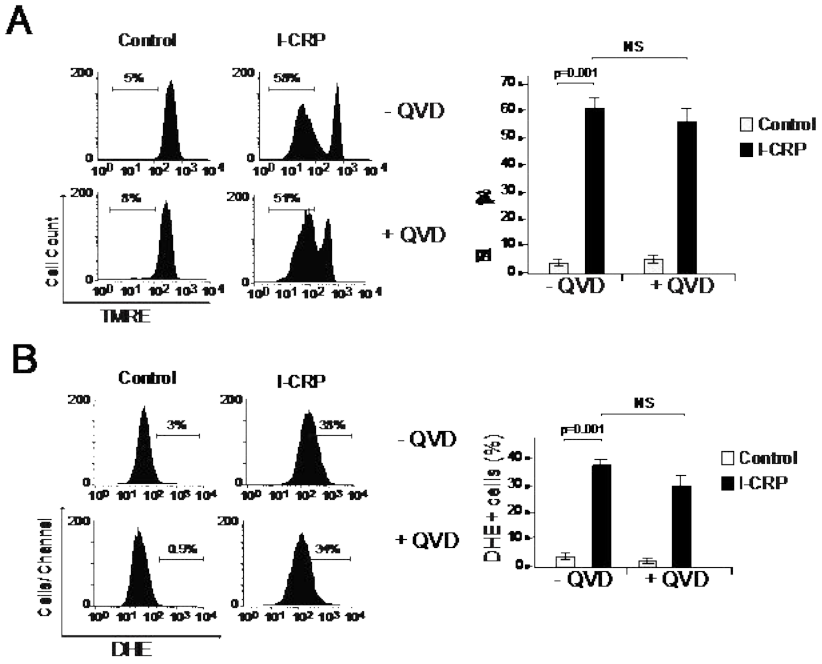

Figure 23. Effect of caspases in mitochondrial damage in Molt-4 cells treated with I-CRP. A) Representative histogram of mitochondrial membrane potential loss analysis and quantification by Flow cytometry using TMRE staining and QVD as a pan-caspase inhibitor. B) Representative histogram of ROS production analysis and quantification by Flow cytometry using DHE staining, and QVD as a pan-caspase inhibitor in Molt-4 cells treated with I-CRP. The results are presented as mean ± standard deviation of three different experiments.

5.2.1.4.7 Immunepotent-CRP induces ROS-dependent cell death in leukemic cells

An excessive amount of intracellular ROS can result in damage to proteins and DNA, stimulating cell death pathways such as apoptosis (Redza-Dutordoir and Averill-Bates 2016). In tumor cells, I-CRP triggers ROS-dependent cell death (Martínez-Torres, Reyes-Ruiz, et al. 2018). To determine the role of ROS in a cell death mechanism, first we tested ROS production in presence of the antioxidant N-acetylcysteine (NAC), and we assessed its implication in mitochondrial and nuclear damage, caspase-3 activation and cell death. In figure 24A, results exhibit that NAC can repress ROS production increased by the CC50 of I-CRP (0.6U/mL), demonstrating that NAC is a potential ROS inhibitor. For this, we assessed LMMP during ROS inhibition, in the analysis we present that NAC avoids LMMP induced by I-CRP (Fig. 24B). The γ-H2AX analysis shows that DNA damage (γ-H2AX) diminishes when we block ROS production (Fig. 24C). The caspase-3 activation analysis showed that the activation of caspase-3 induced by I-CRP is prevented in presence of NAC (Fig. 24D). Due to ROS production induces the molecular characteristics of cell death, we analyzed whether ROS production was playing role in the mechanism of cell death. In figure 7E, we observed that cell death induced by I-CRP is blocked during ROS inhibition with NAC in Molt-4 cells. This reveals that I-CRP induces ROS-dependent cell death in leukemic cells, and that it is a conserved cell death mechanism of cancer cells. Finally, to determine the role of caspases and ROS in cell death, we used NAC and QVD (Fig. 24F). ROS implication in cell death mechanism was confirmed in CEM cells where we also observed ROS, LMMP and cell death inhibition when we used NAC as a ROS inhibitor (Supp. 5). Interestingly, the results reveal that cell death diminished during ROS and caspase inhibition. These results demonstrated that ROS production promotes apoptosis T-ALL cell lines.

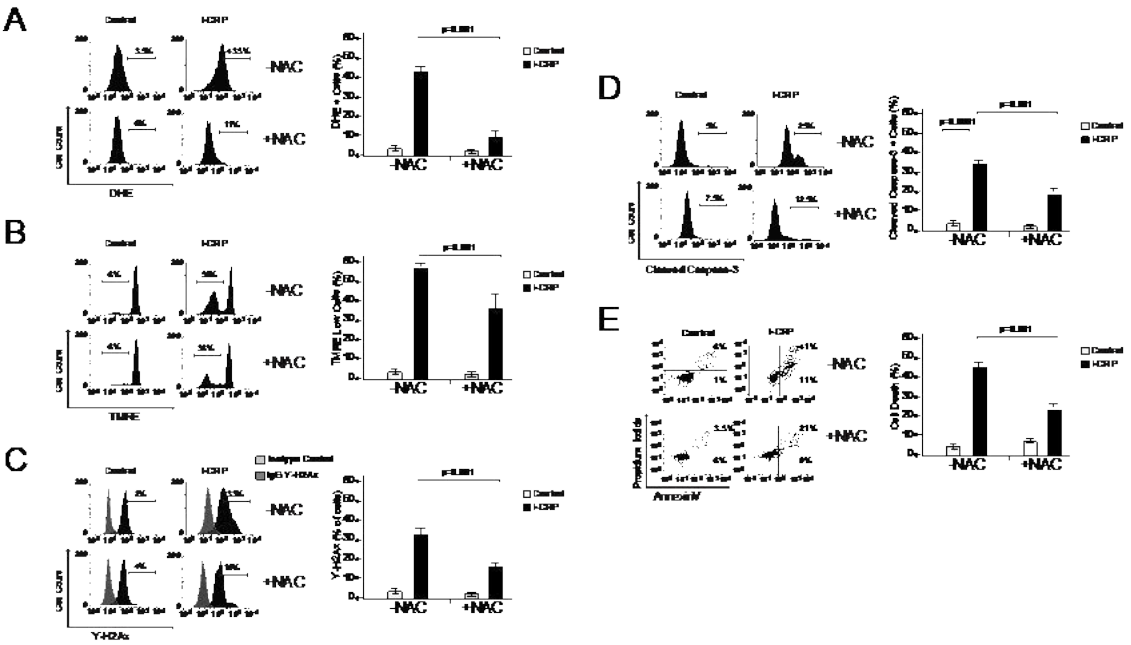

Figure 24. ROS implication in biochemical characteristics of cell death in Molt-4 cells upon I-CRP treatment. A) Representative histograms of ROS analysis and quantification by Flow cytometry using DHE stain and N-Acetyl-cysteine (NAC) as a ROS inhibitor. B) Representative histograms of mitochondrial membrane potential loss analysis and quantification by Flow cytometry using TMRE staining and NAC (ROS inhibitor). C) Representative histograms of nuclear damage analysis and quantification measure γ-H2AX and NAC as a ROS inhibitor by Flow cytometry. D) Representative histograms of caspase-3 activity analysis and quantification by flow cytometry with FITC-DEVD-FMK staining using NAC as a ROS inhibitor. E) Representative dot plots of Cell death analysis and quantification by Flow cytometry using Annexin-V and propidium iodide (PI) staining during ROS inhibition with NAC. The results are presented as mean ± standard deviation of three different experiments.

5.2.1.5 DISCUSSION

The development of selective drugs for cancer treatment is based on the potential of compounds to block cell proliferation and/or induce cell death. Similar treatments for leukemia have been difficult to find because, this being a cancer of the immune system, it is unclear whether the drugs will distinguish between healthy and cancerous cells (Rutering et al. 2016). I-CRP shows selective cytotoxicity to solid cancer cells (Moises A. Franco-Molina et al. 2018; M. A. Franco-Molina et al. 2006; Martínez-Torres, Reyes-Ruiz, et al. 2018; Martinez-Torres et al. 2019), such as MCF-7, BT-474, MDA-MB-453, A-427, Calu-1, U937 and L5178Y-R cell lines (M. A. Franco-Molina et al.

2006; Martínez-Torres, Reyes-Ruiz, et al. 2018; Martinez-Torres et al. 2019). Other types of DLE have also shown antitumor activity in prostate cancer (Hernández-Esquivel et al. 2018), and in combination with chemotherapies (Demečková et al. 2017), and with antibiotics as manumycin in mouse breast cancer (4T1) (Solár et al. 2017). Yet, these cytotoxic analyses were done in solid cancer cell lines, while its effect in cancer lymphocytes was unknown specially because there were doubts about its efficacy in leukemia, as it increases lymphocyte number in lung (Lara et al. 2010) and breast (M. A. Franco-Molina et al. 2008) cancer patients. Here we report for the first time the cell death mechanism of I-CRP or a DLE in leukemic cells.

I-CRP has selective cytotoxicity in the T-ALL cell lines, without affecting PBMCs. Interestingly, in T-ALL cells, I-CRP cytotoxicity is high in comparison with cervical (Martínez-Torres, Reyes-Ruiz, et al. 2018) or lung (Martinez-Torres et al. 2019) cancer cells, suggesting that toxicity is cell lineage–dependent. Of interest, bDLE at high concentrations induce cell death in K562 cells, while at low concentrations it induced differentiation toward the monocyte/macrophage and megakaryocytic lineages (Sierra-Rivera et al. 2016). The explanation could be that I-CRP may induce lymphocyte activation and, as a consequence, cell death, as it occurs in activation-induced cell death in T-cells (Green, Droin, and Pinkoski 2003).

I-CRP showed mitochondrial alterations including high ROS production, loss of MMP, increased Bax (proapoptotic protein) and decreased Bcl-2 (antiapoptotic protein) expression. Similar results were found in curcumin-lead apoptosis, where upregulation of Bax/Bcl2 ratio in SW872 was found (Yao et al. 2015). Bcl-2 plays an important role in progression of cancer, resistance to apoptosis and is usually increased in leukemia (Yao et al. 2015; Okushi et al. 2015; Campbell and Tait 2018). However, I-CRP prevents apoptotic resistance from leukemic cells. This is similar to resveratrol, prednisone (Ghasemi et al. 2018) and prednisolone that induces apoptosis in CCRF-CEM cell line via Bax/Bcl-2 (Ghasemi et al. 2018).

Here we show that I-CRP induces nuclear damage including DNA damage (γ-H2Ax), p53 overexpression, cell cycle arrest in G2 phase and DNA degradation. This mechanism is similar to daunorubicin-induced cell death in human acute lymphoblastic leukemia (CCRF-CEM and Molt-4) (Al-Aamri et al. 2019) and cell death induced by

etoposide in HeLa cells (Soubeyrand, Pope, and Haché 2010), which induce DNA break and overexpression of γ-H2Ax (Soubeyrand, Pope, and Haché 2010). Accordingly, HeLa cells show cell cycle arrest in the G2 phase and low DNA degradation (less than 20%) in response to exposure to I-CRP at 16h and 24h (Martínez -Torres, Reyes-Ruiz, et al. 2018), which means that γ-H2Ax and p53 overexpression may be also involved in HeLa cells.

The activation of caspases is essential for apoptosis; we tested the caspase 3 activity and detected caspase-3 activation, and during the blocking of caspase-3, -8 and -9, cell death was inhibited, indicating apoptosis in the leukemia cell line. Similarly, in murine lymphoblastic leukemia cells (L1210) three different chalcones (flavonoids) induce stress in the endoplasmic reticulum, leading to intrinsic and extrinsic apoptosis (Winter et al. 2014). I-CRP induces caspase-3 activation but caspase-independent cell death mechanisms in cervical cancer cells (Martínez-Torres, Reyes-Ruiz, et al. 2018), while cell death is also caspase independent in lung cancer cells (Martinez-Torres et al. 2019), suggesting, that I-CRP induces different cell death modalities depending to type of cancer.

ROS play a central role in cell signaling, in regulation of the main pathways of apoptosis mediated by mitochondria, death receptors and endoplasmic reticulum (ER) (Redza-Dutordoir and Averill-Bates 2016). In hematological cells ROS promote cell survival, activation, migration and proliferation; but, oxidative stress has been observed in several hematopoietic malignancies (Galadari 2017; Walton 2016; Hole, Darley, and Tonks 2011). I-CRP increases ROS production, which is inhibited in presence of NAC; also, NAC inhibits the loss of MMP, DNA damage, caspase-3 activation and cell death in Molt-4 cells. Similarly, 4-HRP induce ROS-dependent cell death in CCRF-CEM, and CRF-HSB2, Molt-4, and KG-1 (Apraiz et al. 2013). In Jurkat (Jiao et al. 2016) and in hDPCs (Sato et al. 2019) cells ROS inhibition blocked extrinsic apoptosis induced by FAS receptor. CH-AuNPs induce caspase-3 activation, leading to ROS-dependent apoptosis in HeLa (Martínez-Torres, Zarate-Triviño, et al. 2018) and CCRF-CEM (Martínez-Torres et al. 2019) cells.

Our results demonstrate that I-CRP can play different roles in healthy and cancerous lymphocytes. Interestingly, cell death of T lymphocytes following their activation

involves extrinsic apoptosis (Green, Droin, and Pinkoski 2003; Zhang et al. 2005). For this, it is important to evaluate if I-CRP induces Molt-4 activation and, as a consequence, apoptosis.

5.2.1.6 CONCLUSION

Altogether, our results confirm that I-CRP induces selective cytotoxicity in leukemic cells, diminishing cell viability and increasing cell death without affecting healthy blood cells populations. The regulated cell death mechanism induced by I-CRP in non-solid tumors is different in comparison with solid tumors, however, they share characteristics, such as a cell cycle arrest and production of ROS. Additionally, I-CRP promotes ROS production which induces DNA damage (γ-H2Ax) in the nucleus with over expression of p53 to induce cell cycle arrest in G2 phase and finally DNA degradation. ROS production induces mitochondrial damage (loss of mitochondrial membrane potential), including pro and anti-apoptotic protein modulation (Bax and Bcl-2), and finally caspase-3 activation, inducing apoptosis in T-ALL cells (fig.25). This work opens the door to evaluate in more detail the importance of cell lineage in the selectivity and the mechanism of cytotoxicity of I-CRP, which might contribute to its widespread use and clinical application.

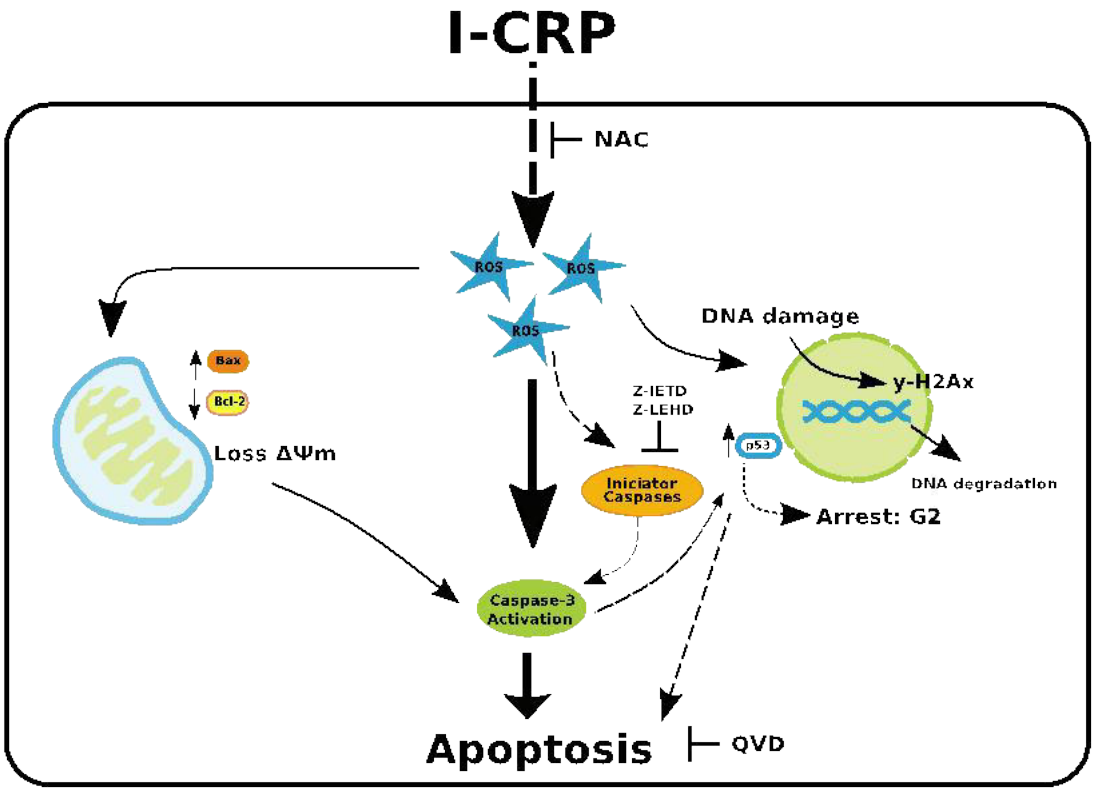

Figure 25. Schematic representation of cell death mechanism in T-ALL cells treated with I-CRP. We propose a cell death mechanism induced by I-CRP in leukemic cells. I-CRP promotes ROS production which induces DNA damage (γ-H2Ax) in the nucleus with over expression of p53 to induce cell cycle arrest in G2 phase and finally DNA degradation. Additionally, ROS production induces mitochondrial damage (loss of mitochondrial membrane potential), including pro and anti-apoptotic protein modulation (Bax and Bcl-2), and finally caspase-3 activation, inducing apoptosis.

5.2.1.7 Data availability

All datasets generated during the current study are available from the corresponding author upon request.

5.2.1.8 Conflicts of interest

The authors declare no competing interests exist.

5.2.1.9 Funding Statement

This work was supported by the Laboratory of Immunology and Virology of the College of Biological Sciences; UANL. HYLA holds a CONACyT scholarship.

