UNIVERSIDAD AUTÓNOMA DE NUEVO LEÓN

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TESIS

STUDY OF Ca2+-IMPLICATION AND IMMUNOGENICITY OF THE REGULATED CELL DEATH INDUCED BY IMMUNEPOTENT CRP ON BREAST CANCER

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The present work was carried out in the Laboratory of Immunology and Virology and was directed by Dr. Ana Carolina Martínez Torres.

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Vanity of vanities! All is vanity.

A generation goes, and a generation comes,

but the earth remains forever.

The eye is not satisfied with seeing,

nor the ear filled with hearing.

I applied my heart to seek and to search out by wisdom.

I have seen everything that is done under the sun, and behold, all is vanity.

I applied my heart to know wisdom and to know madness and folly.

I perceived that this also is but a striving after wind.

For in much wisdom is much vexation,

and he who increases knowledge increases sorrow.

I saw that there is more gain in wisdom than in folly,

as there is more gain in light than in darkness.

Then I said in my heart,

"What happens to the fool will happen to me also.

Why then have I been so very wise?"

And I said in my heart that this also is vanity.

Light is sweet, and it is pleasant for the eyes to see the sun.

Let your heart cheer you in the days of your life.

Walk in the ways of your heart and the sight of your eyes.

Ecclesiastes

To María Neri Morales Ramírez,

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Gone from my sight, but never from my heart.

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II. BACKGROUND

ABBREVIATIONS

$\Delta\psi m$	Mitochondrial membrane potential	NCXs	Na+/Ca2+ exchangers
ACD	Accidental cell death	OMM	Outer mitochondrial membrane
AnnV	Annexin V	PCD	Programmed cell death
APCs	Antigen presenting cells	PD-1	Programmed cell death protein 1
ATF6	Activating transcription factor 6	PE	Phycoerythrin
BECN1	Beclin 1	PERK	Protein kinase RNA-like ER kinase
BMDCs	Bone marrow-derived dendritic cells	PI	Propidium Iodide
CICR	Ca^{2+}-induced calcium release process	PIP_2	Phosphatidylinositol l-4,5-bisphosphate
CRT	Calreticulin	PLC	Phospholipase C
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4	PMCA	Plasma-membrane Ca^{2+}-ATPase
CTLs	Cytotoxic T lymphocytes	PS	Phosphatidylserine
DAMPs	Damage-associated molecular patterns	PTP	Permeability transition pore
DCs	Dendritic cells	RCD	Regulated cell death
ER	Endoplasmic reticulum	ROCs	Receptor-operated channels
ERAD	ER-associated degradation	ROS	Reactive Oxygen Species
ETC	Electron transport chain	RYRs	Ryanodine receptors
FasL	Fas ligand	SERCA	Sarco(endo)plasmic reticulum Ca^{2+}-ATPase
HMGB1	High-mobility group box 1 protein	SMOCs	Second messenger-operated channels
ICD	Immunogenic cell death	SOC	Store-operated channel
ICRP	IMMUNEPOTENT CRP	SOCE	Store operated Ca2+entry
IDO	Indoleamine 2,3-dioxygenase	TAAs	Tumor-associated antigens
IMM	Inner mitochondrial membrane	TCD	Tolerogenic cell death
IP_3	Inositol 1,4,5-trisphosphate	TCL	Tumor cell lysate
IP3Rs	Inositol-1,4,5-trisphophate receptors	TRAIL	TNF-related apoptosis-inducing ligand
JNK	Jun-N-terminal protein kinase	TRP	Transient receptor potential
MAMs	Mitochondrion-associated membranes	UPR	Unfolded protein response
MCU	Mitochondrial calcium uniporter	VDAC	Voltage-dependent anion channel
MDSCa	Myeloid-derived suppressor cells	VOCs	Voltage-operated channels

ABSTRACT

Cancer recurrence is a serious problem in breast cancer patients, and immunogenic cell death (ICD) has been proposed as a strategy to overcome this handicap. IMMUNEPOTENT CRP (ICRP) acts as an immunomodulator and can be cytotoxic to cancer cells. Thus, the immunogenicity of ICRP-induced cell death was evaluated in breast cancer cells. This immunogenicity was evaluated *in vitro*, analyzing the principal biochemical characteristics of ICD in MCF-7 and 4T1 cells. *Ex vivo*, we assessed the ability of the tumor cell lysate (TCL) obtained from ICRP-treated 4T1 cells (ICRP-TCL) to induce DCs maturation, T-cell priming, and T-cell-mediated cancer cytotoxicity. *In vivo,* tumor establishment, tumor regression, and antitumor immune memory after prophylactic and therapeutic ICRP-TCL vaccinations in BALB/c mice was evaluated. ICRP induced an increase in cytoplasmic Ca^{2+} levels, which led to ROS overgeneration, loss of $\Delta\psi m$, CRT exposure, and cell death. Moreover, ICRP treatment provoked autophagosome formation, eIF2α phosphorylation, and release of ATP and HMBG1 in breast cancer cells. Additionally, ICRP-TCL promoted DCs maturation, which triggered T cell-priming, that led to T cell-mediated cancer cytotoxicity. Prophylactic vaccination with ICRP-TCL prevented tumor establishment and induced long-term antitumor memory in BALB/c mice, involving DCs maturation in lymph nodes, CD8+ T-cells augmentation in peripheral blood, and ex vivo tumor-specific cytotoxicity by splenocytes. Finally, ICRP-TCL induced tumor regression in tumor-bearing mice, which also developed long-term antitumor memory. In conclusion, ICRP induces ICD in breast cancer cells, leading to long-term antitumor memory.

RESUMEN

La recurrencia es un serio problema en pacientes con cáncer de mama, y, la muerte celular inmunogénica (MCI) se ha propuesto como una estrategia para sobrepasar esta problemática. El IMMUNEPOTENT CRP (ICRP) actúa como inmunomodulador y es citotóxico en células de cáncer. Por lo tanto, se evaluó la inmunogenicidad de la muerte inducida por el ICRP en células de cáncer de mama. Para ello, *in vitro*, se estudiaron las principales características bioquímicas asociadas con la MCI en células MCF-7 y 4T1. *Ex vivo*, se evaluó la habilidad de el lisado tumoral (TCL) obtenido del tratamiento con ICRP a células 4T1 (ICRP-TCL) para inducir maduración de células dendríticas, estimulación de células T y citotoxicidad en células cancerosas. *In vivo*, se investigó el establecimiento tumoral, regresión tumoral y memoria antitumoral después de vacunaciones profilácticas y terapéuticas con ICRP-TCL. El ICRP incrementó los niveles de Ca^{2+} citoplasmático, desencadenando sobregeneración de ROS, perdida de $\Delta\psi m$, exposición de CRT y muerte celular. Además, el ICRP provocó formación de autofagosomas, fosforilación de eIF2α y liberación de ATP y HMGB1. Por otro lado, el ICRP-TCL promovió la maduración de DCs, desencadenando una citotoxicidad dirigida hacia células cancerosas mediada por células T. La vacunación profiláctica con ICRP-TCL previno el establecimiento tumoral e indujo memoria antitumoral a largo plazo, la cual involucró maduración de DCs en ganglios linfáticos, aumento de células T CD8+ en sangre periférica y citoxicidad tumoral mediada por esplenocitos. Por último, el tratamiento con ICRP-TCL indujo regresión tumoral y memoria antitumoral a largo plazo. En conclusión, el ICRP induce MCI en células de cáncer de mama, que desencadena memoria antitumoral a largo plazo.

I. INTRODUCTION

One of the major homeostatic processes for maintenance of our bodies is phagocytosis of dying cells, this process dictates the immunological consequence that will be triggered, which can be tolerogenic, immunogenic, or silent (Green et al. 2009). In regard to cancer disease, malignant cells are prone to be overlooked by the immune system due to cancer immunoediting. In addition, several therapies and stress in tumor microenvironment trigger tolerogenic cell death, which provokes immune tolerance towards cancer cells, and a dim prognosis as a consequence. A strategy to turn cancer cells immunogenic is through the induction of immunogenic cell death (ICD), hence, the search for therapeutic regimens that render cancer cell death immunogenic and revert immune suppression is important (Kroemer et al. 2013). The bovine dialyzable leukocyte extract IMMUNEPOTENT CRP (ICRP) is cytotoxic to cancer cell lines and induces ICD in the murine melanoma model B16F10 (Rodríguez-Salazar et al. 2017), whereas in HeLa and MCF-7 cells ICRP induced reactive oxygen species (ROS)-dependent autophagosome formation (Alvarez-Valdez, 2018). These are two of the major cellular process associated with ICD, suggesting that ICRP might induce ICD in other cancer models. Furthermore, endoplasmic reticulum (ER) stress, an early ICD biomarker, is associated with deregulation in Ca^{2+} signaling (Kepp et al. 2015), and previous studies in HeLa cells have demonstrated that ICRP induces calpain activation (Ca^{2+}-dependent proteases) (Robles-Reyes 2011), however, Ca^{2+}-implication in ICRP-mediated cell death is not entirely elucidated. Thus, the aim of this study was to understand the role of Ca^{2+} in ICRP-induced cell death and the immunogenicity of this cytotoxic mechanism in a breast cancer model.

II. BACKGROUND

1. CELL DEATH: THE PROCESS THAT RULES LIFE AND DISEASES

"Death occurs so that life can happen and, paradoxically, the extinction of life is imperative for its continuation." A.C. Martínez-Torres, Programed Cell Death…and Cancer, 2013.

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It is estimated that one million cells die per second in our bodies in the course of normal tissue turnover, hence, cell death is one of the principal homeostatic processes and an essential part of life (Griffith and Ferguson 2011). A cell decides among three destinies: to divide, to specialize or to commit suicide. As it is expressed by the irreversibility of time, we undergo continuous changes every second, whereas cell death eliminates abnormal, infected, and unnecessary cells; cell division generates more cells, thus, these opposed processes work together to maintain the balance in multicellular organisms (Melino 2001). Notwithstanding, cell death is implicated in the pathogenesis of several diseases such as cancer, acquired immune deficiency syndrome (AIDS), Alzheimer's disease, Parkinson's disease and neurodegenerative diseases (Hotchkiss et al. 2009). In 1991 was the first clear evidence of defects in the cell death machinery could lead to disease, when a mouse strain was serendipitously discovered, these animals showed a tendency to develop enlarged lymph nodes and spleen as well as autoimmune diseases. These organisms had mutations in the death receptor CD95 (Fas) and its ligand CD95 L (FasL), thus lymphocytes, which proliferated due to the exposure to an antigen, then failed to be eliminated at the end of the immune response because of the mutations that impair cell death pathways, causing T-cell accumulation, and triggering autoimmune diseases

(Cohen and Eisenberg 1991). Evidentially, the study of cell death is pivotal to understand diseases and to find new approaches of therapy.

What is reality, the meaning of death? For a long time, it was difficult to define cell death, is ambitious to accomplish clear definitions of things that are shadows in *Plato's cave*. Death and dying are events strongly influenced by culture and religion; hence, to decrease confusion in scientific community, the Editors of Cell Death and Differentiation have created the Nomenclature Committee on Cell Death (NCCD) where authors propose unified criteria for cell death. The NCCD propose that is important to discriminate between dying as a process and death as an end point. The cells that are succumbing to death are engaged in a process that is reversible until a first irreversible phase is trespassed, these "points-of-no-return" include massive protease activation, loss of $\Delta\Psi m$, complete permeabilization of the mitochondrial outer membrane or exposure of phosphatidylserine (PS) that emit "eat me" signals for neighboring cells. Moreover, they suggests that a cell should be considered dead when any of the following criteria are met: (1) the cell has lost the integrity of the plasma membrane; (2) the cell including its nucleus has undergone complete fragmentation into discrete bodies; and/or (3) its corpse (or its fragments) have been engulfed by an adjacent cell *in vivo*. Furthermore, cells whose cell cycle is arrested would be considered as alive (Kroemer et al. 2009).

Generally speaking, cell death can be classified as programed, regulated, and accidental cell death. Programmed cell death (PCD) stands for those physiological instances of cell death that occur in embryonic or post-embryonic development and tissue homeostasis. Regulated cell death (RCD) indicates cases of cell death (programmed or not) whose

initiation and/or execution is mediated by molecular machinery; thus, it can be inhibited by pharmacological and/or genetic manipulations. Finally, accidental cell death (ACD) refers to cell death triggered by physical conditions (e.g., freeze–thawing cycles, high concentrations of pro-oxidants), which cannot be inhibited by pharmacological and/or genetic interventions (Fig. 1) (Lorenzo Galluzzi et al. 2018). Henceforth, I will refer to regulated cell death.

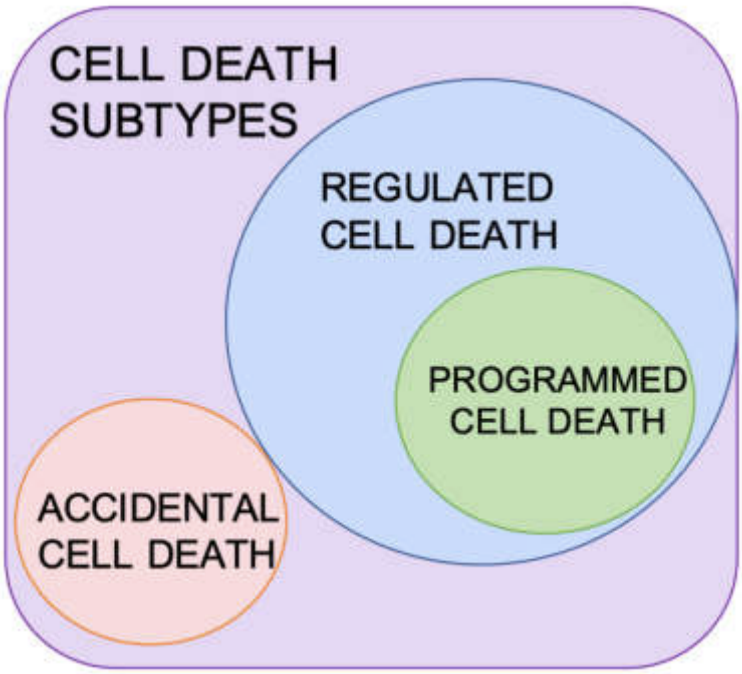

Figure 1. Types of cell death. Regulated cell death can be influenced, at least to some extent, by specific pharmacologic or genetic interventions. The term programmed cell death is used to indicate regulated cell death instances that occur as part of a developmental program or to preserve physiologic tissue homeostasis. Accidental cell death refers to a type of cell death that occurs when cells are exposed to extreme physical, chemical or mechanical stimuli succumbing in a completely uncontrollable manner, reflecting the immediate loss of structural integrity. Adapted from 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. The major actors involved in the signaling pathway define the type of cell death that would be triggered.