5.2.1.10 Acknowledgments

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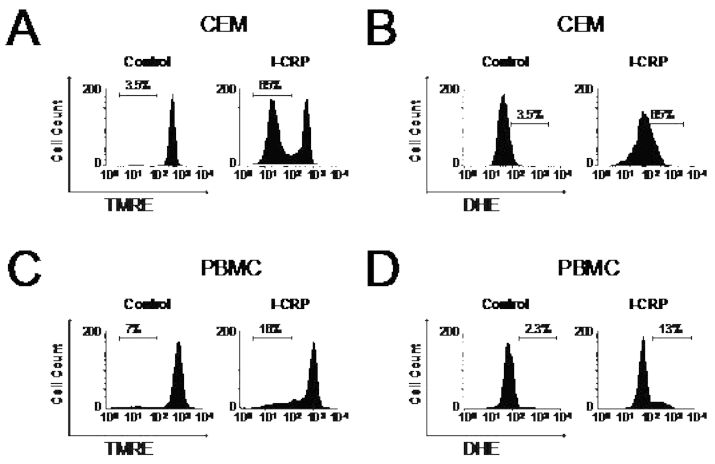
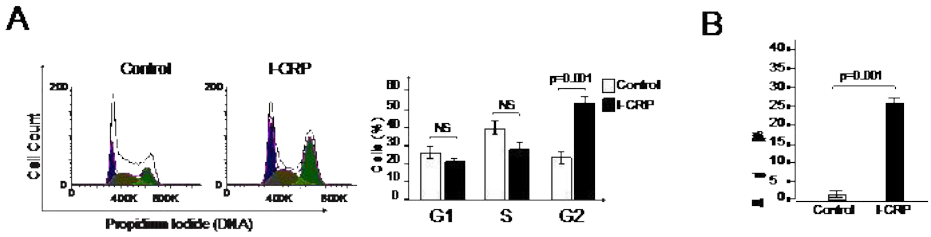
5.2.1.12 SUPPLEMENTARY MATHERIAL

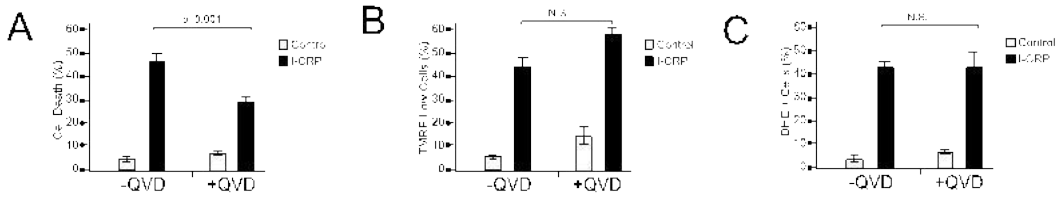
Supplementary 1. Morphological analysis of T-ALL cell lines after I-CRP treatment. Representative images of optical microscopy of CEM and Molt-4 cells treated at different concentrations (0.4, 0.6, 0.8, 1.0U/mL) of I-CRP for 24 and 48 hours.



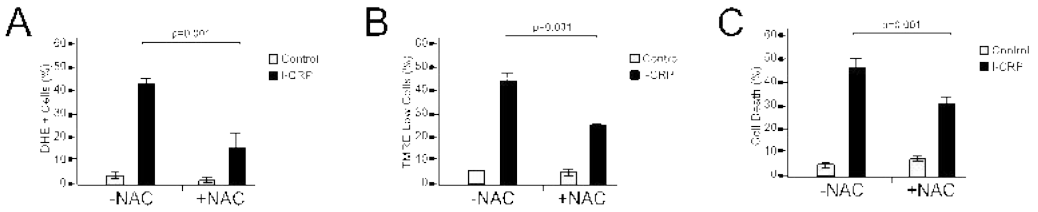
Supplementary 2. Mitochondrial alterations in CEM cell line and PBMC upon I-CRP. A) Representative histogram of mitochondrial membrane potential loss analysis, using TMRE by Flow cytometry in CEM cells. B) Representative histogram of ROS analysis by Flow cytometry using DHE stain in CEM cell line. C) Representative histogram of mitochondrial membrane potential loss analysis, using TMRE by Flow cytometry in PBMC. D) Representative histogram of ROS analysis by Flow cytometry using DHE stain in PBMC.



Supplementary 3. Cell cycle and DNA degradation analysis in CEM cell line after I-CRP treatment. A) Representative histogram of cell cycle analysis and quantification, using RNAse and propidium iodide (PI) stain, by Flow cytometry. B) DNA degradation quantification by Flow cytometry. The results are presented as mean ± standard deviation of three different experiments.



Supplementary 4. Caspases implication in cell death mechanism of I-CRP in CEM cells. A) Graph represents the mean of the cell death quantification by Flow cytometry using Annexin-V and propidium iodide (PI) staining in cells left untreated (control) or treated with ICRP, with or without caspases inhibition with QVD. B) Graph represents the mean of the mitochondrial membrane potential loss quantification by Flow cytometry using TMRE staining in cells left untreated (control) or treated with ICRP, with or without QVD as a pan-caspase inhibitor. C) Graph represents the mean of the ROS production quantification by Flow cytometry using DHE staining, in cells left untreated (control) or treated with ICRP, with or without QVD as a pan-caspase inhibitor. The results are presented as mean ± standard deviation of three different experiments.



Supplementary 5. ROS implication in cell death mechanism of I-CRP in CEM cells. A) ROS quantification by Flow cytometry using DHE staining in cells left untreated (control) or treated with ICRP, with or without N-Acetyl-cysteine (NAC) as a ROS inhibitor. B) Mitochondrial membrane potential loss quantification by Flow cytometry using TMRE staining in cells left untreated (control) or treated with ICRP, with or without NAC (ROS inhibitor). C) Cell death quantification by Flow cytometry using Annexin-V and propidium iodide (PI) staining in cells left untreated (control) or treated with ICRP, with or without ROS inhibition with NAC. The results are presented as mean ± standard deviation of three different experiments.

5.3 CHAPTER III

IMMUNEPOTENT CRP increases intracellular calcium leading to ROS production and cell death through ER-calcium channels in breast cancer and leukemic cell lines

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5.3.1.1 ABSTRACT

IMMUNEPOTENT-CRP (ICRP) is an immunotherapy that induces different cell death mechanisms in leukemic and tumoral cell lines. However, the molecular differences on cell death pathways are not completely elucidated, which limits its application. Here, we evaluated the Ca^{2+} implication in the different cell death modalities induced by ICRP on T-ALL and breast cancer cell lines. Cell death induction was evaluated in T-ALL and breast cancer cell lines. Autophagosome formation, ROS production, loss of mitochondrial membrane potential, ER stress and intracellular Ca^{2+} levels were evaluated. Finally, we assessed the involvement of extracellular Ca^{2+}, and the implication of the ER-receptors IP_3R and RyR in the ICRP-induced cell death by using an extracellular calcium chelator and pharmacological inhibitors. ICRP increases intracellular Ca^{2+} levels as the first step of the cell death mechanism that provokes ROS production, caspase-3 cleaved (in T-ALL cells) and loss of mitochondrial membrane potential. In addition, blocking the IP_3 and ryanodine receptors inhibited ER-Ca^{2+} release, ROS production and ICRP-induced cell death. Taken together our results, demonstrated that ICRP triggered intracellular Ca^{2+} increase leading to different regulated cell death modalities in T-ALL and breast cancer cell lines.

KEYWORDS

Calcium, ryanodine receptor, 2-APB, dantrolene, immunotherapy, cell death

5.3.1.2 INTRODUCTION

Despite the pivotal advances in the treatment of breast cancer and T-cell acute lymphoblastic leukemia (T-ALL); breast cancer is one of the leading cause of death in women (Siegel, Miller, & Jemal, 2019), whereas T-ALL is the most diagnosed cancer in children worldwide (K & B, 2017; Terwilliger & Abdul-Hay, 2017). One of the reasons is the ability of cancer cells to develop different mechanisms of resistance to the current therapies including cell death inducers.

In this direction, IMMUNEPOTENT CRP (ICRP), a bovine dialyzable leukocyte extract (DLE) obtained from disrupted spleen, is cytotoxic in solid cancer cell lines, including cervical, lung and breast cancer (Martinez-Torres et al., 2019;

Martínez-Torres et al., 2018; Reyes-Ruiz et al., 2021a), and hematological malignances, such as T-ALL (Lorenzo-Anota, Martínez-Torres, Scott-Algara, Tamez-Guerra, & Rodríguez-Padilla, 2020b). Despite the difference of the cancer cell linage, some characteristics of the ICRP-cell death remain conserved among the different cancer types, including but not limit to loss of mitochondrial membrane potential, reactive oxygen species (ROS) production, cell cycle arrest and DNA degradation (Martínez-Torres et al. 2018; Martinez-Torres et al. 2019; Martínez-Torres et al. 2020; Lorenzo-Anota et al. 2020). Interestingly, solid cancer cells exposed to ICRP succumb to a ROS-dependent cell death, whereas this same agent induces ROS and caspase-dependent cell death in leukemic cells.

Moreover, we recently reported that ICRP induces characteristics related with endoplasmic reticulum (ER) stress such as autophagosome formation, exposure of chaperone proteins on the plasma membrane, and eIF2a phosphorylation (P-eIF2a) in solid cancers (Martínez-Torres et al., 2020; Reyes-Ruiz et al., 2021b), but less is known about these characteristics in leukemic cells. Ca^{2+} depletion in ER Ca^{2+} pool or Ca^{2+}-overload in this organelle results in disturbances, therefore ER stress (Zhivotovsky and Orrenius 2011).

Moreover, Ca^{2+} is a highly versatile intracellular signal that regulates broad cellular functions and cell death (Monteith, Prevarskaya, & Roberts-Thomson, 2017). For these reasons, the purpose of this study was to evaluate for first time the role of Ca^{2+} and ER-receptors (IP3R and RyR respectively) in the mechanism of cell death induced by ICRP in breast cancer cells of luminal and the triple negative subtypes, and T-ALL.

5.3.1.3 MATERIALS AND METHODS

5.3.1.3.1 Cell culture

MCF-7 human breast adenocarcinoma (ATCC® HTB-22TM) and 4T1 murine mammary adenocarcinoma (ATCC® CRL2539TM) cell lines were obtained from the American Type Culture Collection. MCF-7 cells were cultured in DMEM-F12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (complete DMEM), and 4T1 cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin- streptomycin (complete RPMI) (Life Technologies,

Grand Island, NY) and routinely grown in plastic tissue-culture dishes (Life Sciences, Corning, NY). T-acute lymphoblastic leukemia cell lines CEM (ATCC® CCL-119™) and MOLT-4 (ATCC CRL-1582) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 supplemented with 10 % FBS and 1 % penicillin- streptomycin (complete RPMI) (Life Technologies, Grand Island, NY) and routinely grown in plastic tissue-culture dishes (Life Sciences, Corning, NY). All cell cultures were maintained in a humidified incubator in 5 % CO_2 at 37 °C. Cell count was performed using 0.4% trypan blue (MERCK, Darmstadt, Germany) in a Neubauer chamber.

5.3.1.3.2 Cell Death Induction and inhibition

IMMUNEPOTENT-CRP (ICRP) a bovine dialyzable leukocyte extract was produced as previously described (Coronado-Cerda et al., 2016; M. A. Franco-Molina et al., 2006) and dissolved in media. One unit (U) of ICRP is defined as 24 mg of peptides obtained from 15×10^8 leukocytes. We used different pharmacological inhibitors to determine the cell death mechanism of ICRP in cancer cell lines. QVD-OPh (QVD, 10 μM) as a general caspase inhibitor, N-acetylcysteine (NAC, 5 mM) was used as an ROS inhibitor, Spautin-1 (Sp-1, 15mM) as autophagosome inhibitor, BAPTA (50mM) as extracellular calcium chelator, dantrolene (30 mM) as ryanodine receptors (RyR) inhibitor and 2-APB (30 mM) as inositol triphosphate receptor (IP_3R) inhibitor. The inhibitors were added 30 minutes before ICRP (CC_{50}) treatment.

5.3.1.3.3 Cell Death Analysis

Cell death quantification was determined analyzing phosphatidylserine exposure using annexin V-allophycocyanin (APC) (AnnV, 0.25 μg/mL; BD Biosciences Pharmingen, San Jose, CA, USA) and cell membrane permeability with propidium iodide (PI; 0.5 μg/mL; MilliporeSigma, Eugene, OR, USA) stain. In brief, 5×10^4 cells were seeded and exposed to different concentrations of ICRP in subsequent assays; this allowed to define the median cytotoxic concentration of ICRP required to induce 50% of cell death (CC_{50}). After 24h of treatment, cells were recollected and washed with phosphate-buffered saline (PBS), then resuspended in binding buffer (10 mM HEPES/NaOH pH

7.4, 140 mM NaCl, 2.5 mM CaCl2), and stained during 20 minutes at 4°C. Finally, analysis was assessed by BD Accury C6 flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and analyzed in FlowJo Software (LLC, Ashland, OR).

5.3.1.3.4 Autophagosome formation assay

Autophagosome formation was assessed using Autophagy Detection Kit (Cyto-ID; Abcam, Cambridge, UK). In brief, 5×10^4 breast cancer cells were cultured in 24-well plates (Life Sciences) and 1×10^5 leukemia cells were cultured in 96-well plates (Life Sciences) then cells were treated with ICRP (CC_{50}) for 24h.Thus, cells were detached, washed with PBS, recovered and stained following the manufacturer's instructions. Measurement was determined by flow cytometry and analyzed in a Flowjo Software as mentioned previously.

5.3.1.3.5 EIF2α phosphorylation assay

For this assay, 5×10^4 cells of breast cancer cell lines and 1×10^6 cells of T-acute lymphoblastic cell lines were plated in 6-well dishes (Life Sciences) in complete DMEM or RPMI medium respectively and incubated with ICRP (CC_{50}) for 18 h. Cells were then collected and fixed with methanol for 1h at 4 °C. Cells were then washed with 2%-FACS Buffer (PBS 1× and 2% FBS) and centrifuged twice at 1,800 rpm during 20 min. Next, cells were suspended in 50 µL of 10%-FACS Buffer (PBS 1× and 10% FBS), incubated for 30 min, and shaken at 400 rpm and 25 °C. After this, 0.5 µL of anti-EIF2S1 (phospho S51) antibody [E90] (Abcam, ab32157) was added, incubated for 2 h, and washed with 2%-FACS Buffer. Cells were suspended in 100 µL of 10%-FACS Buffer), incubated for 15 min, and shaken at 400 rpm and 25 °C, 0.5 µL of goat anti-rabbit IgG H&L (Alexa Fluor® 488) (Abcam, ab150077) was then added and incubated for 1 h in darkness. Cells were washed with 2%-FACS Buffer and eIF2α phosphorylation was measured by flow cytometry, as mention before.

5.3.1.3.6 Intracellular Ca^{2+} levels analysis

Intracellular calcium analysis was determine using Fluo-4 AM (Life Technologies). In this assay for breast cancer cell lines 5×10^4 cells were plated in 6-well dishes (Life

Sciences) in complete DMEM and incubated with ICRP CC_{50} for 18 h. After treatment, cells were washed twice with KREBS buffer, suspended in RINGER buffer with 0.001 µg/mL of Fluo-4 AM (Life Technologies) and 0.001 µg/mL of Pluronic F-127 (Life Technologies), and incubated at 37 °C for 30 min in darkness. Next, cells were washed twice with RINGER buffer assessed by confocal microscopy (OLYMPUS X70). For T -acute lymphoblastic cell lines 1×10^5 were plated in 96-well dishes (Life Sciences) in complete RPMI and incubated with ICRP (CC_{50}) for 18 h. Then, cells were recollected, washed and stained as previously described. Cells were placed on microscopy slide with coverslips and assessed. Finally, cells observed using a fluorescence microscope (OLYMPUS IX70) and analyzed with Image-J software.

Intracellular calcium quantification was determined by flow cytometry assays, 5×10^4 breast cancer cells/well in 24-well dishes (Life Sciences) and 1×10^5 T-acute lymphoblastic cells/well in 96-cell dishes (Life Sciences) were incubated with ICRP (CC_{50}) in complete DMEM or RPMI, respectively, for 18h. After treatment cells were recollected, washed and stained as previously described. Finally, analysis was determined by BD Accury C6 flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Results were analyzed using FlowJo Software (LLC, Ashland, OR).

5.3.1.3.7 Mitochondrial membrane potential assessment

To determine mitochondrial damage, we tested loss of mitochondrial membrane potential by tetramethylrhodamine ethyl ester stain (TMRE, 50 nM; Sigma, Aldrich, Darmstadt, Germany). In brief, 5×10^4 cells were incubated with ICRP (CC_{50}) for 24 h in presence or absence of 1.5 mM BAPTA (MERCK). Cells were then harvested, washed with PBS, stained, incubated at 37°C for 30 min, and measured by flow cytometry as described above.

5.3.1.3.8 ROS generation analysis

ROS levels were determined by staining cells with 2′,7′-Dichlorofluorescin diacetate (DCFDA; 2.5 µM; MERCK). In brief, 5×10^4 cells/well were incubated with ICRP (CC_{50}) during 24 h in presence or absence of BAPTA (1.5 mM; MERCK). Cells were

then detached, washed with PBS, stained, incubated at 37 °C for 30 min, and measured using a flow cytometer, as mention above.

5.3.1.3.9 Cleaved Caspase-3 analysis

We used a specific detection kit, FITC-DEV-FMK (ABCAM; Cambridge, UK) to assess caspase-3 activation. In brief, 1×10^5 cells/well were incubated with ICRP (CC_{50}) for 24 h, finally were recovered and stained following the manufacturer's instructions.

5.3.1.3.10 Statistical Analysis

Data were analyzed using GraphPad Prism Software (GraphPad Software Inc., San Diego, CA) and showed as mean ± SD of triplicates from three independent experiments. Statistical analyses were done using the paired Student's t-test. The statistical significance was defined as $p < 0.05$.