For instance, apoptotic cell death is determined by caspases (cysteine-dependent aspartate-directed proteases), while the autophagic machinery rules autophagic cell death. Moreover, executors of cell death may also participate in cell survival; for example, the activation of some caspases play a role in inflammation, proliferation and differentiation, also autophagosome formation is crucial for cell homeostasis through removal of damaged or unnecessary organelles (Galluzzi et al. 2018). Thus, it is thought that the decision of die or survive relies on the nature of the stimuli, and in the status of that particular cell. RCD-inhibitory and -promoting signals coexist and counteract each other, and at some stage one predominate over the other (Galluzzi et al. 2015).

Is there more signaling for death or survival? Where the scales tip, will be the cell fate. For instance, in cancer disease there is a resistance to cell death typically acquired during tumorigenesis, thus in malignant cells RCD-inhibitory signals predominate over RCD-promoting signals. The NCCD has also classified the different cell death modalities described until now, based on the molecular aspects that are essential for the process (Table 1). However, it is important to mention that an interconnection can exist between different cell death modalities (Galluzzi et al. 2018).

Table 1. Principal subroutines of regulated cell death	
Cell death subroutines	Definition
Intrinsic apoptosis.	Type of RCD initiated by perturbations of the extracellular or intracellular microenvironment, demarcated by MOMP (Mitochondrial Outer Membrane Permeabilization), and precipitated by executioner caspases, mainly caspase 3.
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.
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.
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.
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 mainly caspase 1.
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.
Entotic cell death.	A type of RCD that originates from actomyosin-dependent cell-in-cell internalization (entosis) and is executed by lysosomes.
Lysosome-dependent cell death.	A type of RCD demarcated by primary LMP and precipitated by cathepsins, with optional involvement of MOMP and caspases.
Autophagy-dependent cell death.	A form of RCD that mechanistically depends on the autophagic machinery (or components thereof).

Adapted from Galluzzi et al. 2018.

2. Ca^{2+}: THE JANIFORM KILLER

"In the furnaces of the stars the elements evolved from hydrogen. When oxygen and neon captured successive α particles, the element calcium was born."

D. E. Clapham, Calcium Signaling, 2007.

A long time ago, cells had to learn to adapt to changing environments, defying relentless entropy. Hence, cells must trigger signals through messengers whose levels vary over time; in this regard, Ca^{2+} is a highly versatile intracellular signal that regulates broad cellular functions and cell death. Ca^{2+} is not created from an enzymatic reaction or destroyed or converted into an inactive metabolite, thus, its functionality relies on variations in Ca^{2+} concentration in the cytosol and subcellular organelles, which is regulated by Ca^{2+} channels and pumps, and exchangers (Clapham 2007).

Each cell type expresses a unique set of components from the Ca^{2+}-signaling toolkit (Table 2), however, the majority of Ca^{2+}-signaling systems function by generating brief pulses of Ca^{2+}. To trigger specific cellular outcomes, cells decode variations in Ca^{2+} levels; these signals can vary in magnitude as in spatial and temporal characteristics. For instance, localized increment in Ca^{2+} levels in cytosol can regulate cell migration, changes in the frequency and duration of cytosolic free Ca^{2+} oscillations may activate transcription factors, and large sustained increases in Ca^{2+} levels are associated with cell death (Clapham 2007; Monteith, Prevarskaya, and Roberts-Thomson 2017).

Ca^{2+}-signaling toolkit

Receptor-operated, voltage-sensitive and store-operated channels control the influx of Ca^{2+} into the cell, this influx is promoted when Ca^{2+} -permeable channels are open because of the level of free Ca^{2+} in most extracellular fluids (>1mM) is higher in comparison with the level of cytosolic free Ca^{2+} (~100nM), creating a concentration gradient. Once inside the cell, Ca^{2+} can interact with Ca^{2+}-binding proteins or might be sequestered into cellular organelles such as the endoplasmic reticulum (ER) (the largest Ca^{2+} store in cells, reaching millimolar levels) or mitochondrion. Otherwise, the efflux of Ca^{2+} outside the cells requires energy, and it is mediated by the plasma-membrane Ca^{2+}-ATPase (PMCA) or by the Na^{+}/Ca^{2+}-exchanger (NCX), which is electrogenic, exchanging three Na ions for one Ca^{2+} (Berridge, Bootman, and Roderick 2003; Monteith, Prevarskaya, and Roberts-Thomson 2017).

On the other hand, Ca^{2+} level in ER is regulated by sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps, which promotes the entry of Ca^{2+} into ER, whereas IP_3 receptors (IP_3Rs) and ryanodine receptors (RYRs) controls Ca^{2+} efflux from ER. ER Ca^{2+} release is promoted by agonist stimulation through the generation of inositol 1,4,5-trisphosphate (IP_3) through hydrolysis of phosphatidylinositol l-4,5-bisphosphate (PIP_2) carried out by a phospholipase C (PLC). PLC has several isoforms, which are activated by distinct pathways, PLCβ is activated by G-protein coupled receptors, PLCγ is activated by tyrosine-kinase-coupled receptors, an increase in Ca^{2+} concentration activates PLCδ, and PLCε is activated through Ras. The dynamics of IP_3 production varies depending on the PLC isoform that is activated. ER Ca^{2+} release can be also triggered by

calcium-induced calcium release process (CICR), whereby Ca^{2+} promotes its own release from ER. IP_3Rs and RYRs are the principal Ca^{2+} channels that display CICR. IP_3 dictates the sensitivity of IP_3Rs to Ca^{2+}. Also, Ca^{2+} directly activate RYRs, although cyclic ADP ribose can act as a sensitizer, thus, CICR is a process that amplifies microscopic initiation pulses into Ca^{2+} propagating signals (Monteith, Prevarskaya, and Roberts-Thomson 2017).

Another cellular organelle that has Ca^{2+}-transporting proteins is the mitochondrion, these channels include the mitochondrial calcium uniporter (MCU) complex that take up Ca^{2+} electrophoretically inside the mitochondrion, and it can be released through three different pathways: reversal of the uniporter, Na^+/H^+-dependent Ca^{2+} exchange, or as a consequence of permeability transition pore (PTP) (Gómez-Suaga et al. 2018). Ca^{2+} can also interact with different Ca^{2+}-binding proteins, which can function as Ca^{2+}-effectors or Ca^{2+}-buffers. Ca^{2+}-effectors include troponin C, CaM, synaptotagmin, and the annexins, these proteins activate several Ca^{2+}-sensitive cellular processes. Otherwise, Ca^{2+}-buffers, such as calbindin D-28 (CB), and calretinin (CR), function to fine-tune the spatial and temporal properties of Ca^{2+} signals, altering the amplitude and recovery time of individual Ca^{2+} transients (Berridge, Bootman, and Roderick 2003). The components of Ca^{2+}-signaling system create diverse arrays of Ca^{2+} signals that can be different in spatial and temporal properties as shown in Table 2.

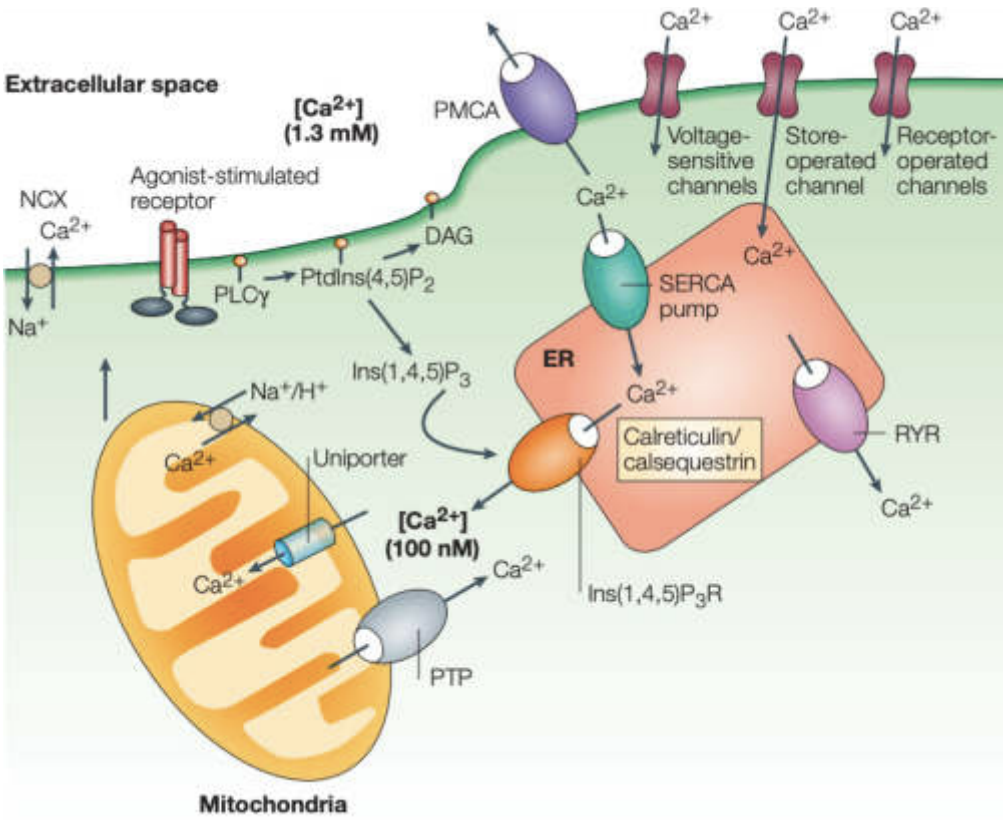

Figure 2. Regulation of intracellular Ca^{2+} compartmentalization. Cellular Ca^{2+} import through the plasma membrane occurs by receptor-operated, voltage-sensitive and store-operated channels. Once inside the cell, Ca^{2+} can interact with Ca^{2+}-binding proteins or be sequestered into the ER or mitochondrion. Ca^{2+} levels in the ER are affected by the relative distribution of SERCA pumps and IPP_3Rs and RYRs, as well as by the relative abundance of Ca^{2+}-binding proteins (calreticulin, calsequestrin) in the ER. The cytosolic Ca^{2+} concentration in unstimulated cells is kept at ~100 nM by both uptake into the ER and Ca^{2+} extrusion into the extracellular space by the PMCA. Ca^{2+}efflux might also be mediated by the Na^+/Ca^{2+} exchanger (NCX). ER Ca^{2+} release is triggered by agonist stimulation through the generation of IP_3 through hydrolysis of PIP_2 operated by a PLC. The mitochondrion takes up Ca^{2+} through a uniport transporter and can release it again through three different pathways: reversal of the uniporter, Na^+/H^+-dependent Ca^{2+} exchange, or as a consequence of permeability transition pore (PTP) opening. Extracted from Orrenius et al. 2003.

Table 2. Ca^{2+}-signaling toolkit	
Receptors	***G-protein-coupled receptors:*** Muscarinic receptors, adrenoceptors, angiotensin receptor, endothelin receptors, metabotropic glutamate receptors, histamine receptor, oxytocin receptor (OT), extracellular Ca^{2+}-sensing receptor (CaR), thrombin receptor (PAR1).
	Tyrosine-kinase-linked receptors: Platelet-derived growth factor receptors (PDGFRα, PDGFRβ), epidermal growth factor receptors.
Transducers	***G proteins:*** Gqα, G11α, G14α, G16α, Gβγ
	Phospholipase C (PLC): PLCβ1–4, PLCγ1, PLCγ2, PLCδ1–4, PLCε, PLCζ.
	ADP ribosyl cyclase
Channels	***Voltage-operated channels (VOCs):*** A plasma-membrane ion channel that is activated by membrane depolarization.
	Receptor-operated channels (ROCs): A plasma-membrane ion channel that opens in response to the binding of an extracellular ligand. MDA receptors (NR1, NR2A, NR2B, NR2C, NR2D), ATP receptor (P2X7), nACh receptor.
	Second messenger-operated channels (SMOCs): A plasma-membrane ion channel that opens in response to the binding of intracellular second messengers such as diacylglycerol, cyclic nucleotides or arachidonic acid.
	Store-operated channel (SOC): A plasma-membrane ion channel, that opens in response to the depletion of internal Ca^{2+} stores.
	Transient receptor potential (TRP) ion-channel family: Plasma-membrane ion channels. This family consists of three groups: the canonical TRPC family, the vanilloid TRPV family and the melastatin TRPM family. TRP channels tend to have low conductances and therefore can operate over much longer time scales without swamping the cell with too much Ca^{2+}.
	Inositol-1,4,5-trisphophate receptors (IP_3Rs): A Ca^{2+}-release channel that is located in the membrane of the endoplasmic reticulum and is regulated by IP_3 and Ca^{2+} itself.
	Ryanodine receptors (RYRs): A Ca^{2+}-release channel that is located in the membrane of the endoplasmic reticulum and is regulated by several factors including Ca^{2+} itself, as well as the intracellular messenger cyclic ADP ribose.
Calcium pumps and exchangers	***Na^+/Ca^{2+} exchangers (NCXs):*** NCX1–3. Plasma-membrane enzyme that exchanges three moles of Na+ for one mole of Ca^{2+}, either inward or outward, depending on the ionic gradients across the membrane.
	Mitochondrion channels and exchangers: permeability transition pore, Na^+/Ca^{2+} exchanger, H^+/Ca^{2+} exchanger, and Ca^{2+} uniporter,

	Plasma membrane Ca^{2+}-ATPases (PMCAs): PMCA1–4. A pump on the plasma membrane that couples ATP hydrolysis to the transport of Ca^{2+} from cytosolic to extracellular spaces.
	Sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCAs): SERCA1–3. A pump located in sarcoplasmic or endoplasmic reticulum membranes that couples ATP hydrolysis to the transport of Ca^{2+} from cytosolic to lumenal spaces.
	Golgi pumps: SPCA1, SPCA2.
Ca^{2+}-buffers	***Cytosolic buffers:*** calbindin D-28, calretinin, parvalbumin.
	ER buffers and chaperones: calnexin, calreticulin, calsequestrin, GRP 78 and 94
Ca^{2+}-effectors	***Ca2+-binding proteins:*** calmodulin, troponin C, synaptotagmin, annexin I–X, neuronal Ca^{2+} sensor family, visinin-like proteins, hippocalcin, recoverin, guanylate-cyclase-activating proteins.