5.3.1.4 RESULTS

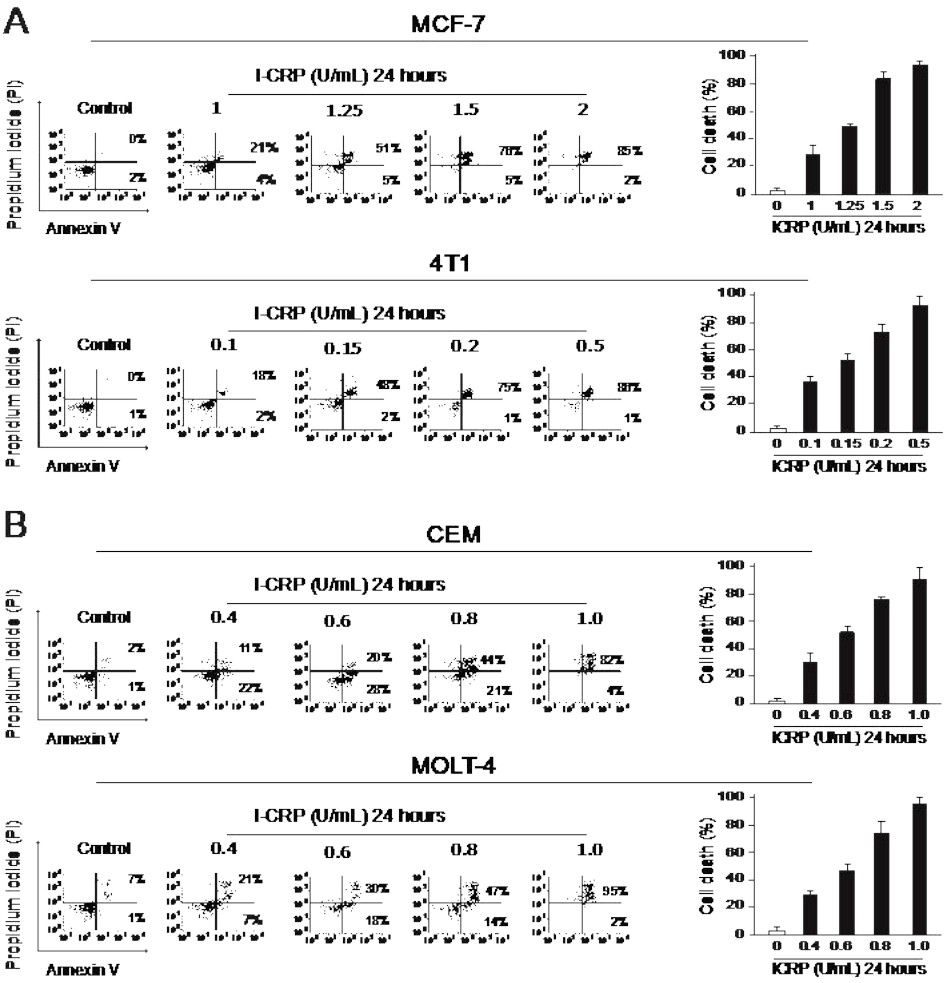
5.3.1.4.1 IMMUNEPOTENT-CRP triggers different ROS-depended cell death modalities depending on cancer cell lineage

ICRP is cytotoxic in a concentration-dependent manner on breast cancer (MCF-7 and 4T1) and T-ALL (CEM and MOLT-4) cell lines through regulated cell death (provoking phosphatidyl serine exposure and cell membrane permeabilization) (Figure 26A-B). Cell death in 30% of cells (CC_{30}) was reached at 1 U/mL, 50% (CC_{50}) at 1.25U/mL, 80% (CC_{80}) at 1.5U/mL, and 100% (CC_{100}) at 2U/mL in MCF-7 (Figure 26A above). In 4T1 30% of cell death (CC_{30}) was reached at 0.1 U/mL, 50% (CC_{50}) at 0.15U/mL, 80% (CC_{80}) at 0.2U/mL, and 100% (CC_{100}) at 0.5U/mL (Figure 26A down). In T-ALL cell lines CEM and MOLT-4 30% of cell death (CC_{30}) was reached at 0.4 U/mL, 50% (CC_{50}) at 0.6U/mL, 80% (CC_{80}) at 0.8U/mL, and 100% (CC_{100}) 1U/mL (Figure 26B).

To further visualize the differences on cell death induced by ICRP on breast and leukemic cell lines. We next evaluated if the ICRP-induced cell death was caspase-

independent on breast cancer cell lines and caspase-dependent on leukemic cell lines, as previously reported (Lorenzo-Anota et al., 2020b; Martínez-Torres et al., 2020; Reyes-Ruiz et al., 2021b). As shown in figure 26C, the percentage of ICRP-induced cell death in MCF-7 cells did not change in presence of QVD (pan caspase inhibitor) 49±4.6% to 49.48±8.8% ($p>0.05$). Interestingly in 4T1 cells QVD significantly potentiated the ICRP-induced cell death from 49.6±5.9% to 59.5±7.1% ($p=0.005$). On T-ALL cell lines ICRP-induced cell death diminishes in presence of QVD, in CEM from 47±5.1% to 18.71±8.8% ($p=0.001$) and in MOLT-4 since 49.5±4.9% to 16.8±4.3% ($p=0.001$). Indicating, that ICRP induced a caspase-independent cell death on breast cancer cell lines, however, provoked apoptosis on leukemic cells.

It is known that ICRP-induced cell death relies on ROS production in solid and leukemic cancer cell lines (Lorenzo-Anota et al., 2020b; Martinez-Torres et al., 2019; Martínez-Torres et al., 2018; Reyes-Ruiz et al., 2021b). The ROS implication on ICRP-induced cell death was confirmed here on breast and leukemic cell lines. In figure 26D we showed that cell death triggered by ICRP diminished in from 49±4.6% to 18.9±9% in MCF-7 ($p=0.0006$), since 49.6±5.9% to 15.65±11.7% in 4T-1 ($p=0.0104$), since 47±5.1% to 21.1±6.3% in CEM ($p=0.001$) and from 49.5±4.9% to 23.4±5.6% in MOLT-4 ($p=0.001$) in presence of the ROS scavenger N-Acetylcysteine (NAC). Importantly, NAC prevents ROS production in MCF-7, 4T1, CEM and MOLT-4 cell lines (Supplementary Fig. 1). These data demonstrated that ICRP induces different ROS-dependent cell death modalities on breast cancer and T-ALL cell lines.


A
MCF-7
I-CRP (U/mL) 24 hours
Control
1
1.25
1.5
2
Propidium Iodide (PI)
Annexin V
Cell death (%)
I-CRP (U/mL) 24 hours
4T1
I-CRP (U/mL) 24 hours
Control
0.1
0.15
0.2
0.5
Propidium Iodide (PI)
Annexin V
Cell death (%)
I-CRP (U/mL) 24 hours
B
CEM
I-CRP (U/mL) 24 hours
Control
0.4
0.6
0.8
1.0
Propidium Iodide (PI)
Annexin V
Cell death (%)
I-CRP (U/mL) 24 hours
MOLT-4
I-CRP (U/mL) 24 hours
Control
0.4
0.6
0.8
1.0
Propidium Iodide (PI)
Annexin V
Cell death (%)
I-CRP (U/mL) 24 hours

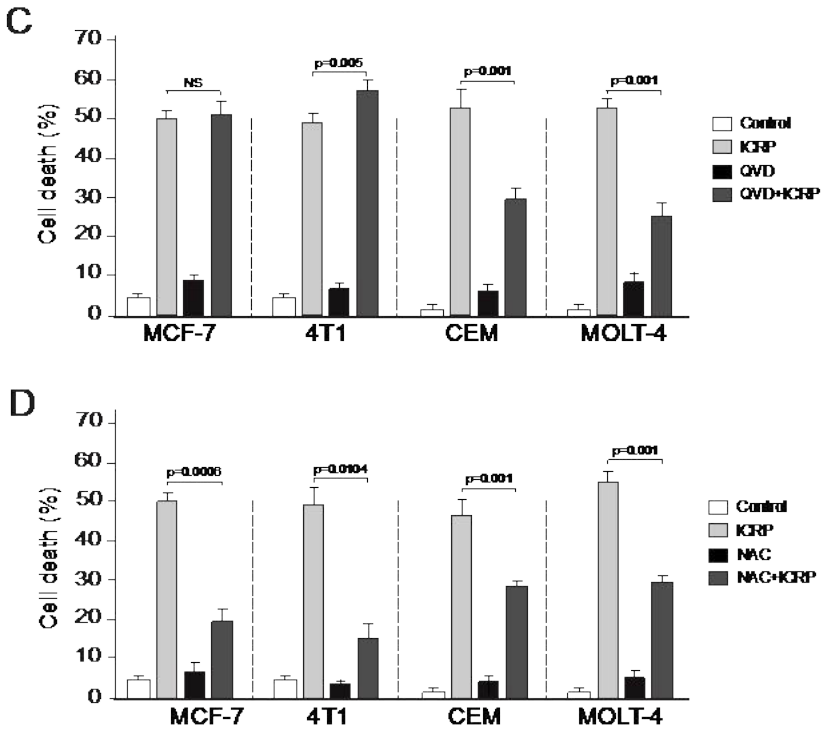

C
Cell death (%)
NS
p=0.005
p=0.001
p=0.001
Control
ICRP
QVD
QVD+ICRP
MCF-7
4T1
CEM
MOLT-4
D
Cell death (%)
p=0.0006
p=0.0104
p=0.001
p=0.001
Control
ICRP
NAC
NAC+ICRP
MCF-7
4T1
CEM
MOLT-4

Figure 26. IMMUNEPOTENT CRP induces different regulated cell death modalities concentration and ROS- dependent manner on breast cancer and in T-ALL cell lines. A-B. Representative dot plots and quantification of cell death measured by flow cytometry using Annexin-V and PI staining in breast cancer cell lines MCF-7 (above) and 4T1 (down) **(A)** and in T-acute lymphoblastic leukemia cells lines CEM (above) and MOLT-4 (down) **(B)** treated with different concentrations of ICRP for 24h. **C.** Cell death quantification of MCF-7, 4T1, CEM and MOLT-4 cell lines left alone or pretreated with a pan-caspase inhibitor QVD before ICRP CC_{50} treatment (24 h). **D.** Cell death quantification in MCF-7, 4T1, CEM and MOLT-4 cell lines left alone or pretreated with the antioxidant NAC before ICRP CC_{50} treatment (24 h). The means (± SD) of triplicates of at least of at three independent experiments were graphed.

5.3.1.4.2 IMMUNEPOTENT-CRP induces ROS-dependent autophagy on breast cancer and T-ALL cells

It has been reported that oxidative stress or ROS production augmentation could induce autophagy that plays an important role on survival (Inguscio, Panzarini, & Dini, 2012). As previously, ICRP demonstrated increase autophagosome formation ROS-dependent in breast cancer cells (Martínez-Torres et al., 2020; Reyes-Ruiz et al., 2021b), we tested if ICRP also induces autophagy on leukemic cell lines as in breast cancer cells. In figure 27A we showed representative histograms (left) and quantification (right) of autophagosome formation. As shown ICRP increased autophagosome formation in breast cancer cell lines since 7.5±2.6% to 45.8±4.1% in MCF-7 ($p<0.0001$), since 10.5±11.7% to 49.6±5.9% in 4T1($p=0351$) and in leukemic cells since 3.5±11.7% to 43.5±2% in CEM ($p<0.0001$), and in MOLT-4 since 7.6±4.0% to 33±9.1% ($p<0.0001$). Interestingly, NAC was able to completely inhibit autophagosome formation in breast and leukemic cell lines (Supplementary Fig.2). Indicating, that the exacerbate ROS production provoked-ICRP increase autophagosome formation in leukemic and breast cancer cell lines.

During specific circumstances autophagy could play two principals roles on cell survival strategy “self-eating mechanism” or cell death “self-killing” (Inguscio et al., 2012). For this, the next step was evaluated if autophagosome formation produced by an exacerbated ROS production could play a fundamental role on survival or death. For

this, we tested cell death in presence of Sp-1, an autophagic inhibitor. In figure 27B we observe the representative dot plots obtained from cell death analyses and in figure 27C showed graphs, the cell death significatively augmented when autophagy was inhibited (in presence of Sp-1) in MCF-7 49.6±4.6% to 59.3±3.8% (p=0.0012), 4T1 49.7±5.9% to 60.5±7.9% (p=0.0032), CEM 48.1±4.1% to 61.8±6.8% (p=0.0019), and MOLT-4 33±9.1% to 69±6.0% (p=0.0002). Importantly, Sp-1 avoids autophagosome formation induced by ICRP in breast cancer and leukemic cell lines (Supplementary Fig. 3). Taken together, the augmentation of ROS production induced by ICRP treatment provokes ROS-dependent autophagy in breast cancer and leukemic cell lines.

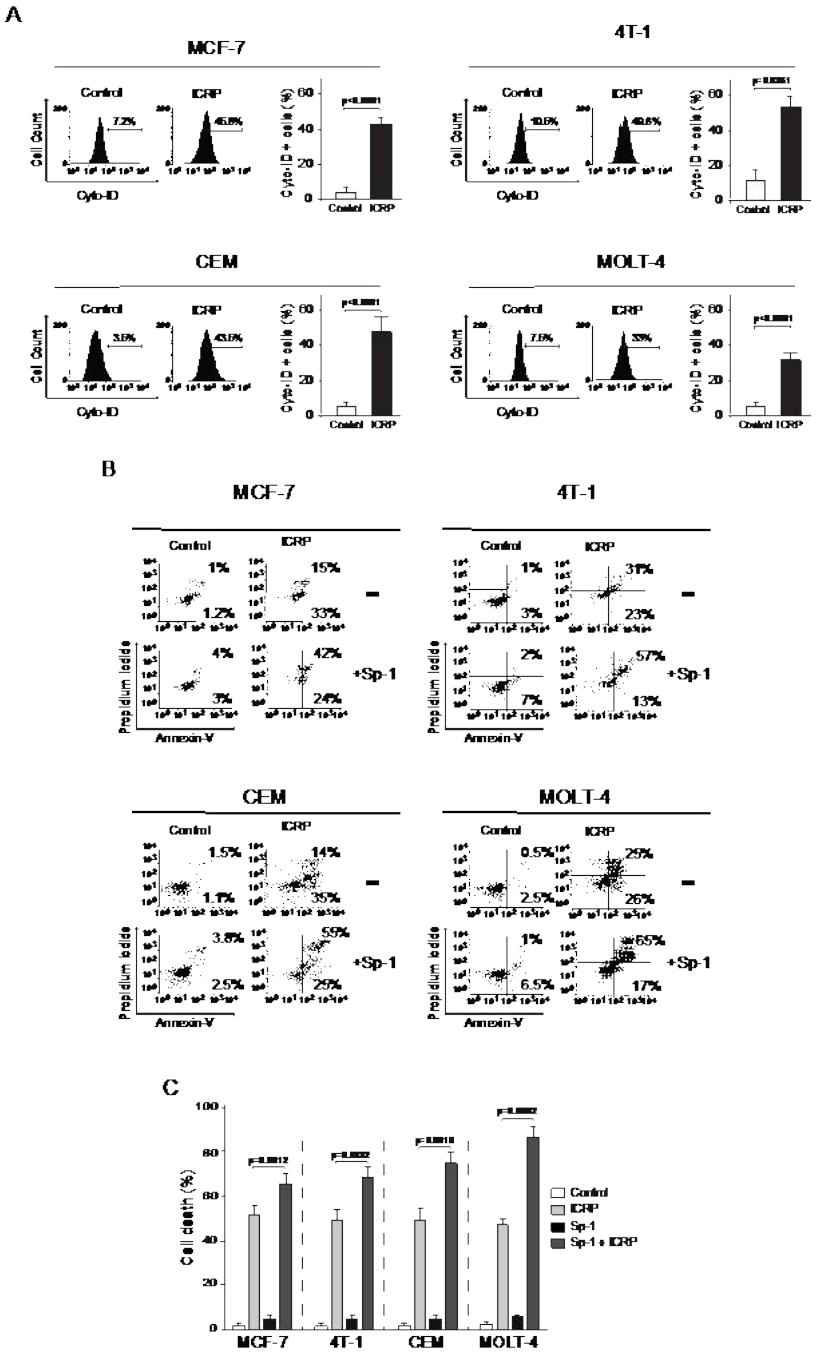


Figure 27. IMMUNEPOTENT-CRP induces prosurvival autophagosome formation on breast cancer and in T-ALL cell lines. A. Representative histograms (left) and quantification (right) of autophagosomes formation measured by flow cytometry using Cyto-ID staining in breast cancer MCF-7 and 4T1 cells, and leukemic CEM and MOLT-4 cell lines treated with ICRP CC_{50} (24 h). **B.** Representative dot plots (left) of cell death analysis in MCF-7, 4T1, CEM and MOLT-4 cell lines left

alone or pretreated with the autophagy inhibitor Sp-1 before ICRP treatment CC_{50} (24 h) and quantification **(C).** The means (± SD) of triplicates of at least of at three independent experiments were graphed.

5.3.1.4.3 IMMUNEPOTENT-CRP induces ER stress and increases cytoplasmic Ca^{2+} levels in breast cancer and T-ALL cells

ROS generation is strongly associated with endoplasmic reticulum alterations which leads to a stress condition in this organelle (Zeeshan, Lee, Kim, & Chae, 2016) promoting to eIF2α-phosphorylation (P-eIF2α) (Rashid, Yadav, Kim, & Chae, 2015). In this context, autophagy could play a crucial role during ER stress as a protective mechanism (Deegan, Saveljeva, Gorman, & Samali, 2013). ER stress and autophagy are two cross-talking processes (Orrenius, Zhivotovsky, & Nicotera, 2003). As previously shown, ICRP induces ROS augmentation promoting autophagosome formation. Hence, we evaluated eIF2α phosphorylation (P-eIF2α) in breast cancer and leukemic cell lines by flow cytometry. As observed in figure 28A, ICRP causes P-eIF2α in MCF-7 (58±8.7%), 4T1 (31±4.2%), CEM (35±7.9%) and MOLT-4 (40±6.5%) cell lines. Revealing, that ICRP induced P-eIF2α, one of the principal biomarkers of ER stress on breast and leukemic cell lines.

In earlier stages of ER stress conditions, intracellular alterations forces calcium (Ca^{2+}) out of the ER increasing the cytosolic concentration (Zeeshan et al., 2016). The presence of autophagy and P-eIF2a suggested alterations in the ER, therefore we evaluated the increase in cytosolic calcium. In MCF-7 and 4T1 Ca^{2+} levels were assessed by confocal microscopy. Results indicated that 18 h-treatment with ICRP induced an augmentation of Ca^{2+} levels in the cytoplasm in comparison with untreated cells (control) (Figure 28B). In CEM and MOLT-4, Ca^{2+} levels were assessed by fluorescence microscopy. We observed that ICRP treatment improves Ca^{2+} rise in cytoplasm in comparison with untreated cells (Figure 28C). The increment in cytosolic level of Ca^{2+} was confirmed by flow cytometry in figure 28D we showed representative histogram (left) and quantification (right). We observed that ICRP increase cytosolic level of Ca^{2+} in breast cancer cells MCF-7 10.7±3.4% to 51.2±4.9% ($p<0.0001$) and 4T1 17.5±3.4% to 42.8±6.0% ($p<0.0001$) and in T-ALL cell lines CEM 9.8±2.2% to

54.2±5.4% ($p<0.0001$) and MOLT-4 5.6±3.1% to 44.7±4.4% ($p<0.0001$). Additionally, in all cancer cell lines (Supplementary Fig. 4) the extracellular Ca^{2+} chelator (BAPTA) significantly inhibited the augmentation of cytoplasmic Ca^{2+} levels. Revealing, that ICRP induces calcium alterations on cancer cell lines.