Adapted from Berridge et al., 2003; Monteith1 et al., 2017.

Ca^{2+}-signaling in cell death induction

Although, Ca^{2+} is required for the functionality of cells, Ca^{2+} overload in the cell or perturbation of intracellular Ca^{2+} compartmentalization can trigger catastrophic events that lead to cell death. Thus, Ca^{2+} can act as a survival factor or as ruthless killer.

Endoplasmic reticulum.

Ca^{2+} depletion in ER Ca^{2+} pool or Ca^{2+}-overload in this organelle results in disturbances in protein folding and, as a consequence in ER stress. Under ER stress, the unfolded protein response (UPR) controls cell fate decisions (Kepp et al. 2015). The UPR acts to reduce unfolded protein load in ER through the attenuation of the influx of proteins into the ER, expansion of the ER membrane, and the synthesis of components of the protein folding and quality control machinery (Grootjans et al. 2016). These cellular processes

are mediated by IRE1, ATF6 (activating transcription factor 6), and PERK (protein kinase RNA-like ER kinase) (Grootjans et al. 2016).

In physiological conditions, the ER chaperone GRP78 binds to ATF6, IRE1 and PERK, staying inactive. Accumulation of unfolded proteins in the ER lumen in response to various stimuli competes with these molecular sensors for the binding of GRP78, resulting in ATF6, IRE1 and PERK derepression. After that, IRE1 suffer a dimerization, followed by autophosphorylation which triggers its RNase activity, this allows the degradation of certain mRNAs, and activation of the transcription factor XBP1s, which controls the transcription of genes encoding proteins involved in protein folding, ER-associated degradation (ERAD), protein quality control and phospholipid synthesis. ATF6 is normally localized in ER, but in UPR this factor is transported to the Golgi apparatus, where is processed and released in the cytosol as ATF6f, which controls genes encoding ERAD and XBP1. PERK is also activated after ER stress; PERK phosphorylates eIF2α (eukaryotic translation initiator factor 2α) to attenuate general protein synthesis, allowing the transcription of ATF4, a transcription factor that controls the transcription of genes associated with autophagy, cell death, and antioxidant responses (Fig. 3) (Hetz 2012; Kepp et al. 2015).

Despite the fact that the UPR is activated to restore the homeostasis of the cells, ER stress can trigger cell death as a *pis-aller* mechanism. ER stress can trigger the activation of caspase-12 by m-calpain (Ca^{2+}-activated cysteine protease), or through the formation of IRE1α-TRAF-2-pro-caspase-12 complex, which is formed as part of the UPR. Once activated, caspase-12 acts on effector caspases to generate cell death. It has also been

reported that IRE α can activate the Jun-N-terminal protein kinase (JNK), which activates the proapoptotic Bcl-2 family member Bim, while inhibiting the antiapoptotic protein Bcl-2 (Orrenius, Zhivotovsky, and Nicotera 2003).

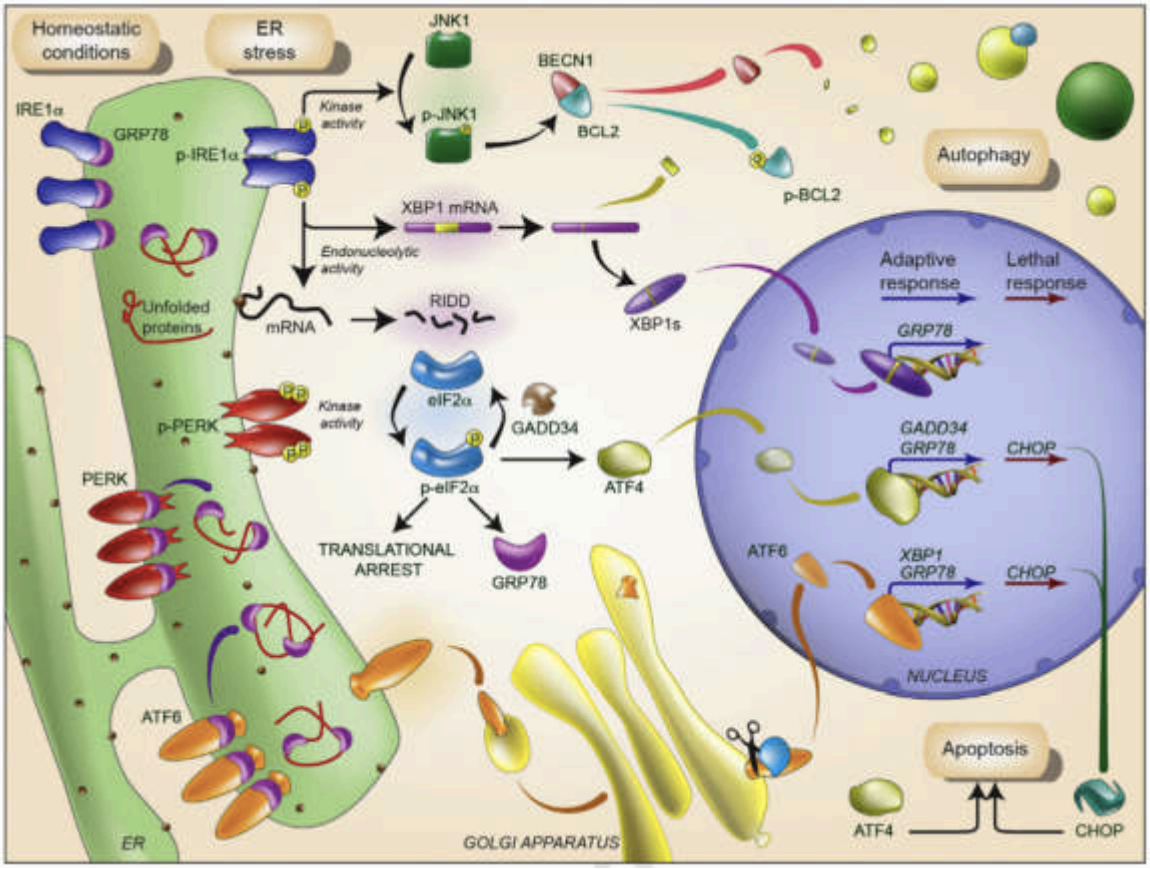

Figure 3. The unfolded protein response (UPR). In physiological conditions, GRP78 binds to, hence inhibiting, activating transcription ATF6, IRE1 and PERK. Unfolded proteins accumulating in the ER lumen in response to various stimuli compete with these molecular sensors for the binding of GRP78, resulting in their derepression. In these conditions, ATF6 is processed by the Golgi apparatus to generate an active transcription factor that activates several proteins involved in the maintenance of reticular homeostasis. IRE1 acquires both an endonucleolytic and a kinase activity, thereby becoming able to catalyze the alternative splicing of XBP1-coding mRNAs, the degradation of other mRNAs localized at the ER, and the phosphorylation of JNK1. Spliced XBP1 codes for a transcription factor that stimulates the synthesis of

proteins implicated in reticular protein handling. Phosphorylated JNK1 promotes autophagy by catalyzing the derepression of the essential autophagic factor beclin 1 (BECN1). PERK phosphorylates eIF2, hence preventing translation at standard start codons while facilitating the use of alternative open reading frames. This results in the post-transcriptional upregulation of ATF4. ATF4 initially promotes the synthesis of GRP78 and GADD34 (an eIF2 phosphatase), hence contributing to the re-establishment of homeostasis. If the protein unfolding-promoting stimulus persists, however, ATF4 (and ATF6) activate CHOP, hence favoring the transition between the adaptive and lethal phase of the ER stress response. Extracted from Kepp 2015.

Along with the UPR, Ca^{2+} depletion from ER Ca^{2+} pool activates the store operated Ca^{2+} entry (SOCE). When the stromal interaction molecules (STIM) (localized in the membrane of the ER away from the plasma membrane when ER Ca^{2+} stores are replete) detects Ca^{2+} depletion through the dissociation of Ca^{2+} from STIM, these proteins are aggregated and translocated to sections of the ER juxtaposed to the plasma membrane and binds Orai, which induces the opening of Orai-CRAC (calcium release activated Ca^{2+}) channels (Fig. 4). This mechanism generates high concentrations of Ca^{2+} inside the cell, which can lead to alterations in plasma membrane potential, PS exposure through the activation of phospholipid scramblases, DNA degradation after the activation of Ca^{2+}-dependent enzymes, the activation of calpains that have a role in cell death, and the crosstalk between calpains and caspases. Moreover, mitochondrion can take up Ca^{2+} electrophoretically from the cytosol through a uniport transporter, a Ca^{2+} overload could

trigger disruptions in the homeostasis of this cellular organelle, and thus, cell death (Monteith, Prevarskaya, and Roberts-Thomson 2017).

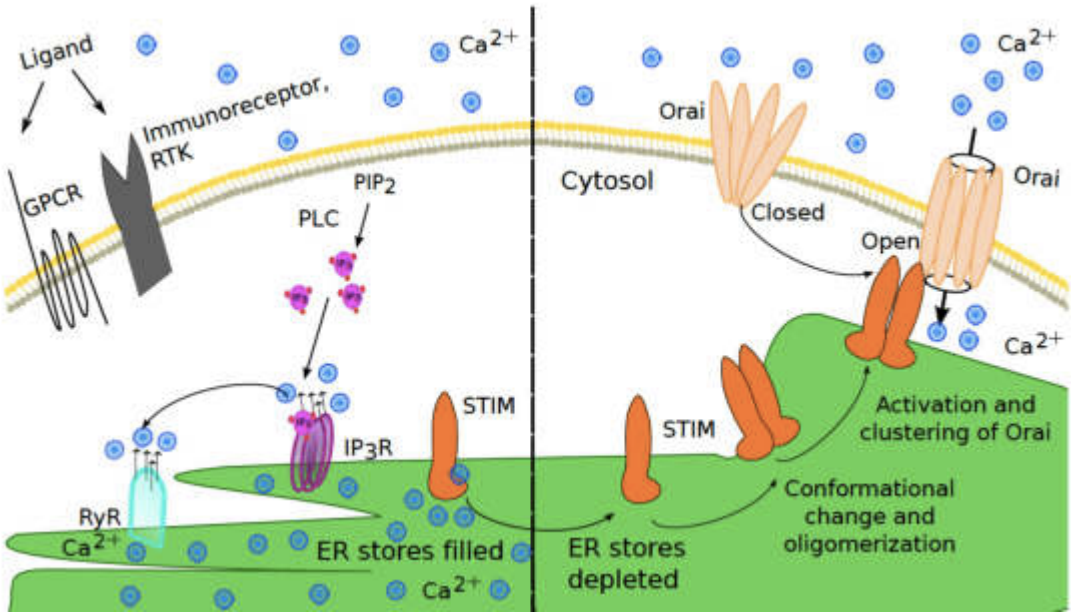

Figure 4. Store operated Ca^{2+}entry. Activation of different types of receptors trigger PLC activation and Ca^{2+} release from the ER, which could lead to depletion of Ca^{2+} ER pool. This results in dissociation of Ca^{2+} from STIM, inducing its aggregation and conformational change (right). STIM then translocates to sections of the ER juxtaposed to the plasma membrane and binds Orai, resulting in the opening of Orai-CRAC (calcium release activated Ca2+) channels. Extracted from Martínez-Torres, 2013.

ER-mitochondrion signaling

The ER and mitochondrion association is the most studied and the first described inter-organelle contact, the ER domain specialized in this association is known as mitochondrion-associated membranes (MAMs), these MAMs are defined as ER membranes that are in close apposition (10–50 nm) to the mitochondrion (Fig. 5) (Kerkhofs et al. 2017). Interactions between mitochondrion and MAMs of ER are

associated with different cellular functions, including inflammasome formation, calcium (Ca^{2+}) signaling, mitochondrion and ER dynamics, autophagy and lipid biosynthesis (Gómez-Suaga et al. 2018).

Ca^{2+} is one of the signals transferred between ER and mitochondrion at the MAMs, this association provide a Ca^{2+} micro-domain, where Ca^{2+} levels are higher than in the bulk cytosol, which is necessary to sustain ER–mitochondrion Ca^{2+} signaling (since the MCU has a low affinity for Ca^{2+}). Once Ca^{2+} is released from ER, it can be transported across the outer mitochondrial membrane (OMM) via the high conductance voltage-dependent anion channel 1 (VDAC1), which is physically coupled to the IP_3R through GRP75, then, Ca^{2+} can cross the inner mitochondrial membrane (IMM) via the mitochondrial Ca^{2+} uniporter (MCU), the poreforming unit in the MCU complex, consisting of MCU itself and its regulators, reaching the matrix (Fig. 6) (Kerkhofs et al. 2018).