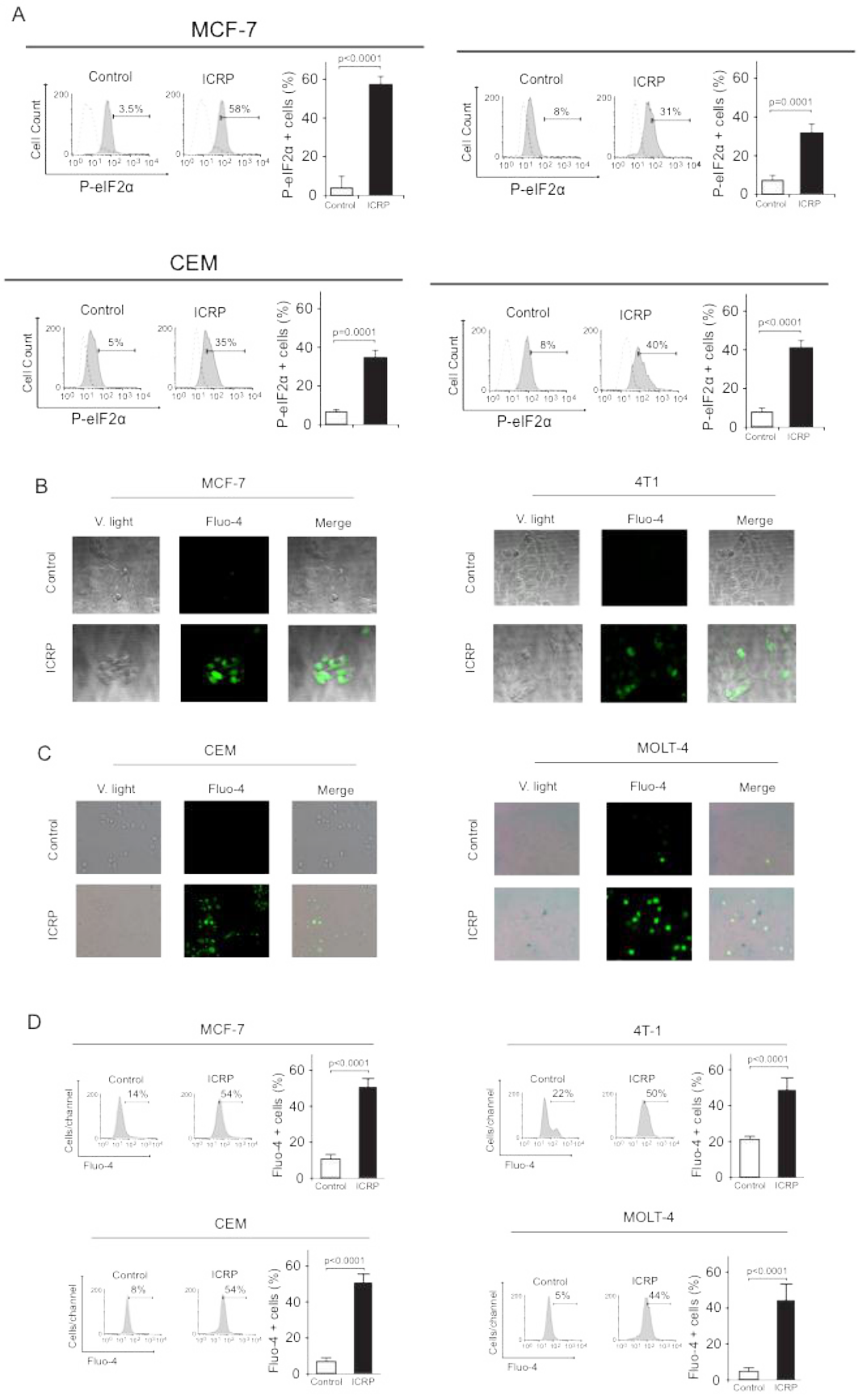

A
MCF-7
Control
ICRP
3.5%
58%
8%
31%
Cell Count
P-eIF2α
P-eIF2α + cells (%)
p<0.0001
p=0.0001
CEM
5%
35%
8%
40%
B
MCF-7
4T1
V. light
Fluo-4
Merge
Control
ICRP
C
CEM
MOLT-4
D
MCF-7
4T-1
CEM
MOLT-4
14%
54%
22%
50%
8%
54%
5%
44%
Cells/channel
Fluo-4
Fluo-4 + cells (%)

Figure 28. IMMUNEPOTENT-CRP induces eIF2α phosphorylation and increase in the cytoplasmic Ca^{2+} levels. A. Representative histograms and quantification of eIF2α phosphorylation measured by flow cytometry using anti-EIF2S1 monoclonal antibody in breast cancer cell lines MCF-7 and 4T1 (left), and leukemic cells lines CEM and MOLT-4 (right). **B.** Confocal microscopy representation of Ca^{2+} cytoplasmic levels measured by Fluo-4AM staining in breast cancer cell lines MCF-7 (left) and 4T1 (right) in absence (control) or presence of ICRP CC_{50} for 18h and visualized using fluorescence microscopy (OLYMPUS X70) (40×). **C.** Fluorescence microscopy representation of Ca^{2+} cytoplasmic levels measured trough Fluo-4AM staining in leukemic cell lines CEM (left) and MOLT-4 (right) in absence (control) or presence of ICRP CC_{50} for 18h and visualized using fluorescence microscopy (OLYMPUS IX70) (40×). **D-E.** Representatives histograms and quantification of Ca^{2+} cytoplasmic levels assessed trough Fluo-4AM staining by flow cytometry in breast cancer cell lines MCF-7 and 4T1 (above) **(D)**, and in T-ALL cell lines CEM and MOLT-4 (down) **(E)** treated with ICRP CC_{50} for 18h. Graphs represent the means (± SD) of triplicates of at least of at three independent experiments were graphed.

5.3.1.4.4 Calcium alterations induced by IMMUNEPOTENT-CRP promotes mitochondrial damage, ROS production and calcium-dependent cell death on breast cancer and in T-ALL cells.

For decades, it has been described that Ca^{2+} plays an important role in cell death regulation. Cytoplasmic Ca^{2+} overload is associated with different cell death modalities. (S, V, & B, 2015; Zhivotovsky & Orrenius, 2011). As an increase of cytosolic Ca^{2+} levels were observed in all cancer cells after ICRP-treatment, the implication of Ca^{2+} augmentation on cell death was analyzed by assessing mitochondrial damage, ROS production, caspase-3 cleaved and cell death in presence or absence of BAPTA. First, cell death was evaluated in presence or absence of BAPTA. As observed in figure 29A, ICRP induced cell death in up to 50% of cells after 24-h of treatment, and the ICRP-cytotoxicity was significantly inhibited in presence of BAPTA from 47.3±4.8% to 20.1±3.9% in MCF-7 (p=0029), since 49.8±5.2% to 14.2±8.7% in 4T-1 (p=0.0106), from 45.5±9.0% to 20.9±7.0% in CEM (p=0.0003) and in MOLT-4 from 48.9±9.3% to 23±3.4% (p=0.0005). These results indicate that ICRP induces calcium-dependent cell death on breast and leukemic cell lines.

As previously shown, ICRP treatment induces caspase-independent cell death in breast cancer cells (Martínez-Torres et al., 2020; Reyes-Ruiz et al., 2021b) however, triggers apoptosis involving caspase-3 cleavage (a principal effector caspase) on leukemic cell lines (Lorenzo-Anota et al., 2020b). Thus, we tested caspase-3 cleaved on CEM and MOLT-4 cell lines treated with ICRP. In figure 29B we showed representative histograms (left) and quantification of caspase-3 cleaved. As shown the pre-treatment with BAPTA prevented caspase-3 cleavage on CEM (39±12.1% to 18.37±5.4%) ($p=0.0031$) and MOLT-4 (57±3.8% to 23±3.6%) ($p=0.0003$) cell lines, suggesting that calcium alteration induced by ICRP promotes caspase-3 activation that culminates in apoptosis pathway in leukemic cells.

The cytoplasmic Ca^{2+} overload has been associated with mitochondrial alterations in different cell death pathways (S et al., 2015; Zhivotovsky & Orrenius, 2011). Thus, we investigate if ICRP-induced calcium alterations promote mitochondrial damage. For this, loss of mitochondrial membrane potential and ROS production was analyzed. ICRP treatment induced loss of mitochondrial membrane potential in all cell lines (Figure 29C) and was inhibited in presence of calcium chelator BAPTA since 59±5.4% to 33±3.4% ($p=0.0037$) in MCF-7, from 60.5±4.8% to 18.4±2.4% ($p<0.0001$), from 41.1±10.8% to 22±7.2% ($p=0.0094$), since 56.2±2.6% to 21.6±6.2% ($p=0.0231$). Also, the ROS production induced by ICRP treatment on MCF-7 (49.7±9.7%), 4T1 (47.7±8.3%), CEM (38.6±7.3%) and MOLT-4 (41.6±3.5%) diminishing in presence of BAPTA being 19.4±6.9%, 10.1±4.7%, 26.2±2.7%, 13.6±7.1%, respectively (Figure 29D). Thus, these data demonstrated that ICRP generated a Ca^{2+}-dependent cell death being the first step of the cell death mechanism induced in breast and leukemic cell lines. Moreover, this characteristic remains independently of the cell death pathway triggered.

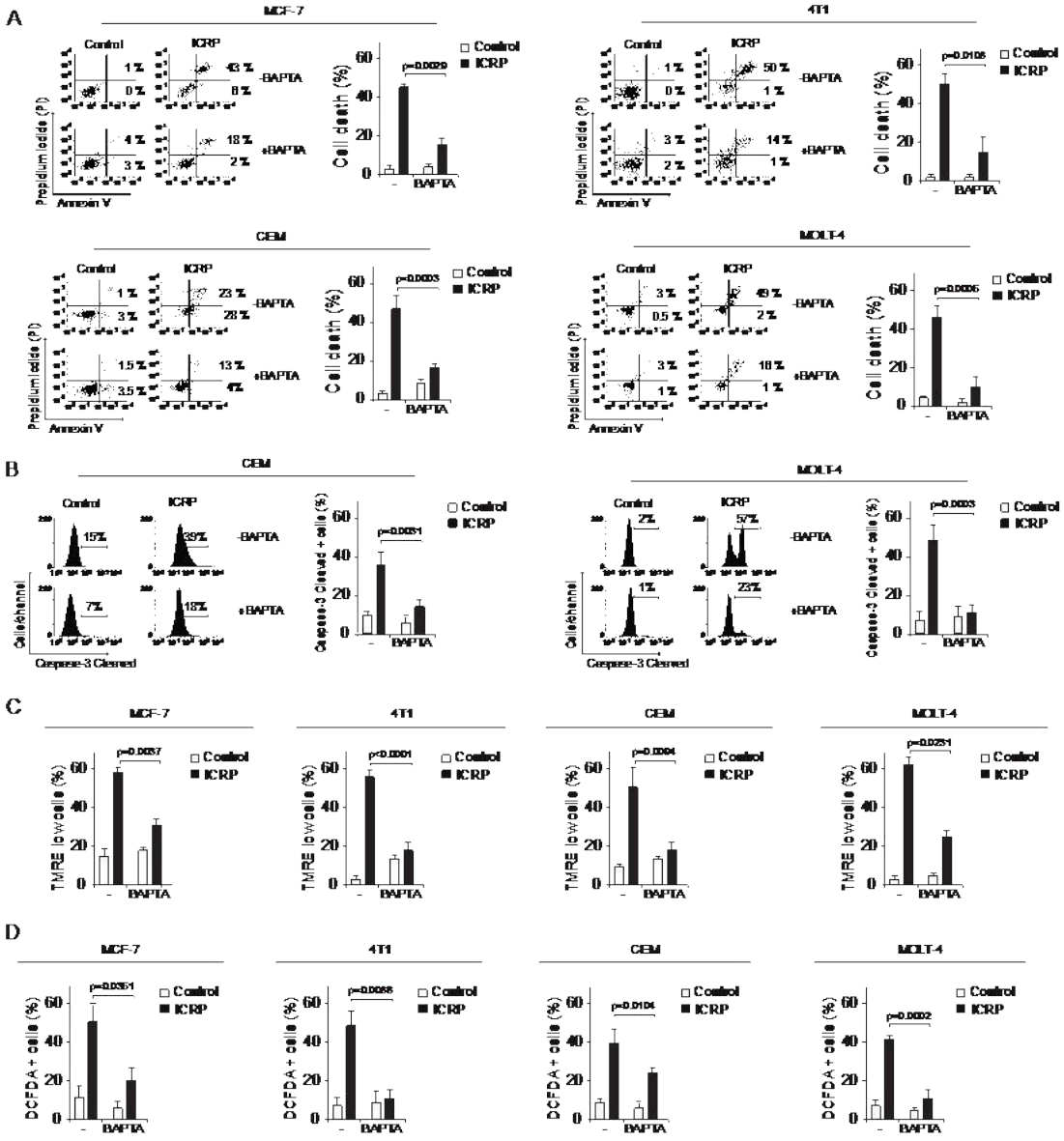


Figure 29. IMMUNEPOTENT-CRP induces Ca^{2+}-dependent cell death in tumoral and leukemic cell lines. A. Representative dot plots and quantification of cell death measured by flow cytometry trough Annexin-V and PI staining in breast cancer cell lines MCF-7 and 4T1 (above), and in T-ALL cell lines CEM and MOLT-4 (down) treated with ICRP CC_{50} for 24h in presence or absence of BAPTA. **B.** Representative histograms and quantification of caspase-3 cleaved by flow cytometry using FITC-DEVD-FMK staining in T-ALL cell lines CEM (left) and MOLT-4 (right) treated with ICRP CC_{50} for 24h in presence or absence of BAPTA. **C-D.** Quantification of loss of mitochondrial membrane potential **(C)** and ROS production **(D)** evaluated trough TMRE and DCFDA staining, respectively, by flow cytometry in breast cancer cell lines MCF-7 and 4T1, and in T-ALL cell lines CEM and MOLT-4 treated with ICRP CC_{50}

for 24h in presence or absence of BAPTA. Graphs represent the means (± SD) of triplicates of at least of at three independent experiments were graphed.

5.3.1.4.5 2-APB and dantrolene inhibit ROS production and cell death induced by IMMUNEPOTENT-CRP

Inositol 1,4,5-trisphosphate receptor (IP3R) and ryanodine receptor (RyR) are the principal mediators of Ca2+ release from intracellular stores (L et al. 2001; Hanson, Bootman, and Roderick 2004; Splettstoesser, Florea, and Büsselberg 2007). We blocked the IP3R and RyR to evaluate their implication on ICRP-induced cell death. Our results showed that 2-APB diminish cell death on MCF-7 since 47.3% to 25.3% ($p<0.0001$), in 4T-1 since 48.6% to 35.83% ($p<0.0001$), in CEM since 44.6% to 21.7% ($p=0.0135$), and MOLT-4 since 52% to 32% ($p=0.0009$). Similar inhibition was observed using dantrolene in MCF-7 cell death diminish since 47.3% to 27.4% ($p<0.0001$), in 4T-1 cells 48.6% decrease to 30.01% ($p<0.0001$), in CEM since 50.9% to 26.8% ($p=0.0337$), and on MOLT-4 since 52% to 30% ($p=0.0003$).

ROS generation is strongly associated with calcium leakage in ER (Zeeshan et al. 2016). Finally, we determined if the IP3R and RyR are involved on ROS production, the principal effectors on ICRP-cytotoxicity. In figure 30B we observed that ROS production induced by ICRP treatment on MCF-7, CEM and MOLT-4 diminished in presence of IP3R and RyR inhibitors. Moreover, we observed by fluorescent microscopy that in presence of 2-APB and dantrolene cytoplasmic calcium inhibition in MCF-7, 4T1, CEM and MOLT-4 cell lines (Figure 30C). Taken together, these results revealed that during RyR and IP3R prevent ER calcium release and to avoiding ROS production and cell death.

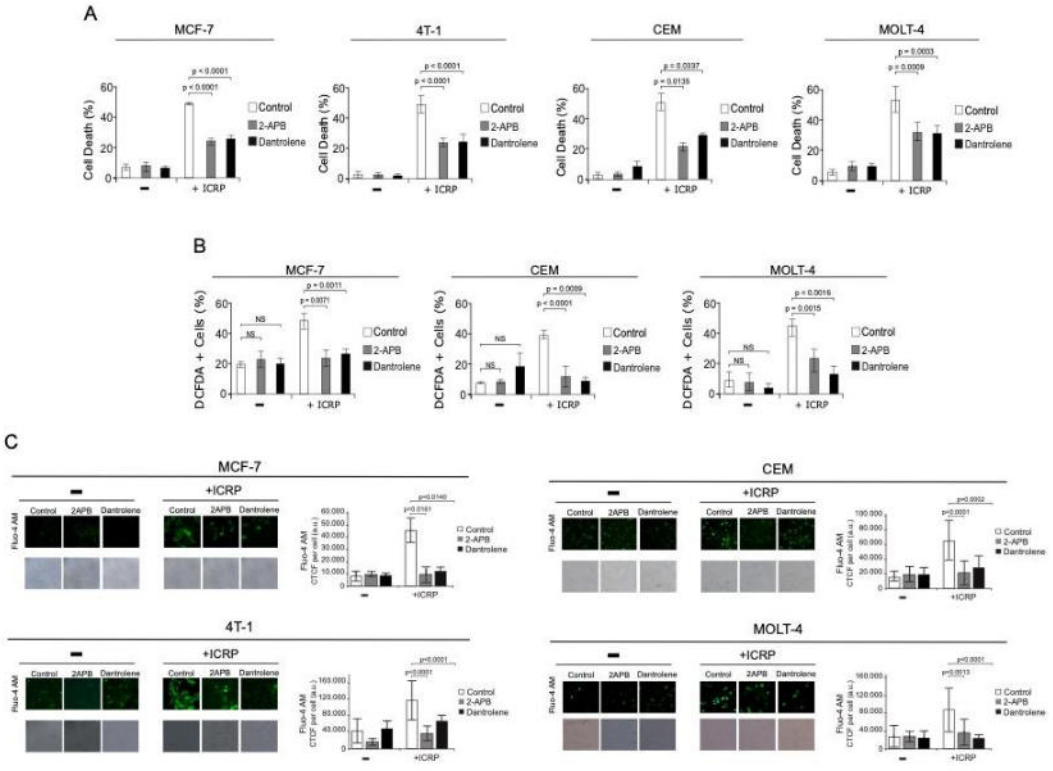


Figure 30. IMMUNEPOTENT-CRP induces ER-Ca^{2+} release throught IP3R and RyR channels leading to ROS production and cell death in tumoral and leukemic cell lines. A. Quantification of cell death measured by flow cytometry trough Annexin-V and PI staining in breast cancer cell lines MCF-7 and 4T1 (above), and T-ALL cell lines CEM and MOLT-4 (down) treated with ICRP CC50 for 24h in presence or absence of 2-APB and Dantrolene. **B.** Quantification of ROS production assessed by DCFDA staining by flow cytometry in breast cancer cell line MCF-7, and in T-ALL cell lines CEM and MOLT-4 treated with ICRP CC50 for 24h in presence or absence of 2-APB and Dantrolene. **C.** Fluorescence microscopy representation (left) and quantification (right) of Ca2+ cytoplasmic levels measured trough Fluo-4AM staining in breast cancer MCF-7 and 4T1 and leukemic cell lines CEM and MOLT-4 trated with ICRP CC50 for 18h and in presence or absence of 2-APB and Dantrolene, visualized using fluorescence microscopy (OLYMPUS IX70) (40×). Graphs represent the means (± SD) of triplicates of at least of at three independent experiments were graphed.