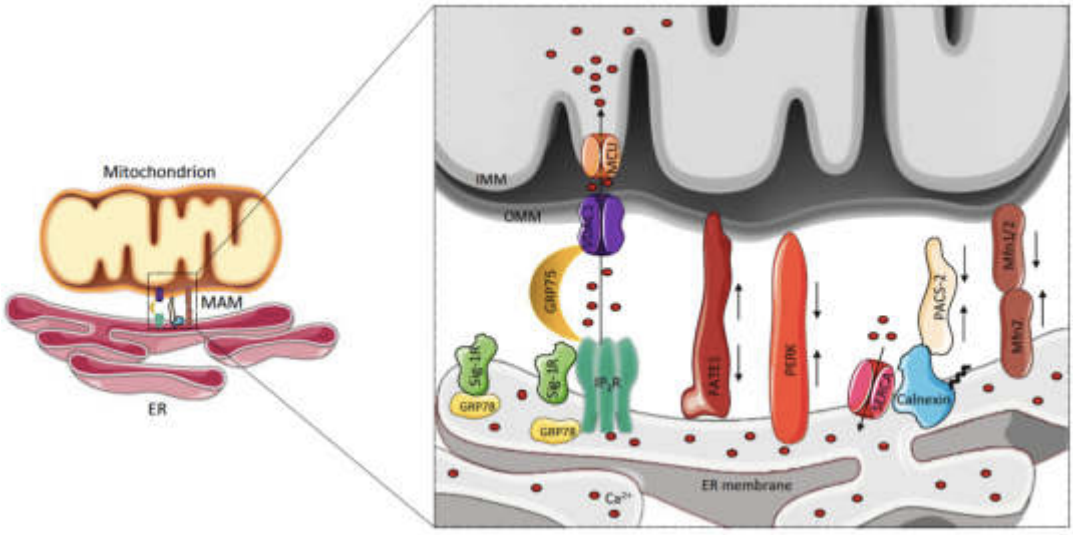

Figure 5. MAM components playing a role in ER-mitochondrial Ca2+ signaling. The principal components of the ER-mitochondrial Ca2+ exchange at the MAMs are the IP3R and VDAC1, which are physically coupled by the chaperone protein GRP75. When Ca^{2+} is released from the ER by the IP_3R, it

freely permeates the OMM via VDAC1, to be transported to the mitochondrial matrix by the MCU, located in the IMM. The chaperone Sig-1R is able to modify IP3Rmediated Ca^{2+} signaling. Binding to GRP78 holds Sig-1R inactive, but under ER stress binding to GRP78 is disrupted and Sig-1R interacts with the IP_3R, stabilizing the IP_3R and enabling proficient Ca^{2+} signaling. The efficiency of Ca^{2+} exchange between ER and mitochondria is influenced by the presence and action of tethering proteins like PERK and Mfn2 and anti-tethering proteins like FATE1. Besides its function as a tethering protein, PACS-2 also contributes to MAM organization, while simultaneously having a role in the enrichment of the chaperone calnexin at the MAMs. Furthermore, calnexin is enriched at the MAMs by palmitoylation, a process that switches calnexin function from quality control/protein folding to ER Ca^{2+}-signaling control by enhancing SERCA activity. Extracted from Kerkhofs et al. 2017.

Mitochondrion

The beauty and cruelty of existence is not something we can handle alone, this struggle extends beyond humans, and the entire organisms in earth compete and help one another. Two billion years ago, some cells evolve to use oxygen as energy source, then, a larger cell engulfed this oxygen-using cell, who survived inside its host, providing energy to the larger cell, these cells became depend one each other for survival. In eukaryote cells, that oxygen-using cell evolve into the mitochondrion, this organelle sequesters the toxic potential of components of the consumed cell, such as the electron-transport chain; for this reason, mitochondrial damage gives rise to signals that can kill the cell (Melino 2001).

The oscillations of Ca^{2+} in the mitochondrion stimulate the mitochondrion's metabolism, for instance, three enzymes that are regulated by Ca^{2+}: pyruvate dehydrogenase, isocitrate

dehydrogenase, and α-ketoglutarate dehydrogenase are part of the TCA cycle. Moreover, Ca^{2+} stimulates the ATP synthase and complex III of the electron transport chain (ETC). In addition, Ca^{2+} influx into the mitochondrion's matrix activates Ca^{2+}-activated K^+ channels and parallel H_2O uptake in the mitochondrion, resulting in cristae compression and H_2O_2 extrusion, which stimulates IP_3R activity (Kerkhofs et al. 2018; Orrenius, Zhivotovsky, and Nicotera 2003).

However, Ca^{2+} overload results in opening of the mitochondrial permeability transition pore (mPTP), either by a direct action of Ca^{2+} on the mPTP or by Ca^{2+} binding to cardiolipin, thereby disrupting complex II of the ETC and subsequent ROS production. mPTP opening leads to mitochondrial swelling, rupture of the OMM, and release of pro-apoptotic factors like cytochrome c and ultimately cell death (Fig. 6) (Kerkhofs et al. 2018; Orrenius, Zhivotovsky, and Nicotera 2003).

As I mentioned before, signals between cells and intracellular signals dictate the cell death modality that will be triggered, also the response to these signals is modulated by the particular components of the molecular machinery present on each cell. In some cells it has been demonstrated that the influx of Ca^{2+} from the extracellular space and the reduction of ER Ca^{2+} levels, leads to an ER stress that favors plasma membrane surface exposure of calreticulin (CRT) (Tufi et al. 2008). CRT exposure facilitates the uptake of dying cells by dendritic cells and the subsequent presentation of tumor-associated antigens to T lymphocytes, hence stimulating an immune response (Krysko et al. 2012). ER stress and Ca^{2+} fluxes has been proposed as common denominators of immunogenic cell death,

thus, these signals are also implicated in the immune responses that will be triggered after cell death.

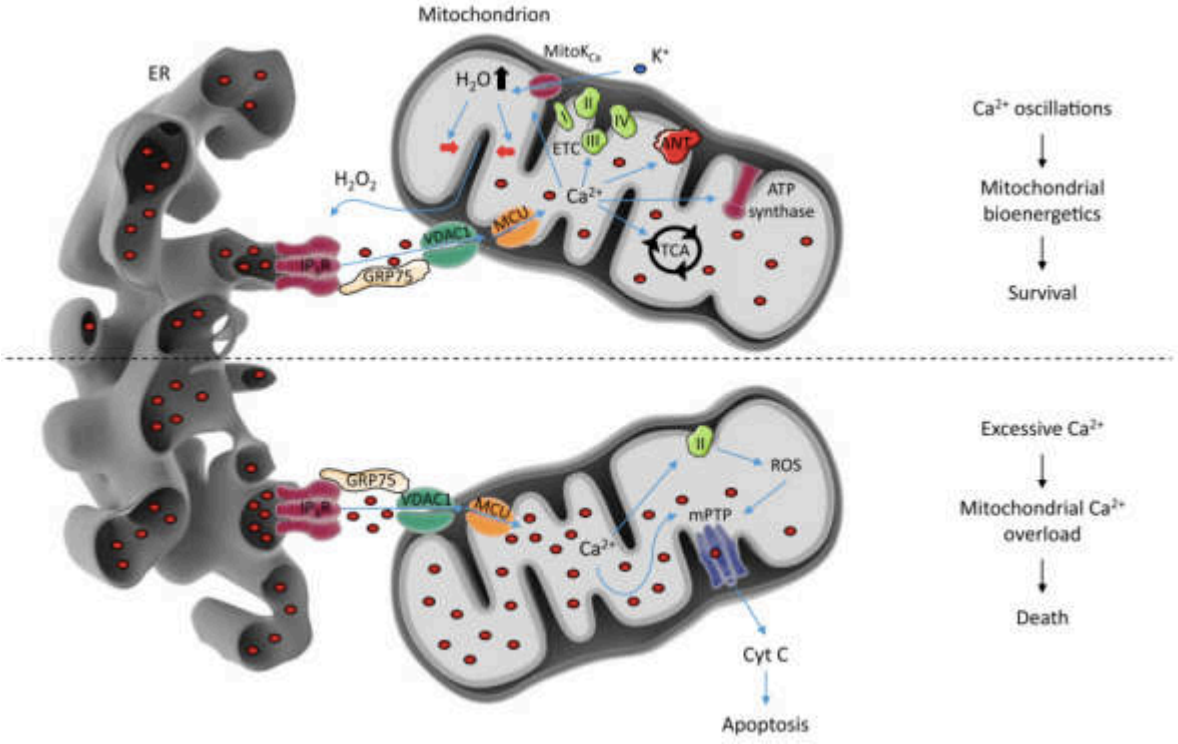

Figure 6. Ca^{2+} signaling at the ER and the mitochondrion in cell death and survival. Arrow-headed lines indicate a stimulatory or consequential effect. The release of Ca^{2+} from ER is mediated by the IP_3R, gated by the intracellular messenger IP_3. Ca^{2+} then travels via VDAC1, which is physically coupled to the IP_3R through GRP75, and MCU to the mitochondrion matrix. Ca^{2+} oscillations targeted to the mitochondrion are able to stimulate mitochondrion's metabolism in several ways. Extracted from Kerkhofs et al. 2018.

3. TOLEROGENIC AND IMMUNOGENIC CELL DEATH

"I have abandoned the belief that the immune system's primary driving force is the need to discriminate between self and non-self. The immune system does not care about self and non-self, its primary driving force is the need to detect and protect against danger."

P. Matzinger, Tolerance, danger, and the extended family, 1994.

The immune system is constantly exposed to dead cells; as part of life, mechanism must exist so that the immune system discriminates between several signals and decide the immunological consequences after cell death. These mechanisms participate in pathogens and cancer cells eradication, healing of tissues while avoiding responses to vital systems of the host. How does the immune system decide? In 2011 Griffith and Ferguson proposed the Five Ws of Dying Cells, saying that the nature of the immune response that develops in the face of dead cells depends on who dies, what it releases, when it dies, where it dies, and why it dies (Griffith and Ferguson 2011). In view of that, cell death can be tolerogenic, immunogenic, or "silent".

Tolerogenic cell death

In a tissue context, phagocytosis of dying cells has a major role in avoiding toxic accumulation of cellular corpses and represents the final stage of cell death. During PCD, dying cells emit "find me" signals (e.g. CX3CL1) to recruit anti-inflammatory cells and ensure their efficient removal, they also release "keep-out" signals (e.g. lactoferrin) to avoid inflammatory cells. Moreover, among these soluble factors, dying cells present a

constellation of “eat me signals” on their surface, which acts on phagocytic receptors on immune cells to facilitating cellular engulfment, whereas living cells avoid phagocytic clearance through surface-associated “don´t eat me” signals (Garg et al. 2016). The best known tolerogenic “eat me” signal is phosphatidylserine (PS), which normally faces the inner lumen of the bilayered plasma membrane in living cells, however during cell death it becomes externalized on the outer leaflet of the plasma membrane owing to the coordinated activity of caspases and scramblases, and inactivation of flippases, this phospholipid binds to a large number of immune receptors in a phagocyte-type manner (Hankins et al. 2015; Nagata, Sakuragi, and Segawa 2020; Segawa and Nagata 2015). Along with PS exposure, externalized cardiolipin, oxidized low-density lipoproteins, thrombospondin, complement C1q and changes in membrane glycosylation status or charges also act as tolerogenic “eat me” signals (Garg et al. 2016).

These tolerogenic “eat me” signals interact with antigen presenting cells (APCs) that exhibits immunosuppressive phenotypes, such as tolerogenic DCs, M2 MΦ, N2 neutrophils, myeloid-derived suppressor cells (MDSCs). As a consequence, APCs that performed tolerogenic phagocytosis do not achieve functional maturation, and present antigens to CD4+ T cells in absence of co-stimulatory signals such as CD80, CD86, CD40 and CD83, and in presence of immunosuppressive cytokines like IL-10 or TGF-β, this promotes the differentiation of naive CD4+ T cells into inducible regulatory T (T_{Reg}) cells and T helper 2 (Th_2) cells, which inhibit pro-inflammatory immune responses. T_{Reg} directly eliminate cytotoxic T lymphocytes (CTLs) through FasL or TNF-related apoptosis-inducing ligand (TRAIL) expression. Additionally, in this scenario CD8+ T cells are stimulated in the absence of activated CD4+ T cells, resulting in the

differentiation of helpless cytotoxic T lymphocytes (CTLs). Helpless CTLs has a short lifespan, and are able to secrete TRAIL which triggers cell death in CTLs and CD4+ T cells, resulting in tolerance (Garg et al. 2016; Green et al. 2009).

Despite the fact that all dying cells can release damage-associated molecular patterns (DAMPs), the mechanism of cell death may modify these immunostimulatory molecules to promote tolerance instead of an immune response. For instance, ROS production can lead to the oxidation of a key cysteine residue in high-mobility group box 1 protein (HMGB1), neutralizing its ability to promote immune responses. Furthermore, some dying cells can release immunosuppressive mediators, such as interleukin-10 (IL-10), transforming growth factor-β (TGFβ), platelet-activating factor (PAF) and prostaglandin E_2 (PGE_2), or promote the release of these factors from the cell that engulfs them (Griffith and Ferguson 2011).

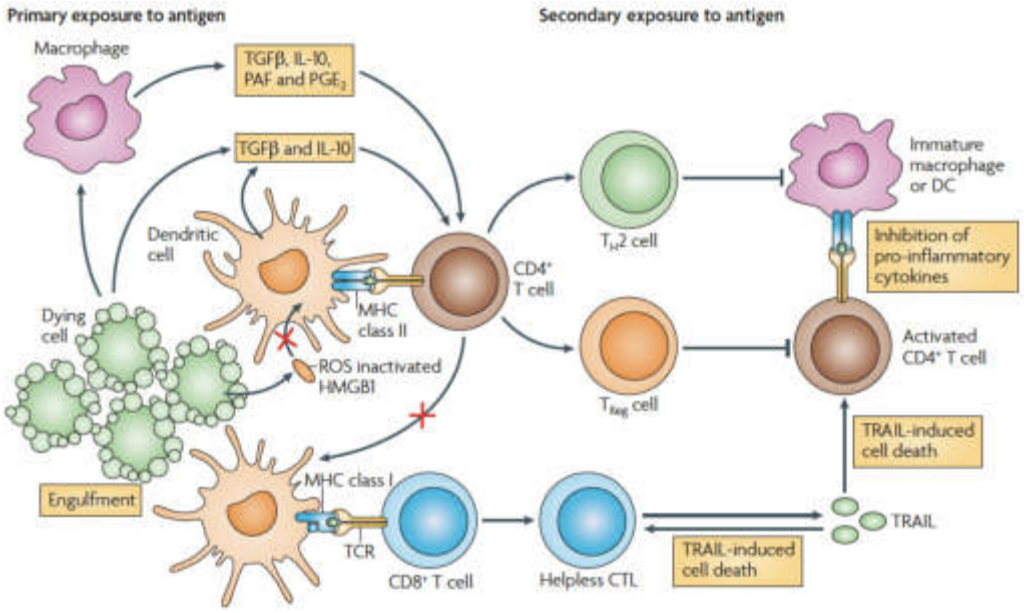

Figure 7. Suggested mechanisms of tolerance induction by dying cells. Cells that undergo apoptosis can release DAMPs, such as HMGB1; however, ROS production during cell death may oxidize HMGB1,

thereby rendering it inactive. In addition, cytokines such as IL-10 and TGFβ can be released from dying cells, or alternatively, dying cells can stimulate macrophages to release these molecules as well as PAF and PGE_2. Production of these cytokines induces the differentiation of naive CD4+ T cells into T_{Reg} cells and Th2 cells, which inhibit pro-inflammatory immune responses. Simultaneously, CD8+ T cells are stimulated in the absence of activated CD4+ T cells, resulting in the differentiation of helpless cytotoxic T lymphocytes (CTLs). The cells can function in a primary cytotoxic response but produce the death ligand TRAIL following secondary exposure to antigen. TRAIL triggers apoptosis of the helpless CTLs and other activated CD4+ T cells, resulting in tolerance. Extracted from Green et al. 2009.

Immunogenic cell death

Immunogenic cell death (ICD) is a cell death modality that does stimulate an immune response against dead-cell antigens. The ability of RCD to drive adaptive immunity relies on two major parameters: antigenicity and adjuvanticity. **Antigenicity** is conferred by the expression and presentation of antigens that fail to induce clonal deletion in the context of central tolerance in a specific host, implying that the host contains naïve T cell clones that can recognize such antigens, hence, normally, healthy cells are limited in their ability to drive ICD, as their antigens are typically expressed by the thymic epithelium during T cell development. **Adjuvanticity** is provided by the spatiotemporally coordinated release or exposure of danger signals that are necessary for the recruitment and maturation of APCs, which are cumulatively referred to as damage-associated molecular patterns (DAMPs); whereas most of these molecules exert non-immunological functions inside the cell, their exposure on the cell surface or their secretion in the extracellular space as a result of cellular stress can function as danger signals for the immune system. Exposure of

calreticulin (CRT), and the release of ATP and HMGB1 are the principal DAMPs associated with ICD (Kroemer et al. 2013).

Different cell death modalities imply a release of **ATP**, this extracellular ATP is a well-known "find me" signal for macrophage and dendritic cells (DCs), upon its binding to P_2Y_2 receptors (widely expressed on cells from the myeloid lineage) (Elliott et al. 2009). Furthermore, it has been demonstrated that ATP stimulates bone marrow–derived DCs maturation with an increase in the expression of CD40, CD80, CD83, and CD86 (Wilkin et al. 2001). Moreover ATP-P_2RX_7 ligation leads to the activation of the inflammasome, which stimulates the proteolytic maturation of pro-inflammatory cytokines, mainly IL-1β and IL-18, and their subsequent release into the extracellular space. As a consequence, IL-1β promotes the functional polarization of IFN-γ-secreting cytotoxic T lymphocytes (CTLs), which are the principal players in the cell-mediated immune response (Ghiringhelli et al. 2009).

Otherwise, cells succumbing to ICD present an array of surface-exposed "eat me" signals, such as **CRT** or HSP90. Interaction of these "eat me" signals with phagocytic receptors on immune cells (e.g. LRP1) aids to remove the cells undergoing ICD (Obeid et al. 2007). Moreover, it has been demonstrated that CRT elicits the production of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) from DCs, thereby facilitating Th1 and/or Th17 polarization. Similarly, HSP90 binding on immune cells facilitates DC maturation and Th1/17 priming (Garg et al. 2016).

HMGB1 is another example of DAMP that can be released by cells succumbing ICD, it has been demonstrated that HMGB1 binds to TLR4, activating the release of pro-inflammatory cytokines by monocytes/macrophages, also, when it is associated with CXCL12 it can mediate mononuclear cells' recruitment. In DCs, HMGB1 increase the expression of pro-IL-1β and avoids the lysosomal degradation of engulfed tumor antigens, which is a major prerequisite for efficient cross-presentation (Guido Kroemer et al. 2013; Krysko et al. 2012).

As a consequence, APCs that carried out immunogenic phagocytosis are able to present the antigens to CD4+ T cells in presence of co-stimulatory molecules and pro-inflammatory cytokines, such as IL-6, IL-12, IL-1β, promoting the differentiation of Th1 cells who orchestrate a type-1 immunity consisting of IFN-γ-driven antigen-directed cytostatic effects and suppression of T_{Reg} differentiation. Also, these APCs stimulate the cross-talk between Th1 cells and CTLs and thereby facilitating CTL-elicited cell elimination through IFN-γ, FasL-CD95 interaction and perforin-granzyme action (Green et al. 2009).

Molecular mechanisms behind Immunogenic cell death

The kinetics and intensity of DAMPs release are dictated by intracellular responses driven by the initiating stressor. Overgeneration of ROS, ER stress, and autophagosome formation are mechanisms that activate danger-signaling pathways, which stimulate the exposure/release of DAMPs. It has been demonstrated that ROS may be crucial for cell death-immunogenicity as this was diminished in the presence of antioxidants (Panaretakis

et al. 2009). Moreover, it is proposed that simultaneous presence of ER stress and ROS production increase the number of DAMPs emitted (Krysko et al. 2012; Martins et al. 2011). Nevertheless, the stress in the ER has been observed in almost all scenarios of ICD described so far. It has been demonstrated that several ICD inductors compromise cells with an intense ER stress which involves an overgeneration of ROS, increased cytoplasmic Ca^{2+} concentrations, and PERK activation. It has been observed that CRT translocation process requires the activation of PERK (Luo and Lee 2013), and as I mentioned before, activated PERK phosphorylates eIF2α (P-eIF2α), which has been established as an early ICD biomarker. P-eIF2α stimulates translational activity of ATF4 that regulates expression of autophagy-associated genes, such as Atg5, Atg7, and Atg10 (Kepp et al. 2013).

In addition, ER stress triggers JNK1 phosphorylation by IRE1α, which promotes autophagy by catalyzing the derepression of the essential autophagic factor beclin 1 (Fig. 3) (Kepp et al. 2013; Kroemer et al. 2013). Moreover, autophagic vesicles loaded with ATP can fusion with the plasma membrane to release this nucleotide into the extracellular medium upon autophagy stimulation in a V-SNARE-dependent manner (Fader, Aguilera, and Colombo 2012).

ICD has been taking great importance in regard to cancer therapy, preclinical and clinical data support the idea that the way in which cancer cells succumb to cell death in response to treatment may be far more important for long-term disease outcome, than the fraction of cells that die. Some scientists believe that, as cancer therapies are unable to eradicate 100% of cancer cells, which is an utopian goal, the efforts in cancer research should

concentrate on the development of combinational therapeutic regimens that render cancer cell death immunogenic and revert immune exhaustion or suppression.

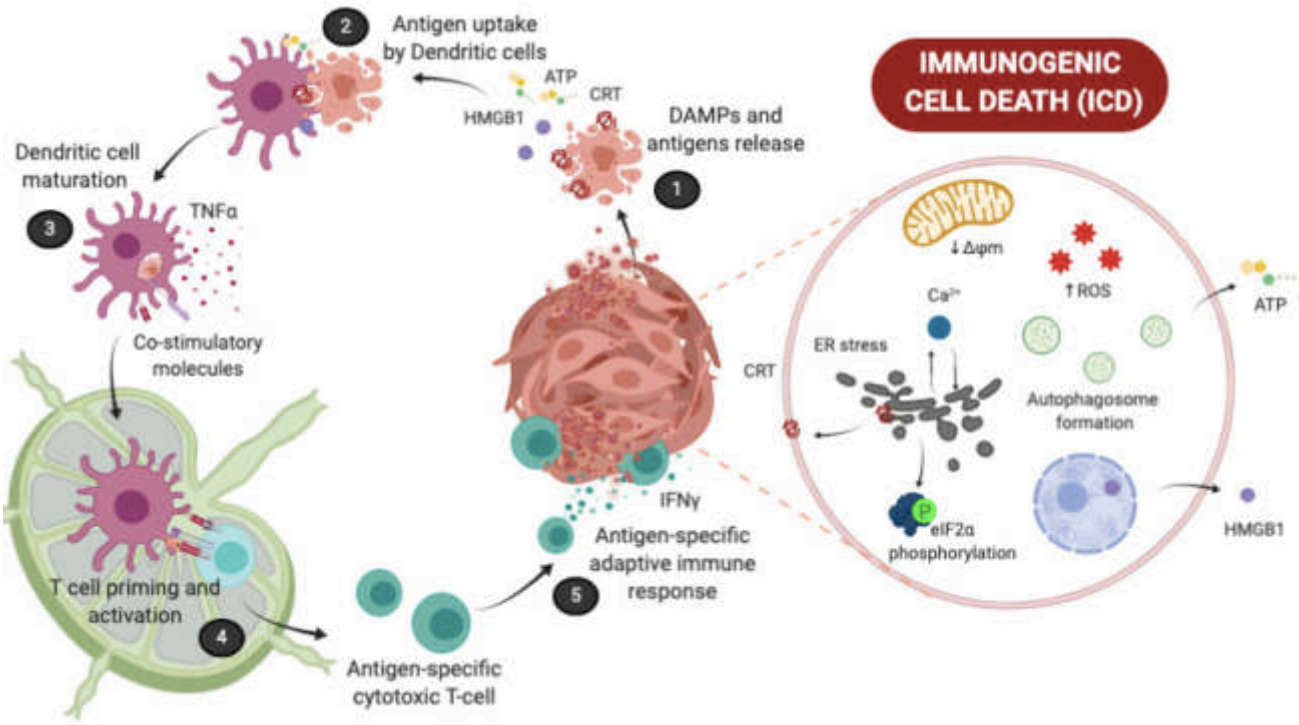

Figure 8. Immunogenic cell death (ICD). In the course of ICD, the cell may suffer deregulation in Ca^{2+} homeostasis that can lead to loss of mitochondrial membrane potential and overgeneration of ROS. This could generate an ER stress, triggering CRT exposure, eIF2α phosphorylation and autophagosome formation. These danger-signaling pathways lead to alterations in the composition of the plasma membrane of dying cells (CRT exposure), as well in their microenvironment (release of ATP and HMGB1), which stimulate DCs maturation. These DCs can then prime CD4+ and CD8+ T cells and thereby trigger immunogenic T helper 1 (Th1) cell and cytotoxic T lymphocyte (CTL) responses, respectively, promoting an immune response against dead-cell antigens.

4. CANCER: THE DISEASE THAT DESTROYS THE HOST WHO NOURISHES IT.

"If prayers are heard in Heaven, this prayer is heard the most: Dear God, please, not cancer. We lack only the will and the kind of money and comprehensive planning that went into putting a man on the moon." Citizens Committee for the conquest of Cancer, Mr. Nixon you can cure cancer, 1969.

Cancer is one of most important unsolved problems we have in medicine, this word encloses a group of diseases that start in almost any tissue of the body when abnormal cells grow uncontrollably, go beyond their usual boundaries to invade adjoining parts of the tissue and/or spread to other organs, this process is called metastasizing and is a major cause of death from cancer. Cancer is the second leading cause of death globally, accounting for an estimated one in six deaths, in 2018. Lung, prostate, colorectal, stomach and liver cancer are the most common types of cancer in men, whereas breast, colorectal, lung, cervical and thyroid cancer are the most common among women (WHO 2020).

Besides its heterogeneity, all cancer diseases share these hallmarks: cell death resistance, deregulation in cellular energetics, sustaining proliferative signaling, growth suppressor evasion, avoidance of immune destruction, replicative immortality, tumor-promoting

inflammation, activation of metastasis and invasion, angiogenesis induction, and genome instability (Hanahan and Weinberg 2011).

Breast cancer

Breast cancer is the most common malignancy and the leading cause of cancer-related death in women worldwide (Fig. 9) (WHO 2020). On the molecular level, breast cancer is a heterogeneous disease, including molecular features such as expression of human epidermal growth factor receptor 2 (HER2), expression of hormone receptors (estrogen receptor and progesterone receptor) and/or BRCA mutations. The classification of Perou and Sorlie in 2000, proposed four subtypes of breast cancer: luminal A and luminal B (expressing hormone receptors), basal-like and HER2-enriched (without hormone receptor expression), and treatment strategies differ according to molecular subtype (Dai et al. 2016).

One of the principal pitfalls leading to the mortality of breast cancer is associated with distant metastasis and its ability to recur up to 20 years after diagnosis (Fig. 10), whereas localized disease is curable in ~60–80% of patients with early-stage, metastatic or recurrent disease carries a dismal prognosis (Richman and Dowsett 2019). This scenario is related to the low immunogenicity of breast cancer cells, as a result of cancer cell release of immune-suppressive factors, which block the cancer-immunity cycle (Gatti-Mays et al. 2019). However, it is now being proposed that with appropriate immune response stimulation, any cancer cell could be turn immunogenic. It has been reported that breast cancer patients treated with anthracyclines (ICD inducers) showed an increment in the

ratio of CD8+ T cells over regulatory T cells intratumorally, and this predict a favorable therapeutic response (Ladoire et al. 2011).