5.3.1.5 DISCUSSION

IMMUNEPOTENT CRP (ICRP) triggers different cell death modalities depending on cancer cell lineage, apoptosis on T-ALL cells and non-apoptotic cell death in breast cancer cells. However, caspase-independent and ROS-dependent regulated cell death in cervical, lung cancer and breast cell lines, involving loss of mitochondrial membrane potential (Dym), cell cycle arrest and DNA degradation (Martinez-Torres et al., 2019; Martínez-Torres et al., 2018; Reyes-Ruiz et al., 2021b). Moreover, in leukemic cell lines ICRP induces apoptosis through ROS production provoking DNA damage, p53 overexpression, cell cycle arrest, DNA degradation, mitochondrial damage including loss of Dym and Bax and Bcl protein modulation and finally caspase-3 activation (Lorenzo-Anota et al., 2020b). Other cell death inductors as gold nanoparticles or photodynamic treatments induce different cell death mechanisms in cancer cells (Martínez-Torres, Lorenzo-Anota, HelenYarimet, García-Juárez, Zarate-Triviño, & Rodríguez-Padilla, 2019; Soriano et al., 2017).

Mitochondria is one of the principal source of ROS due to the cellular metabolism (Yun et al., 2020). At low levels, ROS play a role as redox messengers in intracellular signaling, whereas excessive production of these reactive molecules causes oxidative damage culminating in cell death (Perillo et al., 2020). Autophagy is a major sensor of the redox signaling, playing a role on stress adaptation and cell death (Chang & Zou, 2020; Cordani, Donadelli, Strippoli, Bazhin, & Sánchez-Álvarez, 2019). In this work, it was proved that ICRP induces autophagosome formation, as an adaptive mechanism to avoid cell death. Moreover, this mechanism can be suppressed in presence of the ROS scavenger NAC. These results are consistent with previous observations where other agents such as Salinomycin induced prosurvival autophagy relying on ROS production in breast cancer cell lines MDA-MB-231 and MCF-7 (KY et al., 2017). Also, other drugs as oxaliplatin and Irinotecan promotes ROS augmentation that increases autophagy in gastric cancer cells (Shi et al., 2012) (Zhu et al., 2020).

On the other hand, exacerbated ROS production promotes ER alterations. Where, autophagy plays a crucial role during ER stress to abrogate cellular damage (Deegan et al., 2013; Rashid et al., 2015; Zeeshan et al., 2016). The main source of ROS

production in ER is NADPH oxidase 4 (Nox4) involved in folding proteins, however during intrinsic or extrinsic alteration could provokes UPR initiation (Cao & Kaufman, 2014). ER is under oxidizing conditions during UPR or is somehow opposed to the redox imbalance which argues against a major source of oxidants in ER stress (Zeeshan et al., 2016). Under stressed conditions, Nox4 expression is increased the generation of ROS is also increased also responsible for cause autophagy as a cellular protective mechanism or cell death. pro-apoptotic and could induces cell death (F, J, D, Y, & C, 2015). This could be explained that ROS production induced by ICRP lead to ER stress and autophagy on cancer cell lines. ER stress involves P-eIF2a leading to autophagy and calreticulin exposure, two processes linked with immunogenic cell death induction (Humeau, Bezu, Kepp, & Kroemer, 2020). In this context, conventional chemotherapeutics such as anthracyclines and oxaliplatin induce P-eIF2a, which is determinant for immunogenic cell death induction on U2OS and HCT 116 cancer cells (Bezu et al., 2018). Interestingly, ICRP induces DAMPS emission on B16F10, HeLa, MCF-7, MDA-MB-231, and 4T1 cancer cells, (Reyes-Ruiz et al., 2021b; Rodríguez-Salazar et al., 2017), particularity on murine breast cancer, ICRP treatment induced immunogenic cell death leading to long-term antitumour memory (Reyes-Ruiz et al., 2021b). Suggesting, that ROS production promotes ER stress and autophagy on cancer cell lines and could also be an immunogenic inductor on leukemic cell lines like breast cancer.

Here, it was shown that ICRP induces cytoplasmic Ca^{2+} augmentation which was prevented in presence of BAPTA. Interestingly, the condition of Ca^{2+} chelation in the extracellular space inhibited the loss of mitochondrial membrane potential, ROS production and cell death induced by ICRP. Hence, the increase of intracellular Ca^{2+} levels induced by ICRP is the first step on both cell death modalities described so far on breast and T-ALL cell lines. These results are similar to the cell death pathways described in other treatments. In pancreatic tumor cells, menadione induced ER-Ca^{2+} release, which was accompanied by mitochondrial Ca^{2+} elevation, mitochondrial depolarization, and mitochondrial permeability transition pore (mPTP) opening, leading to cell death (Baumgartner et al., 2009). Ceramide-induced cell death in HeLa cells involves ER-Ca^{2+} release and mitochondrial Ca^{2+} increase, accompanied by

marked alterations in mitochondria morphology (Pinton et al., 2001). Also, cisplatin increased cytoplasmic and mitochondrial Ca^{2+} levels in HeLa cells, which further triggered mitochondrial-mediated and ER stress-associated cell death pathways, moreover, the inhibition of IP_3R decreased calcium release from the ER and inhibited cisplatin-induced cell death (SHEN et al., 2016). In this context, an IP3 receptor antagonist, 2-APB attenuates cisplatin induced $Ca2^{+}$-influx in HeLa-S3 cells and prevents activation of calpain and induction of apoptosis (Splettstoesser, Florea, & Büsselberg, 2007). Dantrolene a selective inhibitor of isoforms RyR1 and RyR3 activation, prevented ER calcium augmentation(Zhao, Li, Chen, Louis, & Fruen, 2001). In this work demonstrated that during RyR and IP_3R receptors inhibition, the ROS production and cell death were inhibited on cancer cell lines using 2-APB and dantrolene inhibitors. It has been reported that during early stages of ER stress, oxidative stress forces calcium out of ER provoking mitochondria reuptake (Li et al., 2009). Consequently, this phenomenon induces calcium augmentation in mitochondria generating major metabolic activities and exacerbate ROS production. Via feedback mechanisms, calcium ions further enhance the sensitivity of calcium channels (Zeeshan et al., 2016; Zhao et al., 2001). The release/migration of calcium is highly associated with ER stress conditions. Under ER stress, calcium release via IP_3R has been suggested to be induced by GSH and xanthine/xanthine oxidase (Cioffi, 2011). ICRP may induce Ca^{2+} depletion in ER, triggering ER stress and autophagosome formation as a consequence (Kepp et al., 2015; Panaretakis et al., 2009). Moreover, Ca^{2+} released by ER could be transferred to mitochondrion through RyR and IP_3R, generating Ca^{2+} overload, resulting in loss of Dym and ROS production (Kerkhofs et al., 2018). Ca^{2+} depletion from ER is well known to activate SOCE allowing the maintenance of high concentrations of Ca^{2+} inside the cell, which can lead to alterations in plasma membrane, mitochondria, nucleus and ER (Orrenius et al., 2003).

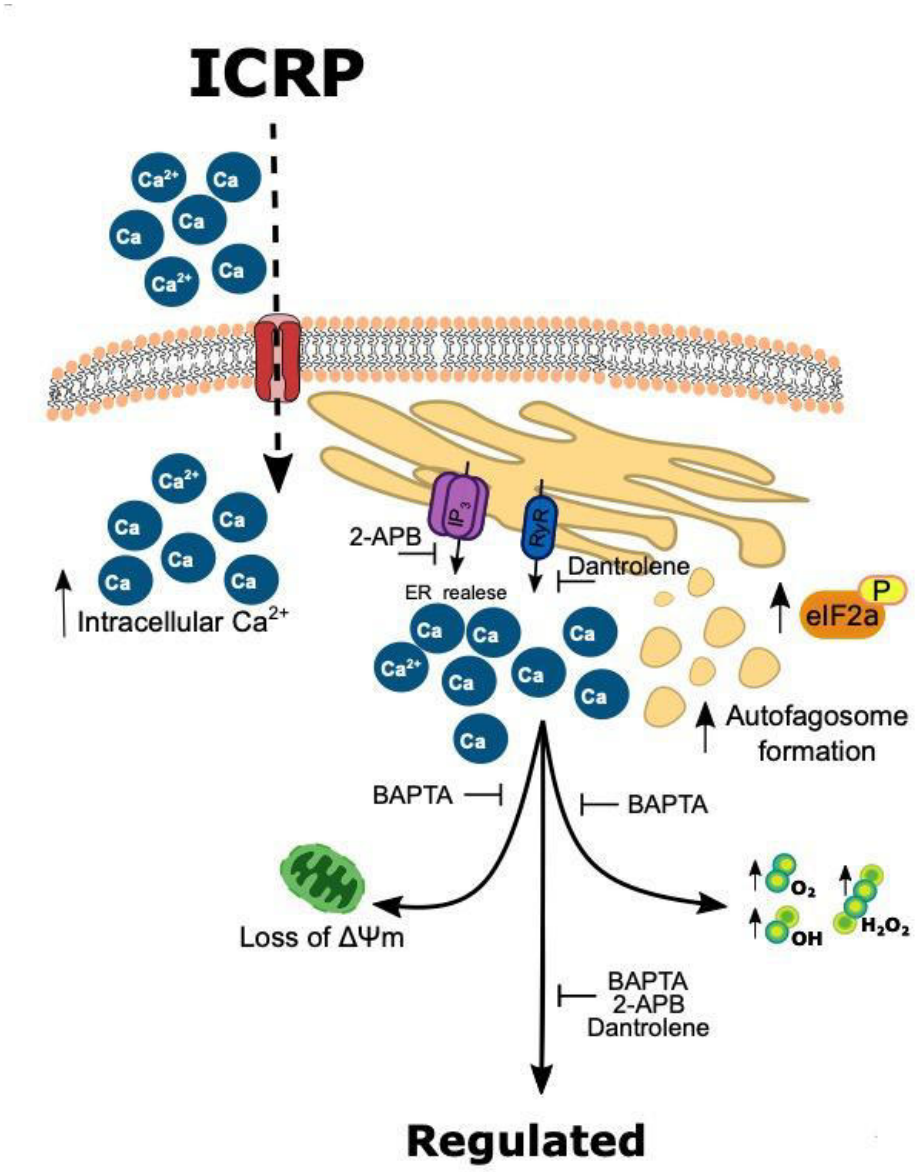


Figure 31. Schematic representation of cell death mechanism induced by IMMUNEPOTENT-CRP on breast cancer and leukemic cell lines. ICRP induces eIF2α-phosphorylation, autophagosome formation and increase intracellular Ca^{2+} levels on cancer cell lines. The intracellular Ca^{2+} augmentation provokes Ca^{2+} release from endoplasmic reticulum (ER) mediated by RyR and IP3R, inhibited using 2-APB and Dantrolene that prevents ICRP cell death and ROS production. Additionally, intracellular Ca^{2+} augmentation is the first step on cell death pathway: exacerbate ROS production and loss of mitochondrial membrane potential (loss of $\Delta\psi m$), provoking to regulated cell death on cancer cell lines.

5.3.1.6 CONCLUSION

Ours results revealed that on cancer cells IMMUNEPOTENT CRP triggers autophagosomes formation and endoplasmic reticulum stress accompanied by the increase of intracellular Ca^{2+} levels. Inositol 1,4,5-trisphosphate receptors (IP_3R) and ryanodine receptors (RyR) are involved (figure 31) on ER alterations and ROS production. Also, Ca^{2+} entry to cancer cells leads to mitochondrial damage and ROS production; and caspase-3 cleaved (on leukemic cell lines) provoking regulated cell death on cancer cell lines. Taken together our results, revealed that Ca^{2+} alterations are the first step on the ICRP-cytotoxicity. This work opens new possibilities to evaluate the cytotoxicity of ICRP on immunogenicity of hematological malignances and others solid cancer.

5.3.1.7 CONSENT TO PUBLISH

Not applicable.

5.3.1.8 DATA AVAILABILITY

All datasets generated during the current study are available from the corresponding author on reasonable request.

5.3.1.9 COMPETING INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5.3.1.10 FUNDING INFORMATION

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5.3.1.11 AUTHOR CONTRIBUTIONS

HYLA, ARR, KMCR, RMR, APUP and KMAV designed and performed all experiments. ACMT directed, conceived, and supervised the project. HYLA, ARR and

KMCR wrote the paper. HYLA, ARR and APUP prepared the figures. HYLA, ARR, KMCR, RMR, APUP and CRP designed experiments, performed statistical analysis, analyzed, and interpreted data and read and approved the final paper.

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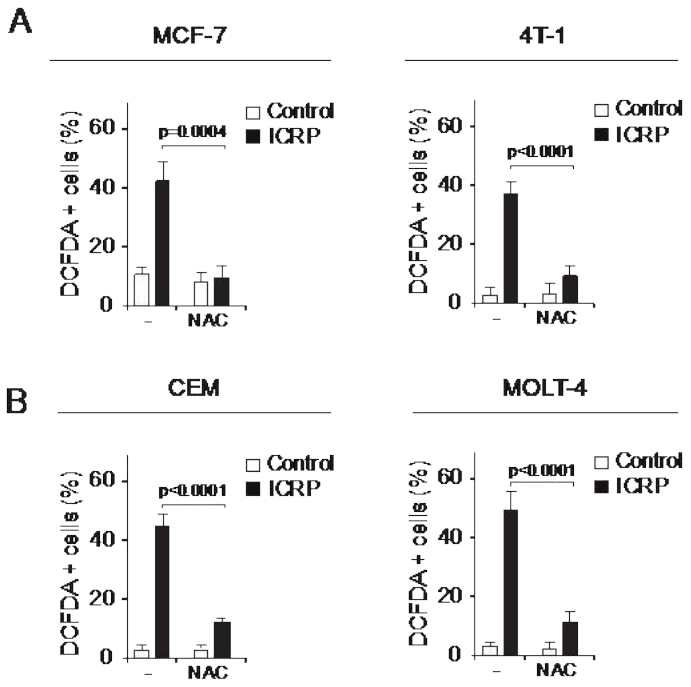
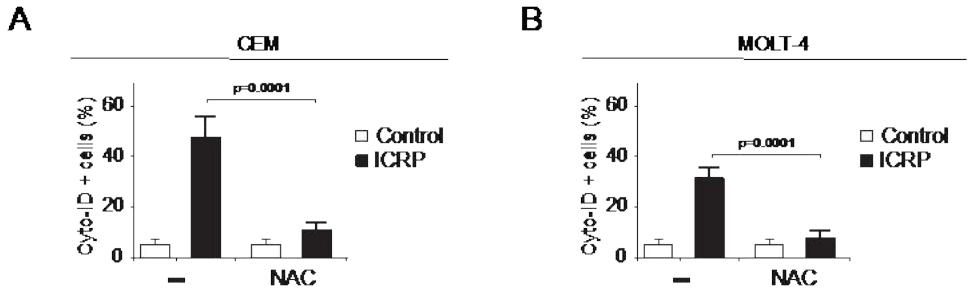
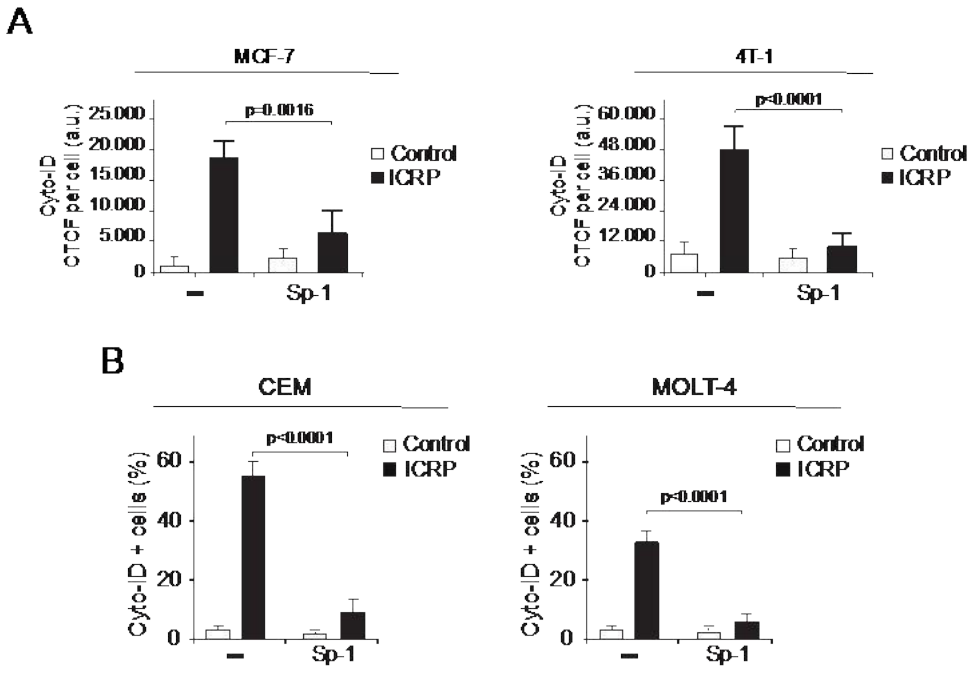
5.3.1.14 SUPPLEMENTARY MATHERIAL



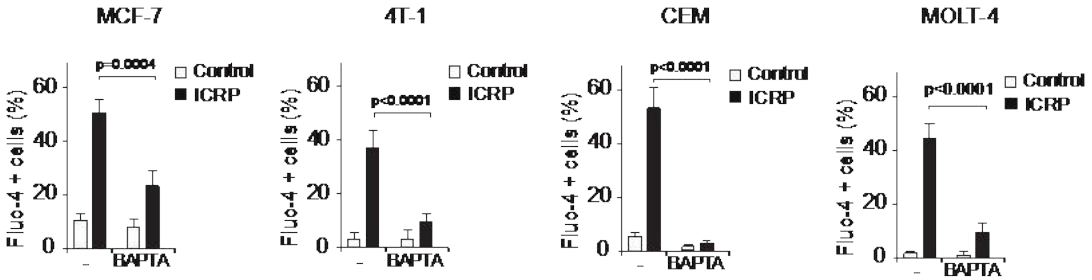
Supplementary 1. NAC inhibit ROS production in Breast and T-ALL cell lines. A) Graph represents the mean of the ROS production quantification by Flow cytometry using DCFDA staining in MCF-7 and 4T-1 analysis, B) CEM and MOLT-4. The results are presented as mean ± standard deviation of three different experiments.



Supplementary 2. NAC inhibit autophagosome formation in CEM and MOLT-4 cell lines. A) Graph represents the mean of the autophagosome formation quantification by Flow cytometry using Cyto-ID staining in CEM and B) MOLT-4. The results are presented as mean ± standard deviation of three different experiments.



Supplementary 3. Spautin-1 (SP-1) inhibit autophagosome formation in Breast and T-ALL cell lines. A) Corrected total cell fluorescence (CTCF) of CYTO-ID staining shown in arbitrary units (a.u.) in MCF-7 and 4T1 cells left untreated (control) or treated with ICRP CC 50 for 24 h without co-treatment (-) or co-treated with Spautin-1 (SP-1). The means (± SD) of triplicates of at least three independent experiments were graphed. B) Graph represents the mean of the autophagosome formation quantification by Flow cytometry using Cyto-ID staining in CEM and MOLT-4 in presence or absence of Sp-1. The results are presented as mean ± standard deviation of three different experiments.



Supplementary 4. BAPTA inhibit intracellular calcium levels in Breast and T-ALL cell lines. A) Graph represents the mean of the cytoplasmic Ca^{2+} quantification by Flow cytometry using Fluo-4-AM staining in MCF-7, 4T-1, CEM and MOLT-4. The results are presented as mean ± standard deviation of three different experiments.