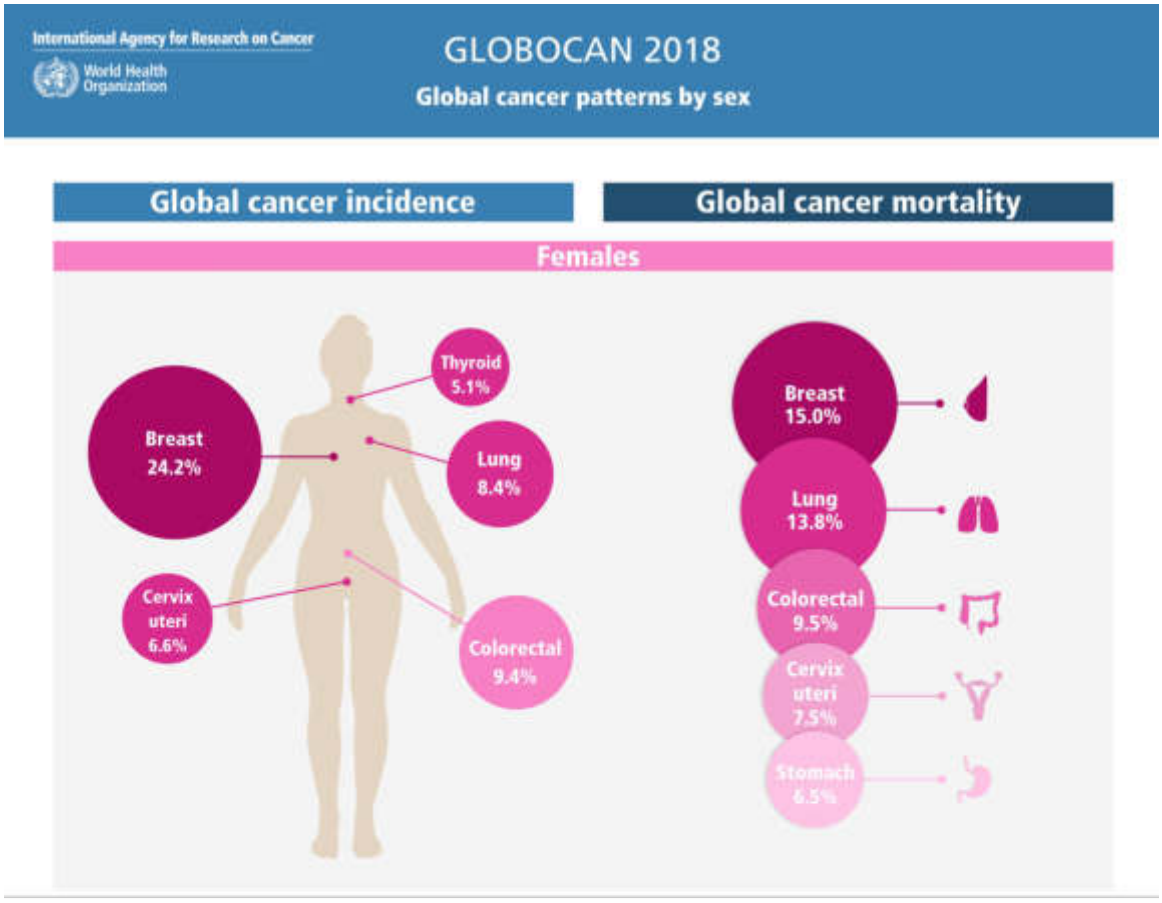

Figure 9. Global cancer incidence and mortality in women. Extracted from Globocan 2018.

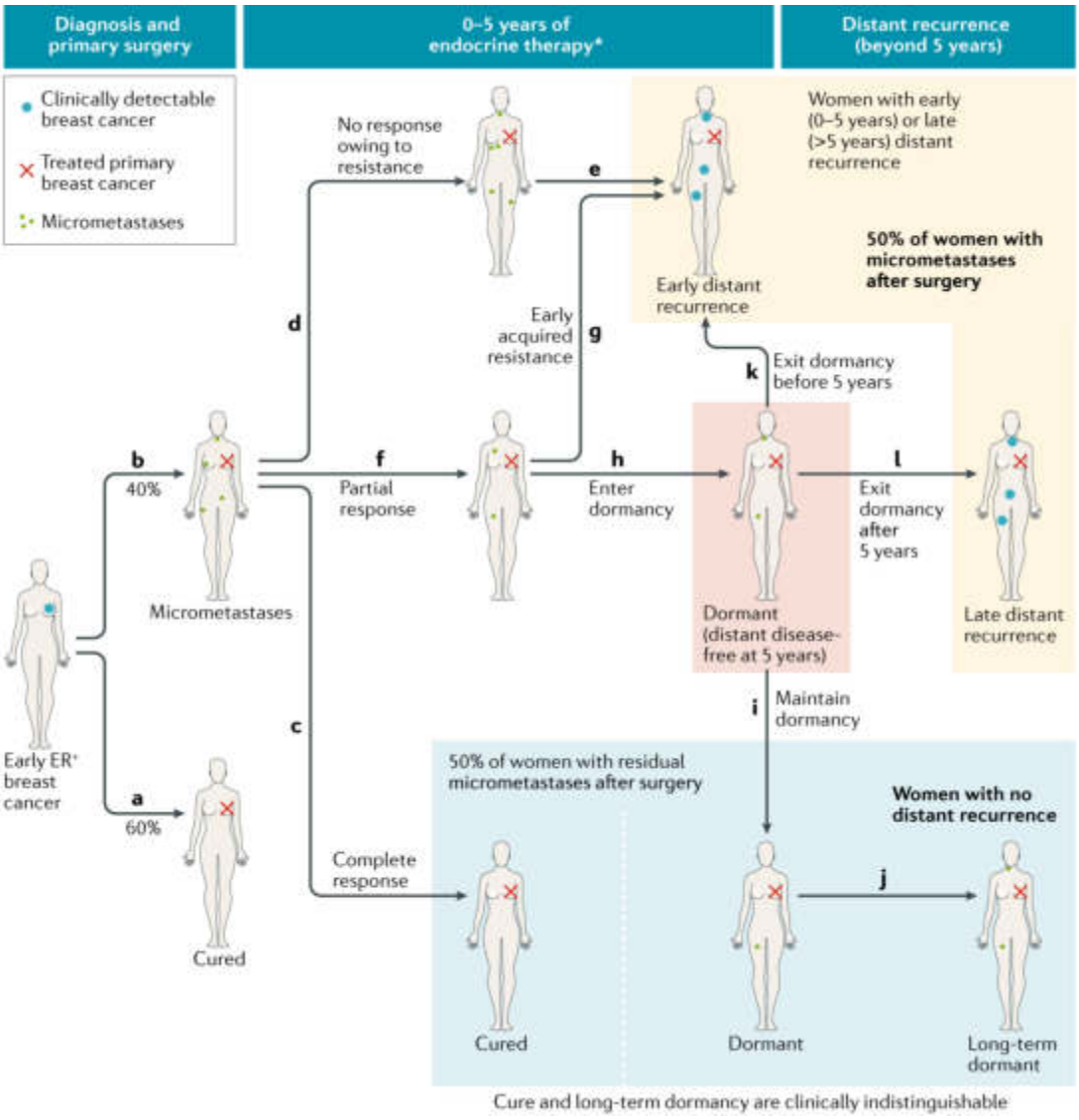

Figure 10. Outcomes of women with ER-positive breast cancer. Out of ~46,000 women diagnosed with ER+ breast cancer annually, ~60% will be cured with surgery alone (part a), and ~40% will have residual micrometastatic disease after surgery (part b). The majority of these women will be treated with endocrine therapy and might subsequently have a complete response (part c), no response (part d), which can lead to metastatic outgrowth (part e) within a short period of time, or a partial response (part f). A partial response would be associated with the presence of residual micrometastatic disease, which would either acquire resistance leading to early recurrence (part g) or enter into a dormant state (part h). Dormant micrometastatic disease (part i) can be maintained, a process that can continue beyond 5 years (part j) or exit from dormancy

can occur within 5 years (part k) or beyond 5 years (part l). The percentages given after 5 years are for the population of women with micrometastatic disease after surgery and not the overall population. *Sometimes preceded by adjuvant radiotherapy and/or chemotherapy. Extracted from Richman and Dowsett 2019.

5. RETURNING CANCER CELLS TO THEIR IMMUNITY CYCLE TRHOUGH IMMUNOGENIC CELL DEATH

Cancer immunosurveillance

In 1891 oncology met immunology when William B. Coley, a renowned surgeon at Memorial Hospital in New York, noticed that cancer patients who got infections after surgery seemed to do better than people who didn't get an infection, thus he tried immunomodulating therapy for cancer with the use of erysipelas in a patient with inoperable sarcoma: Signor Zola, who survived another 8 years after being treated by Coley (Coley 1991). He then created a filtered mixture of bacterial lysates called “Coley’s Toxins” to treat tumors, his first patient named John Ficken with a large inoperable tumor (likely a malignant sarcoma) had a complete remission, lasting until his death 26 years later of a heart attack. Some reports indicate that of 186 patients, in 105 (57%), the treatment was regarded as successful (of these, 35 tumors were operable and 70 tumors were primarily inoperable, 2 of which were treated with apparent success with radiotherapy), today, it is believe that Coley’s Toxins may boost the immune system to attack cancer cells. However, clinical interest diminished in favor of radiotherapy and chemotherapy, which promised a breakthrough in cancer treatment (Kienle 2012; Richardson et al. 1999; Wiemann and Starnes 1994).

Sixteen years later, Paul Ehrlich proposes that the immune system usually suppresses tumor formation, a concept that becomes known as the “immune surveillance” hypothesis (Ehrlich 1909), later confirmed by Robert Schreiber, Lloyd Old, and others in the first decade of the third millennium (Shankaran et al. 2001; Smyth, Thia, Street, Cretney, et al. 2000; Smyth, Thia, Street, MacGregor, et al. 2000). This allowed to define the Cancer-Immunity Cycle concept, which implies that tumor-associated antigens, accompanied by danger signals, are released by cancer cells and then captured by dendritic cells (DCs) for processing, promoting their efficient immunocompetence against cancer (Fig. 11) (Chen and Mellman 2013).

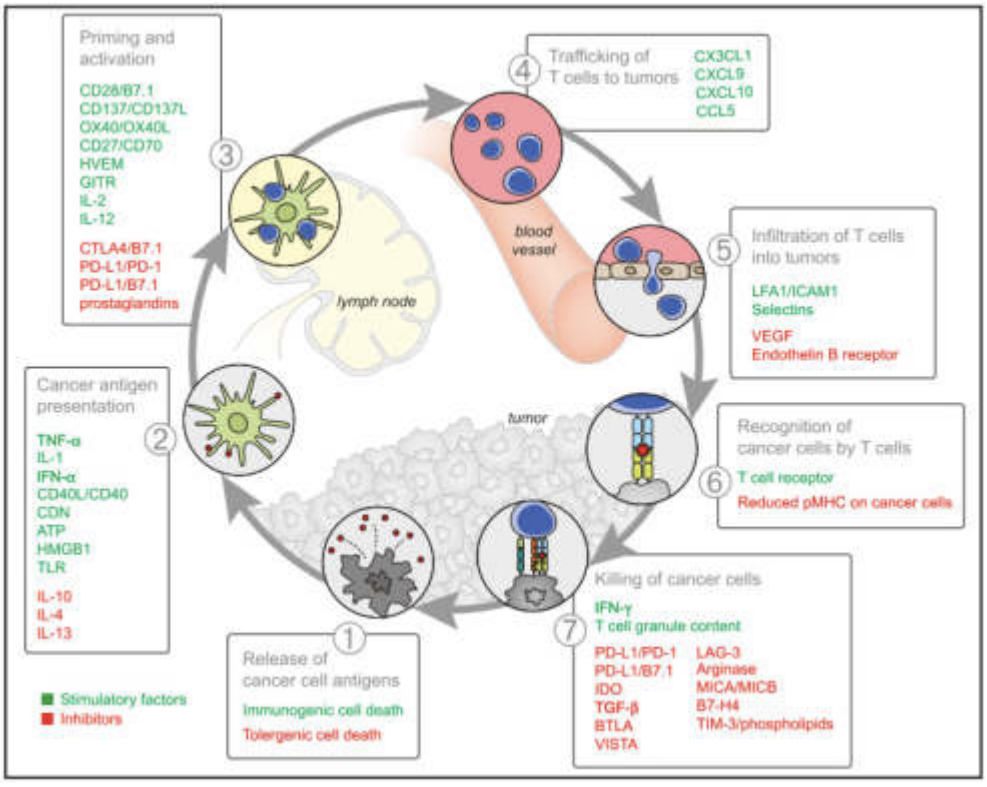

Figure 11. The Cancer-Immunity Cycle. In the first step, neoantigens created by oncogenesis are released and captured by dendritic cells (DCs) for processing (step 1). Next, DCs present the captured antigens on MHCI and MHCII molecules to T cells (step 2), resulting in the priming and activation of effector T cell

responses against the cancer-specific antigens (step 3). The nature of the immune response is determined at this stage, with a critical balance representing the ratio of T effector cells versus T regulatory cells being key to the final outcome. Finally, the activated effector T cells traffic to (step 4) and infiltrate the tumor bed (step 5), specifically recognize and bind to cancer cells through the interaction between its T cell receptor (TCR) and its cognate antigen bound to MHCI (step 6), and kill their target cancer cell (step 7). Killing of the cancer cell releases additional tumor-associated antigens (step 1 again) to increase the breadth and depth of the response in subsequent revolutions of the cycle. Each step of the Cancer-Immunity Cycle requires the coordination of numerous factors, both stimulatory and inhibitory in nature. Stimulatory factors shown in green promote immunity, whereas inhibitors shown in red help keep the process in check and reduce immune activity and/or prevent autoimmunity. Extracted from Chen and Mellman 2013.

Despite the fact that cancer immunosurveillance challenges cells that have undergone neoplastic transformation, the most immunoevasive or highly mutagenic cancer cells are able to escape immunosurveillance and generate a clinically relevant tumor. Thus, cancer cells within an established tumor are able to resist anti-tumor immunity. Along with immunosuppressive microenvironment within the tumor, cancer cells directly employ a number of mechanisms for immunoevasion, such as acquaintance of low immunogenicity by downregulating tumor-associated antigens (TAAs) and MHC class I expression, inciting tolerance by suppressing CD4+/CD8+T cells via immunosuppressive cytokines (e.g. IL-10 or TGFβ) or immune-checkpoints (e.g. programmed cell death 1, programmed cell death-ligand 1, cytotoxic T lymphocyte-associated protein-4), and resisting immune cell-mediated lysis by blunting cell death pathways. Thus, apart from therapies involving direct stimulation of innate or adaptive immune cells, cancer cells are also required to be made immunogenic (Garg and Agostinis 2017; Garg, Dudek, and Agostinis 2013).

Immunological responses triggered by anti-cancer therapy-induced cell death

Induction of cell death by pharmacological means is the basis of almost every non-invasive cancer therapy. Anti-cancer therapy-induced cancer cell death can be subdivided into three distinct profiles: tolerogenic cell death (TCD), immunogenic cell death (ICD), and inflammatory cell death. TCD elicits induction of tolerogenicity by suppressing anti-cancer immunity through the release of anti-inflammatory factors. ICD elicits production of pro-inflammatory cytokines due to co-stimulation conveyed by DAMPs, resulting in immunostimulatory phagocytosis of cancer cell-corpses. Inflammatory cell death elicits acute phase response and other such innate inflammatory reactions that are composed of some tolerogenic and immunogenic signals that create ambiguous immune responses. As I as mentioned earlier, TCD suppress DC activity thereby reducing the possibility of CD4+/CD8+ T-cell activation in general, thereby, it is not surprising that TCD-inducing anticancer therapies (e.g. etoposide, cisplatin, docetaxel or mitomycin C) facilitates accumulation of immunosuppressive immune cells such as M2 MΦ, MDSCs, Th_2 cells, and T_{Reg} cells within the tumor. Otherwise, ICD has received high attention because the exposure of cancer cells to ICD inducers (e.g. anthracyclines, cyclophosphamide, oxaliplatin) is (in most cases) adequate for the activation of robust anti-tumor immunity (Fig. 12) (Dudek et al. 2013; Garg and Agostinis 2017; Zhou et al. 2019).

Therefore, therapeutic success in cancer patients involves an ICD inducer, the response to the immunogenic signals triggered by the ICD, and tumor infiltration by immune effectors, which return cancer cells to the Cancer-Immunity Cycle (Fig. 11) (Zitvogel, Kepp, and Kroemer 2011). Several studies indicate that ICD is a hopeful strategy to

convert cancer cells into their own vaccine, promising a long-term success of anticancer therapies (Li 2017).

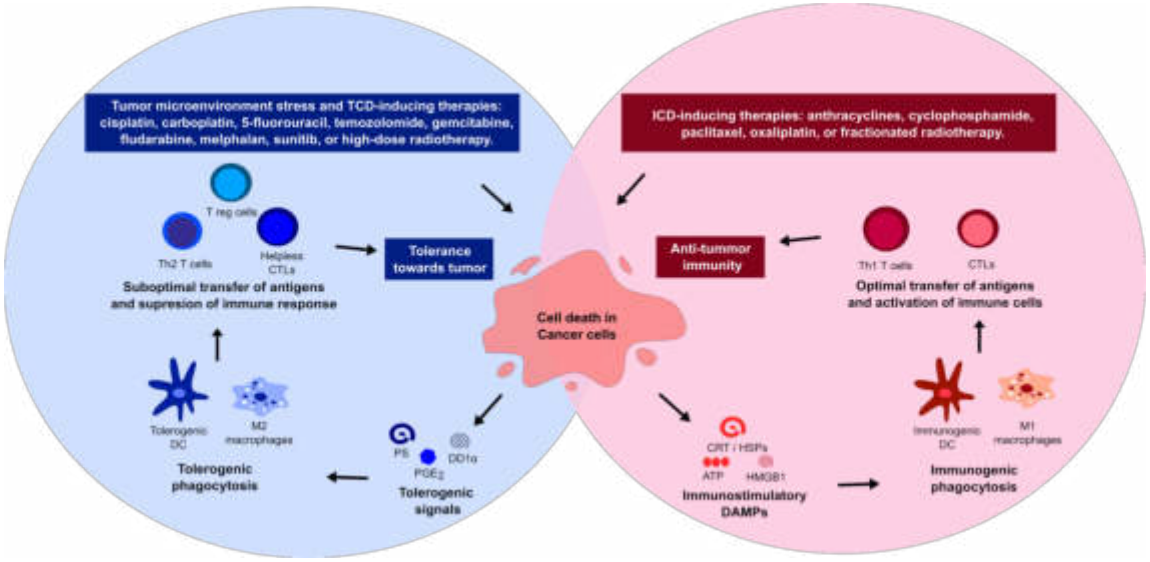

Figure 12. Immunological responses triggered by anti-cancer therapy-induced cell death. Immunological profiles of tolerogenic cell death (TCD) and immunogenic cell death (ICD), and their consequences for anti-tumor immunity or tolerance toward tumor.

Nonetheless, due to the complexity of cancer diseases, ICD may not succeed, because of immunotherapy-resistance mechanisms, such as low presence of tumor-infiltrating lymphocytes (TILs), T-cell anergy or exhaustion, enrichment of the tumor with immunosuppressive factors or immune-checkpoints, low MHC-I expression, unresponsiveness to IFNs, and low burden of TAAs. In addition, resistance mechanisms that directly inactivate immunogenic signaling can also form a hurdle, these mechanisms include mutations in pattern recognition receptors (PRRs), mutations or ablation of proteins mediating danger signaling, and disruption of immunogenic phagocytosis (e.g. by downregulating endogenous levels of CRT) (O'Donnell, Teng, and Smyth 2019).

Hence, it is important to overcome such resistance mechanisms via combinatorial regimens, for an efficient exploitation of ICD in cancer therapy (Fig. 13). Moreover, chemotherapy and radiation are often administered to patients at high doses, which has been associated with immunosuppressive rather than immunostimulatory effects, thereby, in these scenarios cancer cell death is prone to be overlooked by the immune system or to stimulate immune tolerance (Baskar et al. 2012; Zitvogel, Kepp, and Kroemer 2011), hence, the discovery of novel and efficient ICD inducers needs prioritization in cancer research.

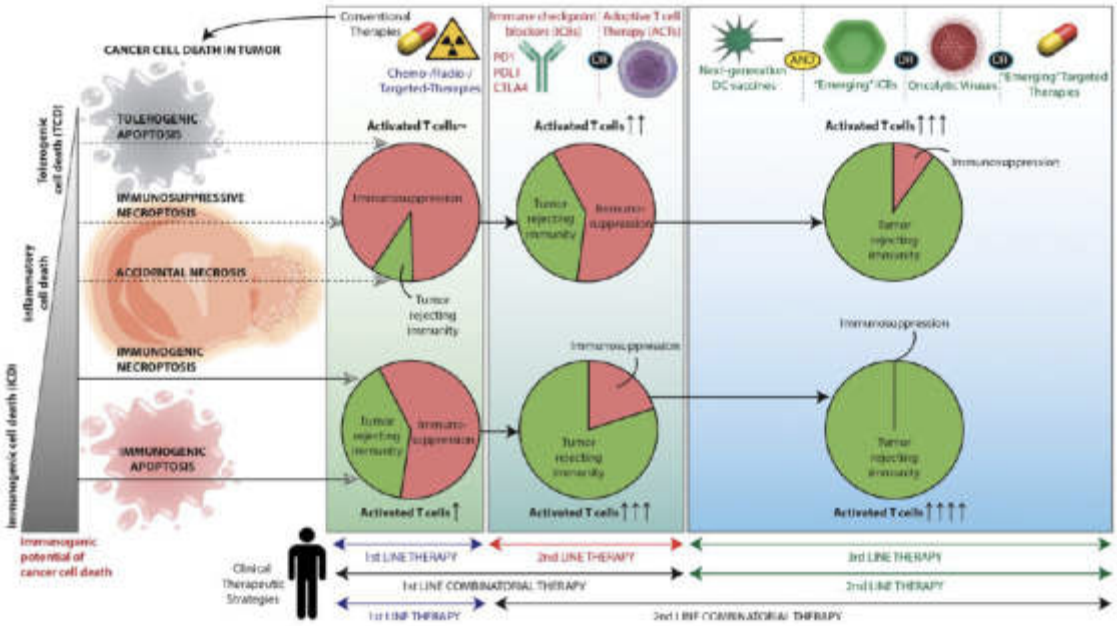

Figure 13. Combinatorial regimens and immunological profiles. Schematic representation of how different immunological profiles of anti-cancer therapy-induced cell death can be exploited for designing highly efficacious cancer immunotherapy regimens. Extracted from Garg and Agostinis 2017.

6. THE BOVINE DIALIZABLE LEUKOCYTE EXTRACT, IMMUNEPOTENT CRP, AS AN IMMUNOGENIC CELL DEATH INDUCER

IMMUNEPOTENT CRP (ICRP) is a bovine dialyzable leukocyte extract (DLE) obtained from disrupted spleen. ICRP is a mixture of substances with biological activity, which provides several applications in human health. Nowadays ICRP has demonstrated to exert immunomodulatory as well as anti-cancer functions that I will further describe.

As for immunomodulatory activities, ICRP improved survival (90%) in BALB/c mice with LPS-induced endotoxic shock, decreasing IL-10, IL-6, and TNF-α levels in serum (Franco-Molina et al. 2004). In addition, ICRP decreased NO, TNF-α and IL-6 but increased IL-10 production in LPS-stimulated murine peritoneal macrophages (Franco-Molina et al. 2005). Moreover, in LPS-stimulated human macrophages ICRP increased endogenous antioxidants activity (catalase, glutathione peroxidase, and superoxide dismutase), and decreased cyclooxygenase-2 activity, PGD_2, NO, and TNF-α production (Franco-Molina et al. 2011). Furthermore, in lung and breast cancer patients undergoing chemotherapy or/and radiation therapy, ICRP administration resulted in improved life-quality and immunomodulatory activity (increasing the total leukocytes and T-lymphocyte subpopulations) (Franco-Molina et al. 2008; Lara et al. 2010).

In regard to anti-cancer proprieties, ICRP induced loss of cell viability in breast (MCF-7, BT-474, MDA-MB-453), lung (A549, A427, Calu-1, INER-51), cervical (HeLa, SiHa), and lymphoid (L5178Y, K562, MOLT-3) cancer cell lines, without affecting the viability

of human monocytes and PBMCs, and murine peritoneal macrophages (Franco-Molina et al. 2006; Martinez-Torres et al. 2019; Martínez-Torres et al. 2018; Sierra-Rivera et al. 2016).

In cervical and lung cancer cell lines, ICRP triggered caspase-independent cell death relying on ROS production, which involves cycle arrest, DNA degradation, and mitochondrial damage (Martinez-Torres et al. 2019; Martínez-Torres et al. 2018). In murine melanoma, ICRP decreased the viability of B16F10 cells, which involves an activation of caspase-3, also, ICRP-treatment decreased tumor weight and improved the survival of tumor-bearing mice, showing a decrease in VEGF production and prevention of metastasis (Franco-Molina et al. 2010), moreover, ICRP combined with oxaliplatin increased DAMPs release and the rate of ICD (Rodríguez-Salazar et al. 2017).

In breast cancer cells, ICRP induced caspase-independent, ROS-dependent cell death and ROS-dependent autophagosome formation (Alvarez-Valdez, 2018); furthermore, breast tumor-bearing mice treated with ICRP present a decrease in tumor volume and increase in survival in comparison with untreated mice. Also, within the tumor, ICRP treatment decreased PD-L1, IDO and Gal-3 expression, IL-6, IL-10, and MCP-1 levels, and increased IFN-γ, and IL-12 levels. Moreover, ICRP treatment increase CD8+ T cells, memory T cells, and innate effector cells in peripheral blood, where they also observed an increase in IFN-γ, and IL-12 levels (Santana-Krímskaya et al. 2020). These results strongly suggest that ICRP may induce ICD in a breast cancer model.

III. JUSTIFICATION

Breast cancer is the most frequently diagnosed cancer and the leading cause of deaths in women. One of the principal pitfalls leading to the mortality of this disease is associated with distant metastasis and its ability to recur up to 20 years after diagnosis, these characteristics are related with the low immunogenicity of breast cancer cells, as a result of cancer cell release of immune-suppressive factors, which block the cancer-immunity cycle. However, it is now being proposed that with appropriate immune response stimulation, cancer cells could be turn immunogenic. In this regard, several studies indicate that immunogenic cell death (ICD) is a hopeful strategy to convert cancer cells into their own vaccine, promising long-term success of anticancer therapies relying on memory immune response induction, which could deal against high recurrence rate in breast cancer patients. IMMUNEPOTENT CRP (ICRP) is cytotoxic to several cancer cell lines, and induces ICD in the murine melanoma model B16F10, whereas in MCF-7 and HeLa cells, ICRP-mediated cell death involves reactive oxygen species (ROS)-dependent autophagosome formation, and loss of mitochondrial membrane potential, which could be associated with deregulations in Ca^{2+} homeostasis. Ca^{2+} fluxes, overgeneration of ROS, and autophagy stimulate intracellular danger signaling pathways that regulate the release of DAMPs and thus, ICD. These results strongly suggest that ICRP might induce ICD in other cancer models, as a conserved mechanism. The aim of this study was to investigate the immunogenicity of ICRP-induced cell death in a breast cancer model as well as the role of Ca^{2+} in this mechanism, using human and murine cell lines, as well as, *ex vivo* and *in vivo* experiments using BALB/c mice.

IV. HYPOTHESIS

IMMUNEPOTENT CRP induces Ca^{2+}-dependent cell death on breast cancer cells, which leads to immune system activation.

V. OBJECTIVES

Main objective:

To evaluate the role of Ca^{2+} and the immunogenicity of IMMUNEPOTENT CRP-induced cell death on breast cancer.

Specific objectives:

To investigate the implication of Ca^{2+} in the mechanism regulating ICRP-induced cell death on breast cancer cells through:

- The analysis of the capacity of ICRP to induce an increase in cytoplasmic Ca^{2+} levels on breast cancer cells.
- The characterization of Ca^{2+}-dependence in the regulated cell death, ROS production, loss of mitochondrial membrane potential, and calreticulin exposure mediated by ICRP on breast cancer cells.

To evidence the immunogenicity of ICRP-mediated cell death in a breast cancer model through:

- The *in vitro* assessment of the principal biochemical characteristics of immunogenic cell death caused by ICRP treatment on breast cancer cells.
- The *ex vivo* evaluation of the capacity of ICRP-induced cell death to stimulate dendritic cell maturation, which triggers an immunocompetence of T cells against cancer cells.
- The *in vivo* demonstration of an antitumor immune response generated by prophylactic and therapeutic vaccinations with tumor cell lysate (TCL) obtained from ICRP-treated 4T1 cells (ICRP-TCL).