5.4 CHAPTER IV

Analysis of IMMUNEPOTENT-CRP on T Cells during activation using anti-CD3soluble

5.4.1.1 INTRODUCTION

T cells are leukocytes usually defined as CD45+ and CD3+, representing 15 to 30% of all CD45+ leukocytes in human peripheral blood, and particularly, on isolated PBMC represent 45 to 70%. T cells originate in bone marrow and mature in thymus, where they differentiate in CD4+ (helper), CD8+ (cytotoxic) among other subsets. These cells play a crucial role on cellular immune response, specially memory response, recognizing antigens through T cell receptor (TCR) (Kumar, Connors, & Farber, 2018) (Velardi, Tsai, & van den Brink, 2021). Their principal role includes directly killing infected host cells, activation of other immune cells, release cytokines, orchestrating the immune response. T cells activation involves complex interactions as TCR signaling, CD28 co-stimulation and cytokines receptors signaling. TCR interacts with foreign antigen in the context of self-MHC. However, itself is insufficient to enable T-Cell activation. Additionally, CD28 receptor (expressed on T Cell), binds to B7-1 (CD80) and B7-2 (CD86) molecules expressed on antigen-presenting cells (APCs) to enhance T cell activation. Also, cytokines secretion by APCs activate signal via cytokine receptors on T cells. Therefore, target cells, as tumor or infected cells, alone are unable to activate T cells. T cells requires target cells to be phagocytosed by APCs, such as dendritic cells, with eventual antigen processing and presentation to T cells through TCR/co-receptor for an appropriate T cell activation, leading to cytokine production, proliferation and active killing of target cells (Sharma, Campbell, Yee, & Goswami, 2015) (Hwang, Byeon, Kim, & Park, 2020).

The IMMUNEPOTENT-CRP (I-CRP) is a dializable leukocyte extract obtained from spleen bovine. Previously, it's been demonstrated an antitumor activity against human and murine cancer cell lines inducing a ROS-dependent regulated cell death and, lately, classified as an immunogenic cell death inductor. In other hand, I-CRP improved life´s quality of lung and breast cancer patients were revealed, where the number of total leukocytes as well as the CD4+, CD8+, CD16+ and CD56+ lymphocytes subpopulations increased. However, the effect of I-CRP on T cells has not been clearly elucidated. Thus, the aim of this study was to analyze the effects of I-CRP in human T cells, and their activation by soluble anti-CD3 monoclonal antibodies (mAbs) *in vitro*.

5.4.1.2 MATHERIALS AND METHODS

5.4.1.2.1 Reagents

IMMUNEPOTENT-CRP (I-CRP) is a bovine dialyzable leukocyte extract (bDLE) produced in the Laboratorio de Inmunología y Virología at Universidad Autónoma de Nuevo León. One unit (U) of I-CRP is defined as 24 mg of peptides obtained from 15×10^8 leukocytes. I-CRP was diluted in culture medium (RPMI 1640 medium, GIBCO Thermofisher, Waltham, Massachusetts, USA). The stock solution was stored at 4°C. The human soluble anti-CD3 mAbs directed to the CD3ε chain (anti-CD3ε, OKT3, 1 μg/mL; BD Biosciences).

5.4.1.2.2 Cell isolation

Blood were collected from healthy donors after a written informed consents were obtained. Peripheral blood mononuclear cells (PBMC) were isolated after centrifugation on Ficoll-Paque™ PLUS (GE Healthcare, Chicago, Ilinois, USA). PBMC were maintained at 1×10^5 cells/mL at 37ºC in 5% CO_2 atmosphere in culture medium.

5.4.1.2.3 T Cells subsets analysis

The principal T cells subsets were implemented on PBMC, by seeding 1×10^5 cells per well in 96-well plates (Corning Inc. Costar, NY, USA) and treated with I-CRP (1.5 U/mL), or soluble anti-CD3 mAbs (anti-CD3ε, OKT3, 1 μg/mL; BD Biosciences) as

positive control or left unstimulated as negative control during 48h. Thereafter, supernatant was discarded, and cells were collected, washed, and stained for 30 min in 100 µL of FACS buffer at 4ºC temperature in darkness with the following mAbs anti-CD3 (CD3-APC; cat; PNM2467 Marseille, France), anti-CD4 (CD4-Pc7; Cat:737660, BD, Maersille, France), anti-CD8 (CD8-Pc7; Cat:737661, BD, Maersille, France), anti-CD27 (CD27-PE, cat:2578, Immunotech Coulter Company, Marseille, France), anti-CD62L (CD62L-PC5, Cat:2655U, BD, Marseille, France), anti-CD45 (CD45-AF750, Cat:A79392, BD, Marseille, France). Analyses were performed by flow cytometry (MACS Quant10) and analyzed on Kaluza 2.1 software (Beckman Coulter). Lymphocytes were gated in FSC/SSC dot plot and at least 30,000 events were collected, then CD45 + were selected and CD3 + lymphocytes. CD3 cells were analyzed for the expression of CD4 and CD8 in an independent manner and CD27+/ CD62L+. T cells and receptor expression analysis was performed in CD3+, CD4+, or CD8 + population.

5.4.1.2.4 Early activation analysis

To determine the activation induced by I-CRP on T cells, we used Vio-Blue staining (Thermofisher, Waltham, Massachusetts, USA) staining combined to CD69. PBMC were seeded at 1×10^5 cells per well in 96-well plates (Corning Inc. Costar, NY, USA) and treated during 48h with 1.5 U/mL of I-CRP or soluble anti-CD3 mAbs (anti-CD3ε, OKT3, 1 µg/mL; BD Biosciences) as positive control. After incubation, cells were collected, washed and then, incubated VioBlue (0.25 µM) and anti-CD69-FITC (CD69 -FITC, cat:560969, BD Biosciences) for 30 min at 4ºC in darkness. Finally, cells were then washed and suspended in FACS buffer to be tested by flow cytometry (Fluorescence-Activated Cell Sorting [FACS]; (MACS Quant10) and analyzed on Kaluza 2.1 software (Beckman Coulter).

5.4.1.2.5 Analysis of the effect of I-CRP in T cells activation

To determine the effect of I-CRP in T cells activation. We used soluble anti-CD3monoclonal antibodies to induces activation on T cells. The experiments were designed in three ways. The first way, to determine if I-CRP augments or decreases

activation of T cells. We seeded PBMC at 1×10^5 cells per well in 96-well plates (Corning Inc. Costar, NY, USA) and treated with 1.5 U/mL of I-CRP for 24h. After this treatment, we added the soluble anti-CD3 mAbs (anti-CD3ε OKT3, 1 μg/mL; BD Biosciences) for additional 24h. Here, we used un-treated cells as a negative control and soluble anti-CD3 mAbs (anti-CD3ε OKT3, 1 μg/mL; BD Biosciences) for 24h as positive control.

The second way, to determine if I-CRP modify the activation of T cells. PBMC were seeded at 1×10^5 cells per well in 96-well plates (Corning Inc. Costar, NY, USA) and treated simultaneously with 1.5 U/mL of I-CRP and with soluble anti-CD3 mAbs (anti-CD3ε OKT3, 1 μg/mL; BD Biosciences) during 48h. Here, we used un-treated cells as a negative control and soluble anti-CD3 mAbs (anti-CD3ε OKT3, 1 μg/mL; BD Biosciences) for 48h as positive control.

And the third way, to determine the effect of I-CRP on activated T cells. We seeded PBMC at 1×10^5 cells per well in 96-well plates (Corning Inc. Costar, NY, USA) and treated with the soluble anti-CD3 mAbs (anti-CD3ε OKT3, 1 μg/mL; BD Biosciences) for 24h. After this treatment, we added 1.5 U/mL of I-CRP to PBMC for additional 24h. After each condition, we analyzed subsets of T cells and early action by flow cytometry (Fluorescence-Activated Cell Sorting [FACS]; (MACS Quant10) and analyzed on Kaluza 2.1 software (Beckman Coulter). Here, we used un-treated cells as a negative control and soluble anti-CD3 mAbs (anti-CD3ε OKT3, 1 μg/mL; BD Biosciences) for 48h as positive control.

5.4.1.3 RESULTS

Previously, we observed that I-CRP (1.5U/mL) induces early activation on NK cells improving their effector activity against target cell. Thus, we tested the effect of I-CRP on T cells, analyzing the major subsets on PBMC (Figure 32). In figure 32A, it is shown the gating strategy used to determine CD4 and CD8 subsets on T cells derived from healthy donors. In figure 32B, representative dot plots of CD4 subsets are shown, a

decrement in I-CRP treated cells fluorescence were observed (p=0.0286) when compared to the negative control and with soluble anti-CD3 mAbs (anti-CD3s). Similarly, in CD8 subset, the $CD8^{high}$ subset diminishes (p=0.002) when compared to control, however, $CD8^{low}$ subset increased (p=0.0286) in I-CRP treated cells compared to un-treated cells (Figure 32C). Thus, naïve ($CD27^{+}$ and $CD62L^{+}$) and central memory ($CD27^{+}$ and CD62L–) subsets in PBMC derived from healthy donors were evaluated, the gating strategy is shown in figure 32D. Decremented fluorescence to CD27 and CD62L were observed in I-CRP treated cells when compared the untreated cells and anti-CD3s treated cells, indicating that I-CRP diminish the major markers of naïve and central memory subsets in PBMC. Our results demonstrated that I-CRP induces immunophenotype changes on T cells derived from healthy donors.

Figure 32. Immunophenotypes of T cells in peripheral blood derived from healthy donors. **A)** Representative gating strategy for flow cytometry analysis to CD4 and CD8 subsets analysis on T cells (CD3+). **B)** Representative dot plots (left) and quantification (right) of CD4+ T cells (CD3+). **C)** Representative dot plots (left) of CD8+ T cells (CD3+) and quantification (right) of $CD8^{high}$ and $CD8^{low}$ subsets. **D)** Representative gating strategy for flow cytometry analysis to naïve ($CD27^+$ and $CD62L^+$) and central memory ($CD27^+$ and CD62L–) on T cells (CD3+). **E)** Representative dot plots of naïve ($CD27^+$ and $CD62L^+$) and central memory ($CD27^+$ and CD62L–) subsets analysis (left) and quantification (right). All data shown is median and range. Statistics were performed by non-parametric Mann–Whitney U-test ($p<0.05$).

To determine if I-CRP (1.5U/mL) induces early activation on T cells, CD69 expression on T cells were tested, using similar gating strategy as in figure 33A. A representative dot plot analysis it´s shown in figure 33A, were incremented fluorescence on cells treated by soluble anti-CD3 mAbs increase fluoresce it's observed compared to un-treated cells (p=0.0002), similar to I-CRP treated cells (p=0.0002). As previously demonstrated that I-CRP enhances cytoplasmic Ca^{2+} levels on T-ALL cell lines inhibited in presence of extracellular Ca^{2+} chelator (BAPTA), we tested cytoplasmic Ca^{2+} levels on T cells. We showed in supplementary 1, that I-CRP increases cytoplasmic Ca^{2+} levels on CEM 59%, MOLT-4 (43%), PBMC (15%) and T cells (25%) that inhibits in presence of BAPTA. Additionally, we observed that I-CRP induces early activation on T-ALL cell lines and T cells, being 41% on CEM, 47% on MOLT-4 cell lines and on 38% in PBMC and 17% in T cells, interestingly, in presence of BAPTA the activation diminishes. Demonstrating, that I-CRP induces low activation on T cells in comparison to T-ALL cell lines and PBMC.

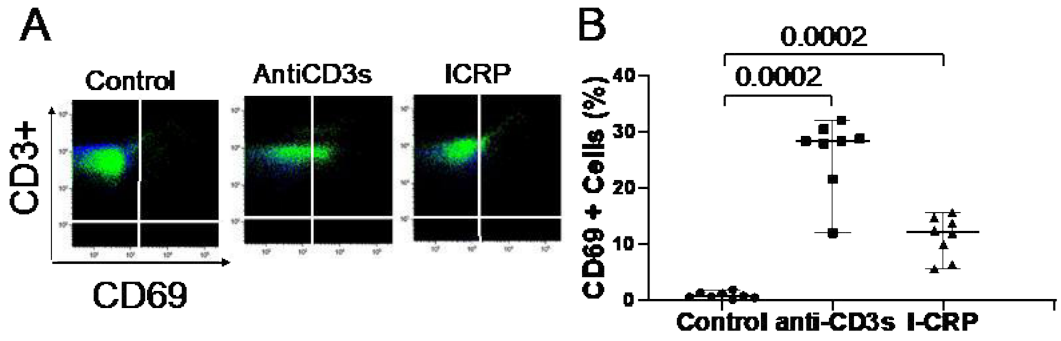


Figure 33. Early activation analysis on T cells derived from healthy donors. A) Representative dot plots of early activation (CD69) analysis on T cells (left) and quantification (right). All data shown is median and range. Statistics were performed by non-parametric Mann–Whitney U-test ($p<0.05$).

As we observed that I-CRP induces phenotypic changes and early activation on T cells, but lower than PBMC and T-ALL cell lines. We tested the effect during activation using a soluble anti-CD3 mAbs as a trigger. First, we determine the effect of I-CRP on T cells previous an activation signal. Thus, we treated T cells by I-CRP (1.5U/mL) during 24h and after we add a soluble anti-CD mAbs. We observed in figure 34A that I-CRP pre-treatment (p=0.0379) decrease CD69 expression on T cells compared to

soluble anti-CD3 mAbs alone (p=0.0002). Similar observations were observed on $CD4^+$ (figure 34B), $CD8^+$ (figure 34C and D), $CD27^+/CD62L^+$ (figure 34E), and $CD27^+/CD62L-$ (figure 34F), subsets. Demonstrating, that I-CRP pre-treatment decrease the activation induced by soluble anti-CD3 mAbs on T cells.

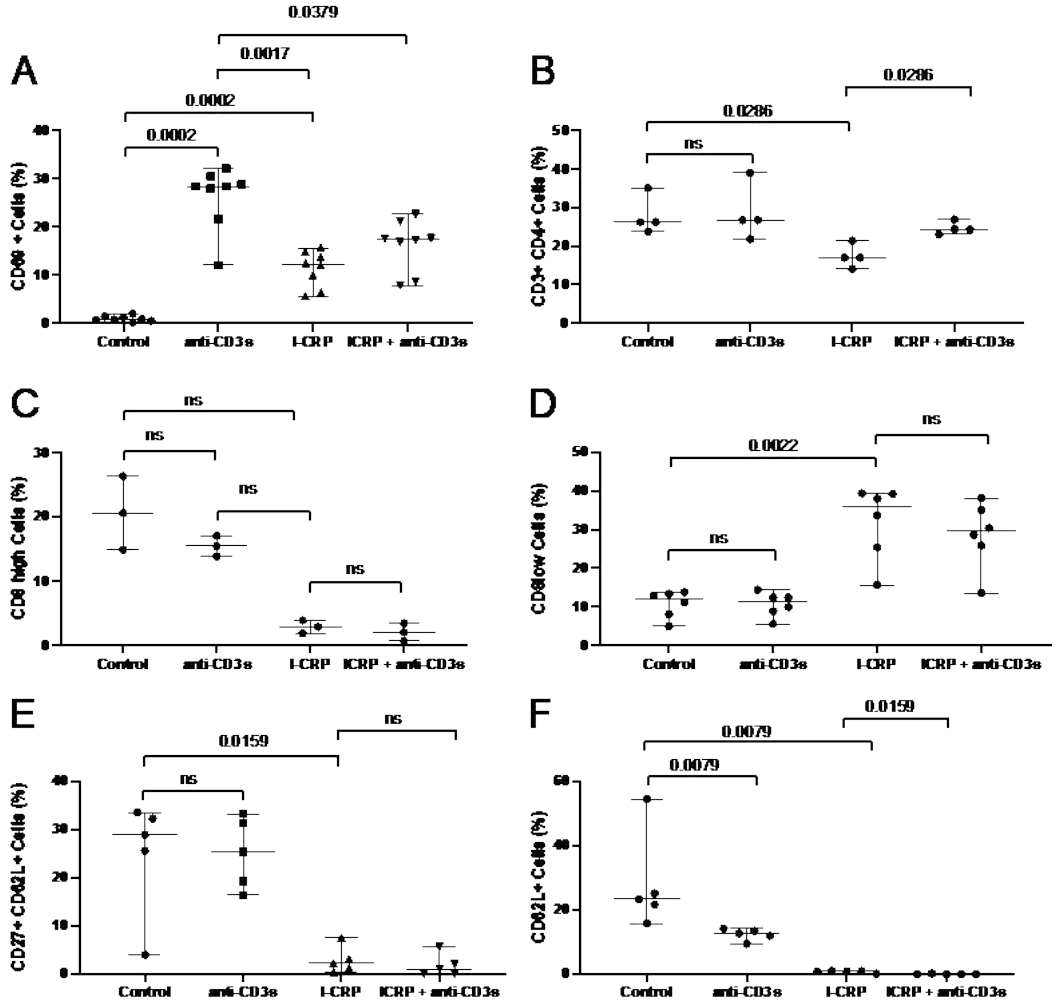


Figure 34. Early activation and immunophenotype analysis on T cells derived from healthy donors after I-CRP treatment after activation using anti-CD3soluble. A) Quantification of CD69 expression on T cells (CD3+), **B)** CD4 **C)** $CD8^{hight}$, **D)** $CD8^{low}$**, E)** CD27+/CD62L+, and **F)** CD27+/CD62L– subsets treated with I-CRP after activation by anti-CD3soluble. All data shown is median and range. Statistics were performed by non-parametric Mann–Whitney U-test ($p<0.05$).

As we observed that I-CRP decreases activation induced by anti-CD3s on T cells. We decided to evaluate the effect of I-CRP on T cells during an activation signal by soluble

anti-CD3 mAbs. Thus, T cells were treated by I-CRP (1.5U/mL) during 24h and after soluble anti-CD3 mAbs were add. In figure 35A it´s observed that I-CRP plus soluble anti-CD3 mAbs shown less CD69 expression on T cells than soluble anti-CD3 mAbs alone. Similar conditions were observed on $CD4^+$ (figure 35B), $CD8^+$ (figure 35C and D), $CD27^+/CD62L^+$ (figure 35E), and $CD27^+/CD62L-$ (figure 35F), subsets. Demonstrating, that I-CRP decrease the activation induced by anti-CD3s on T cells.

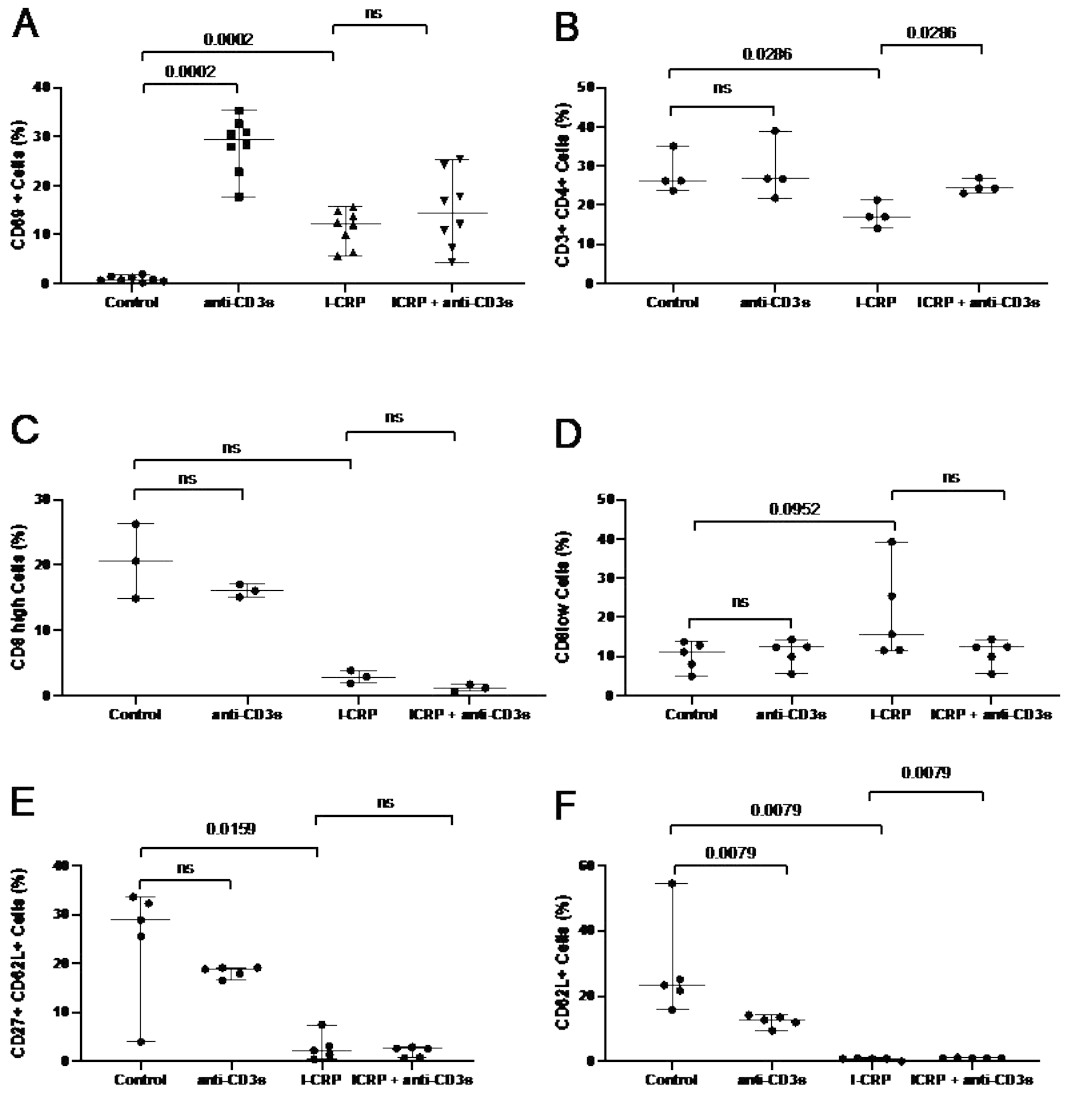


Figure 35. Early activation and immunophenotype analysis on T cells derived from healthy donors treated with I-CRP treatment during activation using anti-CD3soluble. A) Quantification of CD69 expression on T cells (CD3+), **B)** CD4 **C)** $CD8^{hight}$, **D)** $CD8^{low}$, **E)** CD27+/CD62L+, and **F)**

CD27+/CD62L− subsets treated with I-CRP after activation by anti-CD3soluble. All data shown is median and range. Statistics were performed by non-parametric Mann–Whitney U-test ($p<0.05$).

The next step, was evaluated the effect of I-CRP on activated T cells by soluble anti-CD3 mAbs. Thus, we pre-treated T cells by soluble anti-CD3 mAbs during 24h and after we treated I-CRP (1.5U/mL) for 24h. Interestingly, in figure 36A I-CRP plus soluble anti-CD3 mAbs increase CD69 expression ($p=0.0002$) on T cells compared to I -CRP treated cells ($p=0.0002$). However, in figure 36B we observed that $CD4^{+}$ subset ($p=0.0286$) diminishes in compared to soluble anti-CD3 mAbs ($p=0.0286$), in contrast $CD8^{high}$ increased ($p=0.0002$) compared to soluble anti-CD3 mAbs ($p=0.002$). In contrast $CD8^{low}$ (figure 37D), $CD27^{+}/CD62L^{+}$ (figure 36E), and $CD27^{+}/CD62L-$ (figure 36F), subset does not show significant difference. Our results demonstrated that I-CRP increase the activation induced by soluble anti-CD3 mAbs on T cells.

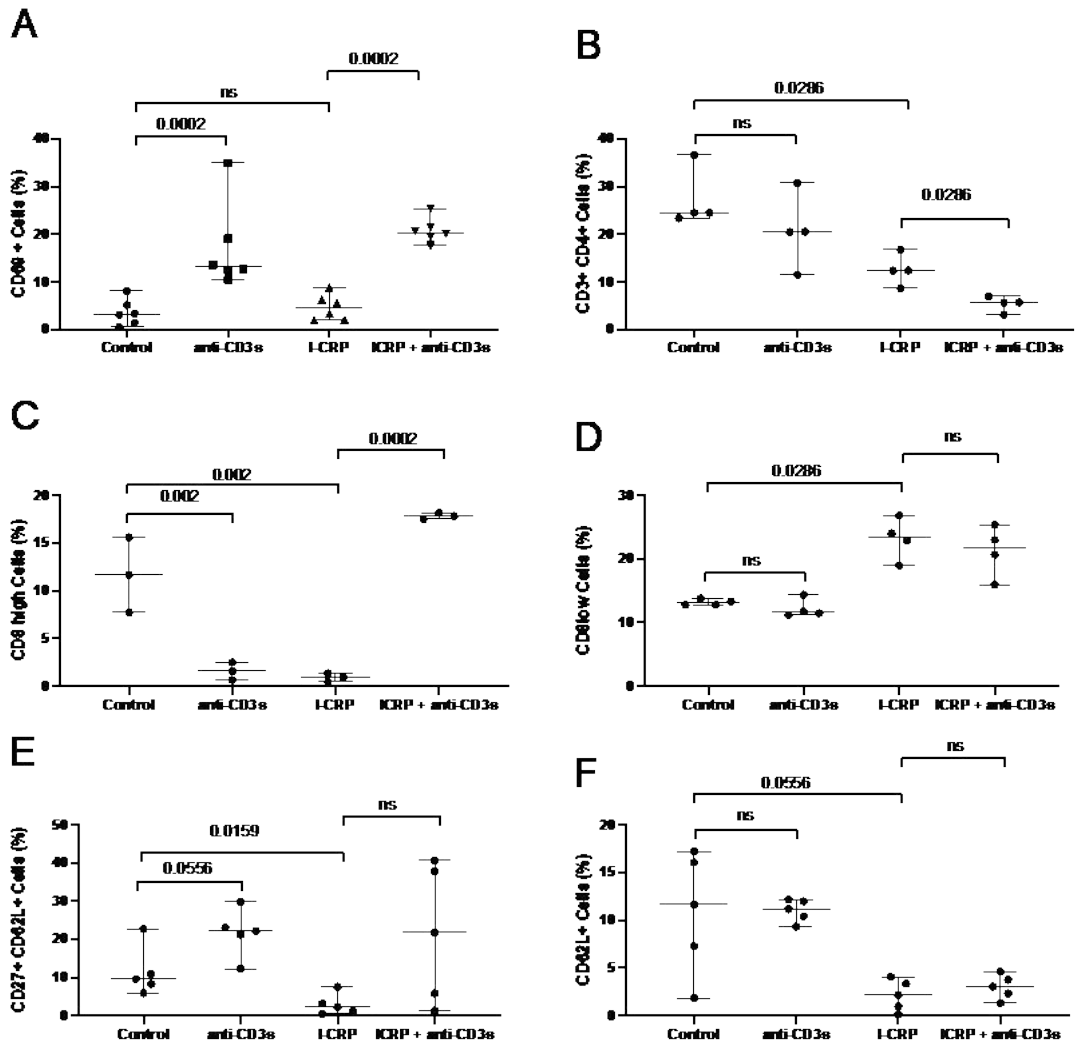


Figure 36. Early activation and immunophenotype analysis on T cells derived from healthy donors treated with I-CRP treatment after activation with anti-CD3soluble. A) Quantification of CD69 expression on T cells (CD3+), **B)** CD4 **C)** $CD8^{hight}$, **D)** $CD8^{low}$**, E)** CD27+/CD62L+, and **F)** CD27+/CD62L− subsets treated with I-CRP after activation by anti-CD3soluble. All data shown is median and range. Statistics were performed by non-parametric Mann–Whitney U-test ($p<0.05$).

Finally, we evaluated if I-CRP could induces senesce or exhaustion on T cells. We tested CD57, PD-1 and CD160 expression on T cells. In figure 37A, it is shown the gating strategy used to determine CD57, PD-1 and CD160 expression on T cells derived from healthy donors. Interestingly, we do not observe CD57 expression on I-CRP treated cells ($p=0.8413$) compared to control cells (Fig. 37B). Additionally, I-CRP treated cells do not show PD-1 ($p=0.6991$) and CD160 ($p=0.0952$) expression

compared to control cells (Fig. 37C and 37D, respectively). Our results revealed that I-CRP do not induces senesce or exhaustion on T cells.

In conclusion, our results demonstrated that I-CRP induced phenotypic changes on T cells decreasing, CD4, CD8, naïve and central memory subsets. Additionally, induces early activation, interestingly, in presence of anti-CD3s induces an immunomodulator effect (figure 39). Particularly, I-CRP promotes activation induced by anti-CD3s just after stimulation, but not previous or during stimulation.

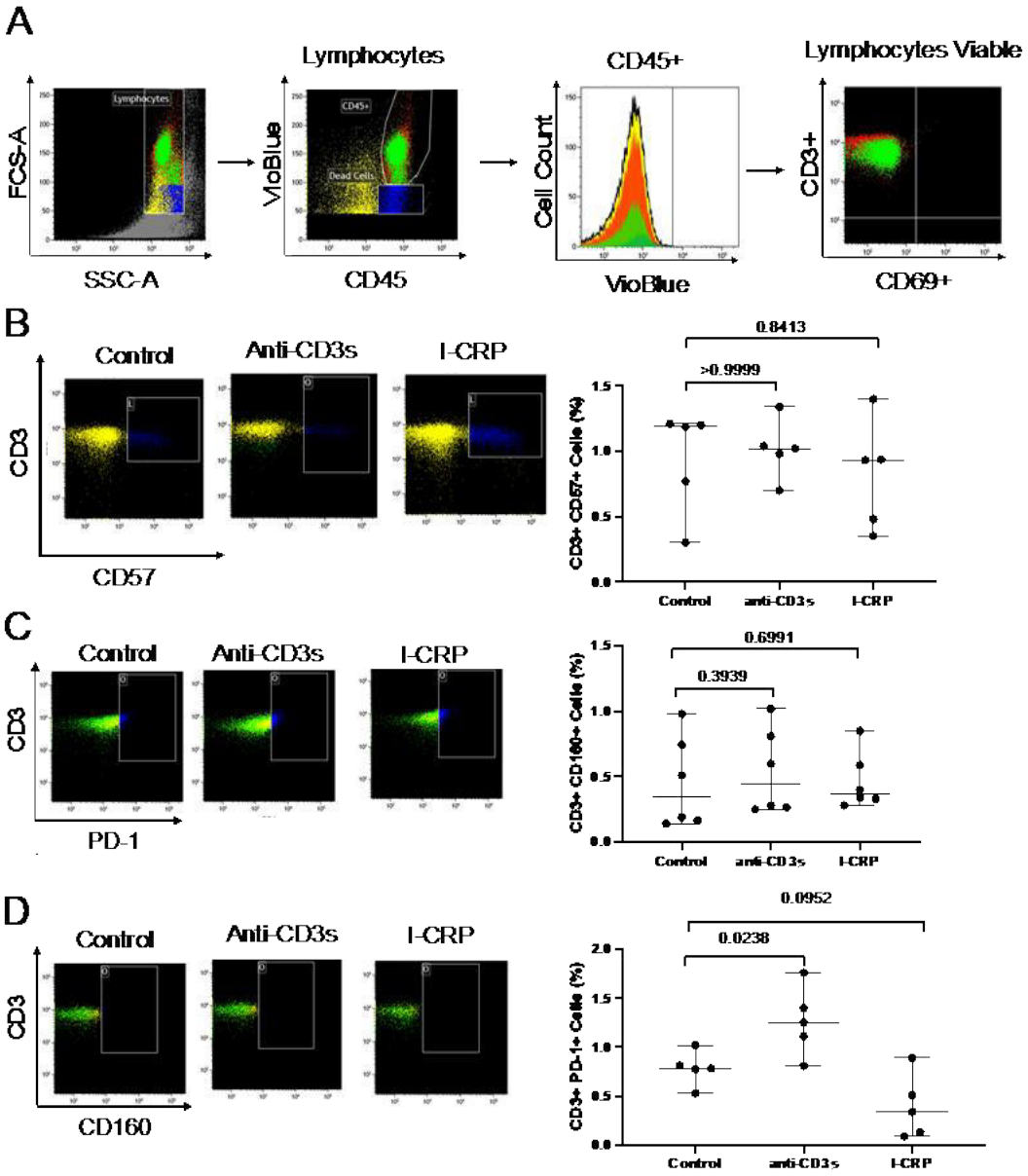


Figure 37. Senesce and exhaustion analysis on T cells derived from healthy donors treated with I-CRP treatment. A) Representative gating strategy for flow cytometry analysis to determine senesce (CD57) or exhaustion (PD-1 and CD160) expression on T cells (CD3+). **B)** Representative dot plots (left) and quantification (right) of CD57 expression on T cells (CD3+). **C)** Representative dot plots (left) of PD-1 expression on T cells (CD3+) and quantification (right). **D)** Representative dot plots (left) of CD160 expression on T cells (CD3+) and quantification (right). All data shown is median and range. Statistics were performed by non-parametric Mann–Whitney U-test ($p<0.05$).

5.4.1.4 DISCUSSION

Currently, it has been described any methods to stimulate T cells *in vitro*. Most of them take advantage of T cell biology, primarily targeting the engagement of the T cell receptor (TCR) that engage sufficient intracellular signal transduction and drives productive activation, proliferation, and differentiation. Poltorak et al., 2020. A wide variety of reagents have been developed to achieve T cell activation, from nonspecific such as PHA mitogen, Concanavalin A, LPS, to a directed stimulation like anti-CD3 mAbs. Zanin-Zhorov, et al., 2007 Alsalamah et al., 2019. Typical polyclonal stimuli (that can activate a heterogeneous primary T cell population) are the ones based on at least bi-valent anti-CD3 and anti-CD28 mAbs. Multi-valent binding is necessary, because ligation of the TCR alone (defined as signal 1) will not induce full T cell activation but will rather result in a non-responsive state. Therefore, in addition to the TCR stimulation, co-stimulatory receptors—most notably CD28—must deliver supporting signals (so called signal 2). CD28-mediated co-stimulation synergizes with TCR signals promoting survival, clonal expansion, and differentiation. Poltorak et al., 2020. In addition to TCR- and CD28-mediated signaling (signal 1 and 2), cytokines such as IL-2 (signal 3) facilitate later stages of T cell stimulation.

Here, we observed that I-CRP treatment decrease CD4, CD8, CD27 and CD62L expression on T cells, interestingly, promotes early activation on T cells (CD69 expression). Others reports demonstrated CD4 downregulation on murine T helper cells during chronic *in vitro* stimulation using anti-CD3/CD28 beads Grishkan et al., 2013. Naji et al., reported that HLA-G expressed by APCs or as soluble protein downregulates CD4 and CD8 expression promoting to immunosuppressive subpopulations with potent implications in peripheral tolerance. These CD3+CD4low

and CD3+CD8low T cell subsets, decreased CD62L expression but increased CD45RA and HLA-DR expression, and high levels of IL-10 (Naji Abderrahim, et al., 2007). Suggesting, that I-CRP could be used on autoimmune diseases.

In this context, it has been reported that CD62L (L-selectin) is delayed from the T cell surface after TCR engagement to avoid T cell re-entering on lymphoid nodes. ADAM17 a metalloproteinase has shown to shed CD62L on activated T cells. Mohammed et al., 2019 Moritz et al., 2017. To date, several members of the ADAM (a disintegrin and metalloproteinase) family included ADAM-17 can be rapidly stimulated by calcium influx, activation of extracellular signal-regulated kinase, protein kinase C (PKC) and tyrosine kinases such as VEGFR2, and also via G-protein-coupled receptors Gooz M., 2010, Le Gall, et al., 2010. In this context, I-CRP have been demonstrated increase calcium influx on T-ALL cell lines, PBMC and on T cells (Supplementary material 1). This suggest that I-CRP could increase intracellular calcium on T cells that promotes the ADAM-17 shed and the CD62L downregulation.

In other hand, it is important tested exhaustion or senesce on T cells, due to these conditions could involve the loss of biological function of T cells. Exhausted T cells display high levels of CD43 (1B11), CD69 and inhibitory receptors as PD-1 but low levels of CD62L and CD127, which is an expression profile typically associated with effector T cells (Yi et al., 2010). Here, we observed that I-CRP do not induces CD57, CD160 and PD-1 expression on T cells, discarding the exhaustion or senesce on T cells. In other reports, on *in vivo* model I-CRP demonstrated decreased tumor suppressor molecules such as PD-L1, Gal-3, and IL-10 induced by the combination of conventional chemotherapies doxorubicin/cyclophosphamide Santana-Krímskaya, et al., 2020. In contrast, promotes the infiltration of CD4 and CD8 T cells on lymphoid tissue and on tumor microenvironment. Santana-Krímskaya, et al., 2020, Reyes-Ruíz et al., 2021. Suggesting, that I-CRP could activated T cells and promotes tumor regression.

5.4.1.5 CONCLUSION

Overall, our results show that I-CRP induces early activation on T cells and downregulation of CD4, CD8, CD27 and CD62L expression even on activated T cells. Interestingly, improves the activation of T cells. This work helps to understand the mechanism of I-CRP as immunostimulant on T cells and on activated T cells.

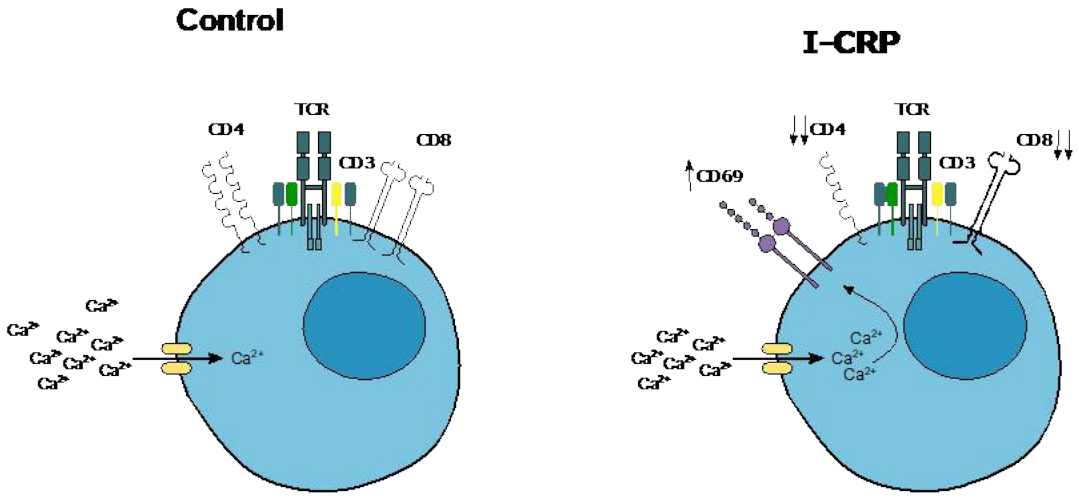


Figure 38. Schematic representation of I-CRP on T cells derived from healthy donors. I-CRP decrease CD4, CD8, CD27 and CD62L expression on T cells, additionally induces early activation on T cells. Interestingly, I-CRP increases cytoplasmic Ca^{2+} levels inhibited in presence of BAPTA, required by early activation.

5.4.1.6 REFERENCES

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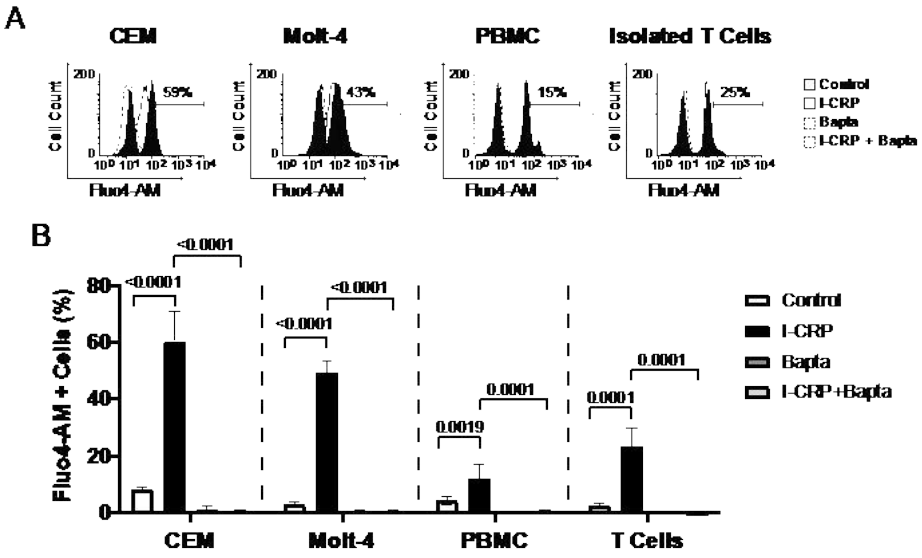
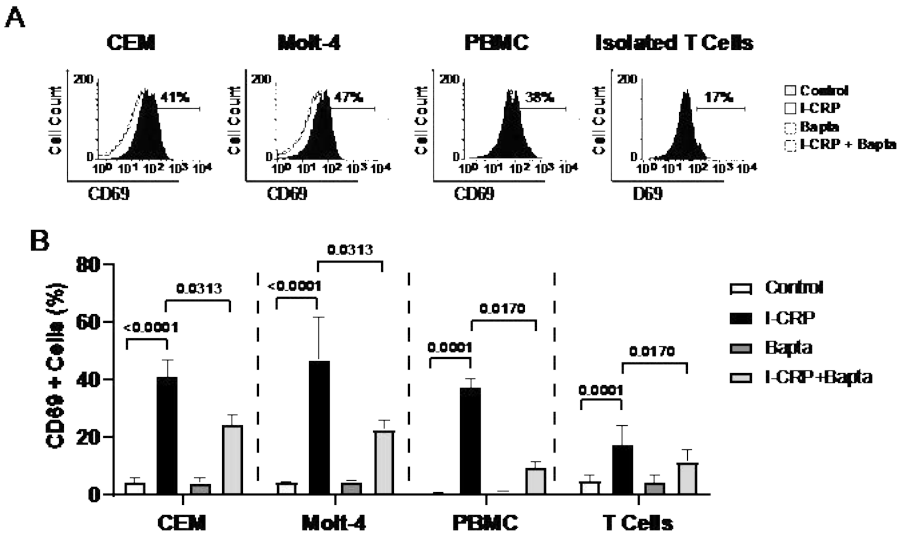
5.4.1.7 SUPPLEMENTARY MATERIAL



Supplementary 1. A) Representative histograms of cytoplasmic Ca^{2+} levels and **B)** Graph represents the mean of the cytoplasmic Ca^{2+} quantification by Flow cytometry using Fluo-4-AM staining in CEM and MOLT-4 cell lines, and PBMC and T cells derived from healthy donors, in presence or absence of Bapta. The results are presented as mean ± standard deviation of three different experiments.



Supplementary 2. A) Representative histograms of CD69 expression and **B)** Graph represents the mean of the CD69 expression by Flow cytometry in CEM and MOLT-4 cell lines, and PBMC and T cells derived from healthy donors, in presence or absence of Bapta. The results are presented as mean ± standard deviation of three different experiments.

6 CONCLUSIONS

Overall, there results demonstrate the immunomodulator effect of IMMUNEPOTENT-CRP (I-CRP) on lymphoid cells. On NK cells, induces early activation, increasing $CD56^{dim}$ CD16– subset and changes in the Natural Killer cell repertoire enhancing to effector activity against target cell. Additionally, on T cells induces changes on immunophenotype even during activation stimuli, increasing to early activation and without senesce and exhaustion. However, on T-Acute Lymphoblastic leukemia cell lines, triggers an endoplasmic stress accompanied by the increase of cytoplasmic Ca^{2+} levels. Ca^{2+} entry to leukemic cells provokes Ca^{2+} release from ER through IP_3R and RyR receptors lead to ROS production, mitochondrial and nuclear damage, and caspase -3 activation. ROS production augmentation induces mitochondrial damage through proapoptotic and antiapoptotic protein modulation (Bax and Bcl-2), and loss of mtochondiral membrane potential, and nuclear alterations including DNA damage (γ-H2Ax), overexpression of p53, cell cycle arrest, and DNA degradation. This intracellular signaling pathway promotes autophagosome formation. However, I-CRP induces calcium and ROS-dependent apoptosis in leukemic cells. Overall, here, we show that I-CRP cytotoxicity is selective to leukemic cells, inducing calcium and ROS-dependent apoptosis. In general, we demonstrated the immunomodulator effect of IMMUNEPOTENT-CRP on NK and T Lymphocytes derived from healthy donors and the cytotoxic effect on T-ALL Cell lines (Figure 39). This research opens the door to further exploration of their role in the immune system and the cell death mechanism that could potentially work in conjunction with other therapies including hematological malignances.

Figure 39. Schematic representation of IMMUNEPOTENT-CRP on peripheral mononuclear cells derived from healthy donor and on T-ALL cell lines. I-CRP do not indices changes on cell viability on PBMC derived from healthy donor, thus induces early activation, and modify pro- and anti-inflammatory cytokines release, improving to $CD56^{dim}$ CD16– NK subset. Particularly, on NK cells modify the natural cytotoxicity receptors improving their effector activity against target cell. On T Lymphocytes induces phenotypic changes that includes activations without senescence and exhaustion. IN other hand, on T-ALL Cell lines induces eIF2α-phosphorylation, autophagosome formation and increase intracellular Ca^{2+} levels on cancer cell lines. The intracellular Ca^{2+} augmentation provokes Ca^{2+} release from ER mediated by RyR and IP3R, inhibited using 2-APB and Dantrolene that prevents ICRP cell death and ROS production. Additionally, intracellular Ca^{2+} augmentation is the first step on cell death pathway: exacerbate ROS production, loss of mitochondrial membrane potential (loss of $\Delta\psi m$), provoking to caspase-3 cleaved, nuclear damage leading to apoptosis.

7 PERSPECTIVES

- Elucidate if ICRP enhance the effector activity in NK cells during viral infections or in cancer models.
- Elucidate the molecular and cellular mechanism involved on NK activation during ICRP treatment.
- Investigate if ICRP could reverse exhausted phenotype of NK cells during chronic infections.
- Determine if ICRP enhance the effector activity in T cells during chronic infections.
- Evaluated the cytotoxic effect of ICRP on peripheral mononuclear cells derived from T-ALL patients.
- Analyze the anti-tumoral effect *in vivo* of ICRP on leukemia or lymphoma models.
- Elucidate Ca^{2+} signaling differences between normal cells and malignant cells treated with ICRP.
- Study Ca^{2+} signaling in the activation of immune cells triggered by ICRP.
- Evaluate immunogenic potential of ICRP on leukemia or lymphoma models.

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9 BIOGRAPHYCAL ABSTRACT

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Theis: "Study of IMMUNEPOTENT-CRP effect on different subpopulations of lymphocytes and tumoral t cell lines"

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Awards:

- Best Poster of Microbiology/Immunology at Forum of Presentation and Advances in Research. UANL, Nuevo León, México, 2020.
- Special distinction for the quality and originality of the work. VIII Encuentro de Jóvenes Investigadores en el Estado de Nuevo León, 2020.
- Master thesis with honors, 2018.
- Second Place of Posters at the National Symposium of Pharmaceutical Sciences and Biomedicine, hosted by the College of Chemical Sciences, UANL, 2018.

Professional experience:

- Instructor of Molecular Biology. School of Biological Sciences (FCB), UANL, (México), 2020.
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List of Scientific Communications

Scientific publications on Indexed Journals JCR:

- **Helen Yarimet Lorenz-Anota*,** Alejandra Reyes-Ruiz*, Kenny Misael Calvillo-Rodríguez, Rodolfo MendozaReveles, Andrea Paola Urdaneta-Peinado, Ana C. Martínez-Torres*, Cristina Rodríguez-Padilla. IMMUNEPOTENT-CRP increases intracellular calcium in breast cancer and leukemic cell lines leading to divergent regulated cell death modalities. *co-first authors. In revision. Experimental Cell Research.
- **Helen Yarimet Lorenz-Anota*,** Alan Benancio Martínez-Loria*, Daniel Scott -Algara, Ana C. MartínezTorres, Cristina Rodríguez-Padilla1 . Bovine Dialyzable leukocyte extract enhance cytotoxic activity of lymphocytes NK. *co-first authors. In revision. Cellular Immunology.
- **Helen Yarimet Lorenz-Anota,** Diana G. Zarate-Triviño, Jorge A. Uribe-Echeverría, Andrea Ávila-Ávila, José Raúl Rangel-López, Ana C. Martínez-Torres, Cristina Rodríguez-Padilla. Chitosan-Coated Gold Nanoparticles Induce Low Cytotoxicity and Low ROS Production in Primary Leucocytes, Independent of Their Proliferative Status. Pharmaceutics. Impact factor 6.3.
- **Helen Yarimet Lorenz-Anota,** Ana C. Martínez-Torres, Daniel Scott-Alagara, Cristina Rodríguez-Padilla. Bovine Dialyzable Leukocyte Extract IMMUNEPOTENT-CRP Induces Selective ROS-Dependent Apoptosis in T-

Acute Lymphoblastic Leukemia Cell Lines. Journal of Oncology. Impact factor 4.3.

- **Helen Yarimet Lorenzo-Anota*,** Ana C. Martínez-Torres*, Martín G. García-Juárez*, Diana G. ZarateTriviño, Cristina Rodríguez-Padill1 . Chitosan gold nanoparticles induce different ROS-dependent cell death modalities in leukemic cells. International Journal of Nanomedicine. *co-first authors. International Journal of Nanomedicine. Impact factor 5.02.
- Ana Carolina Martínez-Torres, Diana G. Zarate-Triviño, **Helen Yarimet Lorenzo-Anota,** Andrea ÁvilaÁvila, Carolina Rodríguez-Abrego, Cristina Rodríguez-Padilla.Chitosan gold nanoparticles induce cell death in HeLa and MCF-7 cells through reactive oxygen species production. International Journal of Nanomedicine. Impact factor 5.02.

Scientific publications in Arbitrated Journals:

- "La selectividad citotóxica de las nanopartículas de oro cubiertas de quitosano está mediada por ROS en células cancerosas sin afectar células sanas independientemente de su estado proliferativo". VII Simposio Nacional de Ciencias Farmacéuticas y Biomedicina y V Simposio Nacional de Microbiología Aplicada (2020).
- "Estudio de la implicación del Ca2+ y estrés del retículo endoplásmico en la muerte celular inducida por el Immunepotent CRP en células de cáncer de mama". VIII Encuentro de Jóvenes Investigadores en el Estado de Nuevo León (2020).
- "Estudio del efecto de la estimulación con dieta hipercalórica sobre la actividad mitocondrial, la autofagia y poblaciones de linfocitos T de la descendencia adulta de un modelo de programación materan nutricional". VIII Encuentro de Jóvenes Investigadores en el Estado de Nuevo León (2020).
- "Evaluación de las poblaciones de Linfocitos T en sangre periférica, y de la función mitocondrial y la autofagia en células derivadas de organos linfoides de

un modelo de programación fetal por dieta hipercalórica". VIII Encuentro de Jóvenes Investigadores en el Estado de Nuevo León (2020).

- "Las nanopartículas de oro recubiertas de quitosano presentan citotoxicidd hacia células tumorales sin afectar a células del sistema inmune, independientemente de su estado proliferativo". VIII Encuentro de Jóvenes Investigadores en el Estado de Nuevo León (2020).
- "El Immunepotent-CRP induce apoptosis en células leucémicas Molt-4". VI Simposio Nacional de Ciencias Farmacéuticas y Biomedicina y IV Simposio Nacional de Microbiología Aplicada (2019).
- "Chitosan-coated gold nanoparticles induce reactive oxygen species-dependent cell death in leukemic cell lines". V Simposio Nacional de Ciencias Farmacéuticas y Biomedicina y III Simposio Nacional de Microbiología Aplicada (2018).

Oral and poster presentations:

- Poster Presentation: IMMUNEPOTENT-CRP increases intracellular calcium leading selective ROS-dependent apoptosis in T-Acute Lymphoblastic Leukemia cell lines. Cell la Vie - the Online Edition. (2021).
- Oral presentation. Bovine Dialyzable Leukocyte Extract IMMUNEPOTENT-CRP Induces Selective ROS-Dependent Apoptosis in T-Acute Lymphoblastic Leukemia Cell Lines. 31° Congreso Nacional de Investigación e Innovación en Medicina (2021).
- Oral presentation. IMMUNEPOTENT-CRP increases intracellular calcium leading selective ROS-dependent apoptosis in T-Acute Lymphoblastic Leukemia cell lines. XII Congreso Internacional de Investigación UVM. (2021).
- Poster presentation: La selectividad citotóxica de las nanopartículas de oro cubiertas de quitosano está mediada por ROS en células cancerosas sin afectar PBMC sana en proliferación. #LatinXChem Twitter Conference. (2020)

- Poster presentation: La selectividad citotóxica de las nanopartículas de oro cubiertas de quitosano está mediada por ROS en células cancerosas sin afectar células sanas independientemente de su estado proliferativo. VII Simposio Nacional de Ciencias Farmacéuticas y Biomedicina y V Simposio Nacional de Microbiología Aplicada (2020).
- Oral presentation: Estudio de la implicación del Ca2+ y estrés del retículo endoplásmico en la muerte celular inducida por el Immunepotent CRP en células de cáncer de mama. VIII Encuentro de Jóvenes Investigadores en el Estado de Nuevo León (2020).
- Oral presentation: Estudio del efecto de la estimulación con dieta hipercalórica sobre la actividad mitocondrial, la autofagia y poblaciones de linfocitos T de la descendencia adulta de un modelo de programación materan nutricional. VIII Encuentro de Jóvenes Investigadores en el Estado de Nuevo León (2020).
- Oral presentation: Evaluación de las poblaciones de Linfocitos T en sangre periférica, y de la función mitocondrial y la autofagia en células derivadas de organos linfoides de un modelo de programación fetal por dieta hipercalórica. VIII Encuentro de Jóvenes Investigadores en el Estado de Nuevo León (2020).
- Oral presentation: Las nanopartículas de oro recubiertas de quitosano presentan citotoxicidd hacia células tumorales sin afectar a células del sistema inmune, independientemente de su estado proliferativo. VIII Encuentro de Jóvenes Investigadores en el Estado de Nuevo León (2020).
- Poster presentation: IMMUNEPOTENT-CRP induces selective apoptosis in T-ALL cell lines. Organized for Young researches in life sciences (2019).
- Poster presentation: Bovine Dialyzable Leukocyte Extract induces selective ROS-dependent Apoptosis in T-Acute Lymphoblastic Leukemia. VII Congreso de la Rama Transduccion de Senales. (2019).
- Poster presentation: Chitosan gold nanoparticles induce different ROS-dependent cell death modalities in leukemic cells. VII Congreso de la Rama Transduccion de Senales (2019).

- Oral presentation. El IMMUNEPOTENT-CRP induce apoptosis en células leucémicas Molt-4. 30° Congreso Nacional de Investigación e Innovación en Medicina (2019).
- Oral presentation. Immunepotent-CRP: dialyzable leukocyte extract induces selective ROS-dependent apoptosis in T-acute lymphoblastic leukemia cell line. VII Congreso de Especies Reactivas del Oxigeno en Biologia y Medicina. 2019.
- Poster presentation: CH-AuNPs are selective cytotoxic agents ROS-dependent in cancer cell lines. VII Congreso de Especies Reactivas del Oxigeno en Biologia y Medicina (2019).
- Oral presentation. El Immunepotent-CRP induce apoptosis en células leucémicas Molt-4. VI Simposio Nacional de Ciencias Farmacéuticas y Biomedicina y IV Simposio Nacional de Microbiología Aplicada (2019).
- Oral presentation. Chitosan-coated gold nanoparticles induce reactive oxygen species-dependent cell death in leukemic cell lines. V National Symposium of Pharmaceutical Sciences and Biomedicine. School of Chemical Sciences, UANL (2018).
- Oral presentation. Chitosan-coated gold nanoparticles induce reactive oxygen species-dependent cell death in leukemic cell lines. Organized for Young research in life sciences (2018).
- Poster presentation. Chitosan-coated gold nanoparticles induce reactive oxygen species-dependent cell death in leukemic cell lines. The 11th European Workshop on cell death (2018).
- Poster presentation. Chitosan-coated gold nanoparticles induce reactive oxygen species-dependent cell death in leukemic cell lines. VI Congress of Reactive Oxygen species in biology and medicine. Organized for the Mexican Biochemistry Society (SBM) (2017).