VI. MATERIALS AND METHODS

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). All cell cultures were maintained in a humidified incubator in 5 % CO_2 at 37 °C. Cell count was performed in a Neubauer chamber, using 0.4% trypan blue (MERCK, Darmstadt, Germany).

Animals

The Animal Research and Welfare Ethics Committee (CEIBA), of the School of Biological Sciences approved this study: CEIBA-2019-006. All experiments were conducted according to Mexican regulation NOM-062-ZOO-1999. Female BALB/c mice (Six-to-eight-week-old; 20 ±2 g weight) were provided by the animal house at the Universidad Autonoma de Nuevo Leon, Mexico. Animals were housed in plastic cages and given seven days to acclimate to the housing facility. Environmental conditions were temperature 21°C ±3°C, humidity 55% ±10%, and 12 h light/dark cycle. Animals were supplied with rodent maintenance food (LabDiet, St. Louis, MO) and water *ad libitum*,

and they were monitored twice daily for health status, no adverse events were observed. Mice were randomly assigned to different groups for all studies.

Intracellular Ca^{2+} levels analysis

For this assay, $5x10^5$ cells were plated in 6-well dishes (Life Sciences) in complete DMEM or RMPI to a final volume of 2 mL and incubated with ICRP CC_{50} for 18 h (MERCK). Then, 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. Next, cells were washed twice with RINGER buffer assessed by confocal microscopy (Olympus X70). For flow cytometry assays, $5x10^4$ cells/well in 24-well dishes (Life Sciences) were incubated with ICRP CC_{50} for 18 h (MERCK) in complete DMEM or RPMI to a final volume of 400 µL. Cells were then detached, washed with KREBS buffer, and suspended in 200 µL of 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. Cells were then washed with RINGER buffer and assessed by BD Accury C6 flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Results were analyzed using FlowJo Software (LLC, Ashland, OR).

Cell death assay

Cell death was measured using 1 µg/mL APC Annexin V (BD Pharmingen, San Jose, CA) and 0.5 µg/mL propidium iodide (PI) (MERCK). In brief, $5x10^4$ cells/well in 24-well dishes (Life Sciences) were incubated with different doses of ICRP for 24 h to stablish ICRP CC_{50} or ICRP. CC_{50} for 24 h in presence or absence of 1.5 mM BAPTA (MERCK)

in complete DMEM or RPMI to a final volume of 400 µL. Cells were then detached, washed twice with PBS, and suspended in 100 µL of binding buffer (10 mM HEPES/ NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl2), after which they were stained and assessed by flow cytometey as described above.

ROS generation analysis

ROS levels were determined by staining cells with 2.5 µM 2′,7′-Dichlorofluorescin diacetate (DCFDA) (MERCK). In brief, $5x10^4$ cells/well were incubated with ICRP CC_{50} for 24 h in presence or absence of 1.5 mM BAPTA (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.

Mitochondrional membrane potential analysis

Mitochondrional membrane potential was measured using 500 nM TMRE (MERCK). In brief, $5x10^4$ cells in 24-well dishes 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.

Calreticulin exposure analysis

For this, $5x10^4$ cells were seeded in 24-well plates and treated with ICRP CC_{50} for 24 h in presence or absence of 1.5 mM BAPTA (MERCK). Cells were detached, washed, stained with Calreticulin-Phycoerythrin antibody (0.1 µL of a 1 to 1000 dilution) (Calreticulin-PE, FMC-75; Enzo Life Science, Farmingdale, NY) in 2%-FACS buffer, and incubated 1

h in darkness at 25 ºC, after that, cells were washed and suspended in 100 µL of 2%-FACS buffer to be assessed by flow cytometry as described above.

Autophagosome formation assay

For this evaluation, $5x10^4$ cells were cultured in 24-well plates (Life Sciences) with ICRP CC_{50} for 24 h. Cells were then detached, washed with PBS, stained with Autophagy Detection Kit (Abcam, Cambridge, UK), and measured by flow cytometry, as explained above.

EIF2α phosphorylation assay

For this assay, $5x10^5$ cells were plated in 6-well dishes (Life Sciences) in complete DMEM to a final volume of 2 mL, and incubated with ICRP CC_{50} for 18 h. Cells were then collected and fixed with eBioscience Foxp3/transcription factor fixation/permeabilization concentrate and diluent (Life Technologies) for 1 h at 4 °C. Cells were then washed with 2%-FACS Buffer (PBS 1x 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 1x 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 1h in darkness. Cells were washed with 2%-FACS Buffer and eIF2α phosphorylation was measured by flow cytometry, as mention before.

ATP release assay

Supernatants of ICRP CC_{50}-treated cells ($2x10^5$) were used to assess extracellular ATP by a luciferase assay (ENLITEN kit, Promega, Madison, WI), following manufacturer's instructions. Bioluminescence was determined in a Synergy HT microplate reader, using the Software Gen5 (BioTek, Winooski, VT) at 560 nm.

High-mobility group box 1 release assay

For this assay, $2x10^5$ were treated with ICRP CC_{50} for 24 h. Supernatants were used to assess extracellular HMGB1 using the HMGB1 BioAssay ELISA Kit (Human) for MCF-7 and the HMGB1 BioAssay ELISA Kit (Mouse) for 4T1 cells (US Biological Life Science), following manufacturer's instructions, in a spectrophotometer at a wavelength of 450 nm.

Generation of mouse BMDCs

To obtain bone marrow-derived dendritic cells (BMDCs), seven-to-eight-week-old BALB/c mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg body weight) and xylazine (10 mg/kg body weight) and were euthanized by cervical dislocation (n= 5 mice). Bone marrow was removed from femur and tibia after mouse death by flushing into complete RPMI (Life Technologies). Eluted cells were cultured at 37 ºC in a controlled humidified atmosphere with 5 % CO_2 for 5 days in complete RPMI

and 20 ng/mL IL-4 and GM-CSF (R&D Systems, Minneapolis, MN), until approximately 50 % of cells were CD11c+.

T cells-isolation

Seven-to-eight-week-old BALB/c mice were anesthetized as mentioned above. Blood was obtained by cardiac puncture, and then cervical dislocation was performed. Peripheral blood mononuclear cells (PBMCs) isolation was performed by density gradient centrifugation, using Ficoll-Hypaque-1119 (MERCK). CD3+ cells were isolated from total PBMCs by positive selection using magnetic-activated cell sorting microbead technology with anti-CD3ε-biotin and anti-biotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), following manufacturer's instructions. Primary murine CD3+ cells were maintained in complete RPMI and incubated at 37 ºC in a controlled humidified atmosphere with 5 % CO_2.

ICRP-TCL-mediated BMDCs maturation

BMDCs were suspended in complete RPMI at a concentration of $1x10^6$ cells/mL and stimulated with ICRP-tumor cell lysate (ICRP-TCL) ($3x10^6$ cells/mL at a ratio of 1:3 BMDCs to ICRP-treated 4T1 cells (BMDCs-ICRP-TCL)). Control BMDCs were left untreated or stimulated with 1 µg/mL lipopolysaccharide (LPS) (MERCK). After 24 h, culture supernatants were removed and stored at -80 ºC to analyze TNF-α release by flow cytometry (BD CBA Mouse Th1/Th2 Cytokine Kit, BD Biosciences, San Jose, CA), and wells were washed twice with PBS before the next co-culture (with the addition of T

lymphocytes). Additionally, some BMDCs-Control and BMDCs-ICRP-TCL wells were collected to analyze DCs markers expression.

DCs markers expression

For this assay, $1x10^5$ BMDCs were suspended in 100 µL of 2%-FACS buffer, and maturation was analyzed by immunostaining using anti-CD11c-Alexafluor 488 (R&D Systems, Minneapolis, MN), anti-CD80-FITC, and anti-CD86-APC (BD Biosciences) at 25 °C for 30 min, and washed twice with PBS. Cell surface markers were evaluated by flow cytometry, as mention above.

BMDCs-T-lymphocytes co-culture

BMDCs-Control or BMDCs-ICRP-TCL were maintained in complete RPMI at a concentration of $1x10^6$ cells/mL. Allogeneic BALB/c mCD3+ cells were then added to each well at $3x10^6$ cells/mL (at a ratio of 1:3 DCs to CD3+ cells), and incubated for 96 h at 5 % CO_2 and 37 °C. Supernatants were then removed, stored at -80 ºC to further analyze TNF-α and IFN-γ levels by flow cytometry (BD CBA Mouse Th1/Th2 Cytokine Kit, BD Biosciences), and lymphocytes were washed with PBS, and suspended in complete RPMI to be used in the next co-culture (T-lymphocytes with cancer cells).

T-Lymphocytes-4T1 cells co-culture

Viable 4T1 cells were seeded at a concentration of $1x10^5$ cells/mL. Cells were then stained with 0.1 µL/mL of calcein-AM (BD Biosciences) for 30 min and washed twice with PBS. Next, unprimed (previously co-cultured with BMDCs-Control) or primed (previously co-cultured with BMDCs-ICRP-TCL) allogeneic BALB/c CD3+ cells were added to each

well at 5x105 cells/mL (at a ratio of 1:5 cancer cells to CD3+ cells); 4T1-T-lymphocytes co-culture was incubated at 37 ºC and 5 % CO_2 for 24 h. Supernatants were removed and stored at -80ºC to further analyze IFN-γ levels, as mentioned above. Cancer cells were then washed and detached to analyze 4T1-calcein negative cells by flow cytometry, as described above.

Prophylactic vaccination with ICRP-TCL

For this, 4T1 cells were treated with 0.5 U/mL ICRP for 24 h; cells were then washed, detached, and cell death was confirmed using trypan blue staining and flow cytometry, as previously indicated. Seven-to-eight-week-old BALB/c mice were inoculated subcutaneously (s.c.) with $1.5x10^6$ dying 4T1 cells (n=10 mice) or with PBS (n=6 mice) on the left flank side. On day 7 after vaccination, the mice were challenged s.c. on the opposite flank with $5x10^5$ living 4T1 cells.

Therapeutic vaccination with ICRP-TCL

For this evaluation, mice were inoculated s.c. with $5x10^5$ live 4T1 cells on the right flank side. When tumors reached 100 mm^3 (on day 3 after cancer cell inoculation), mice were treated s.c. with $1.5x10^6$ dying 4T1 cells on the opposite flank during two weeks (two times per week) (n=9 mice); mice injected with PBS were used as control (n=5 mice).

Tumor volume measurements

Tumor volume was measured three times a week, using a caliper (Digimatic Caliper Mitutoyo Corporation, Japan), this was determined with the following formula: tumor volume (mm3) = 4π / 3 ∗ A (length) ∗ B (width) ∗ C (height). When tumors exceeded

1000 mm^3 mice were anesthetized as described above and were euthanized by cervical dislocation.

Long-term memory assays

Mice in complete remission after ICRP-TCL prophylactic (n=9 mice) and therapeutic vaccinations (n=8 mice) were re-challenged with $5x10^5$ living 4T1 cells in 100 µL of PBS into the opposite flank, and naïve mice were used as control (n= 5 mice). Tumor volume and mice survival was evaluated, as described above.

Additionally, tissues from tumor-draining lymph nodes (TDLN) and tumor re-challenge sites were obtained from naïve mice and mice in complete remission after ICRP-TCL prophylactic vaccination, three days after tumor re-challenge. Tissues were fixed in 3.7% neutral formalin, embedded in paraffin, sectioned (5 µm thickness) and stained with H&E (MERCK). Histopathological analyses were done by an external veterinarian pathologist (National professional certificate 2593012). Blood was obtained by cardiac puncture from anesthetized mice as described above, and PBMCs isolation was performed by density gradient centrifugation, using Ficoll-Hypaque-1119 (MERCK). Cells from TDLN and spleen were isolated using 70 µm cell strainers (MERCK) and suspended in 2%-FACS buffer. PBMCs were stained with Mouse T lymphocyte antibody cocktail: PE-Cy7 CD3e, PE CD4, and FITC CD8 (BD Pharmingen) following manufacturer's instructions. Maturation of DCs was analyzed by immunostaining of cells from lymph nodes using anti-CD11c-Alexafluor 488 (R&D Systems), and anti-CD86-APC (BD Biosciences) at 25 °C for 30 min and washed twice with PBS. Cell surface markers were evaluated by flow cytometry, as mention above. Viable 4T1 cells were seeded at a concentration of $1x10^5$

cells/mL, stained with 0.1 μL/mL of calcein-AM (BD Biosciences) for 30 min and washed twice with PBS. Next, splenocytes from naïve or ICRP-TCL mice were added to each well at 40x105 cells/mL (at a ratio of 1:40 cancer cells to splenocytes); 4T1-splenocytes co-culture was incubated at 37 ºC and 5 % CO_2 for 24 h. Supernatants were removed and stored at -80 ºC to further analyze IFN-γ levels, as mentioned above. Cancer cells were then washed and detached to analyze 4T1-calcein negative cells by flow cytometry, as described above (n=6 mice per group).

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. For in vitro studies, statistical analysis was done using paired Student's t-test, and for *in vivo* and *ex vivo* studies Mann-Whitney tests and two-tailed unpaired Student's t-tests were performed.

VII. RESULTS

1. IMMUNEPOTENT CRP induces regulated cell death in a concentration-dependent manner on breast cancer cells.

To archive the objectives of the project, the first step was to determinate the cytotoxic concentration of ICRP that induced 50% of cell death (CC_{50}) in MCF-7 and 4T1 cells, that were then used for next evaluations. ICRP induced regulated cell death in all cell lines after 24 h of treatment in a concentration-dependent manner, as shown in Figure 14. Cell death in 30% of the cells (CC_{30}) was reached at 1 U/mL in MCF-7, and 0.1 U/mL in 4T1 cells. CC_{50} by ICRP was caused at 1.25 U/mL in MCF-7, and 0.15 U/mL in 4T1 cells, whereas CC_{80} was induced at 1.5 U/mL in MCF-7, and 0.2 U/mL in 4T1 cells, and CC_{90} was reached at 2 U/mL in MCF-7 and 0.5 U/mL in 4T1 cells (Fig. 14).

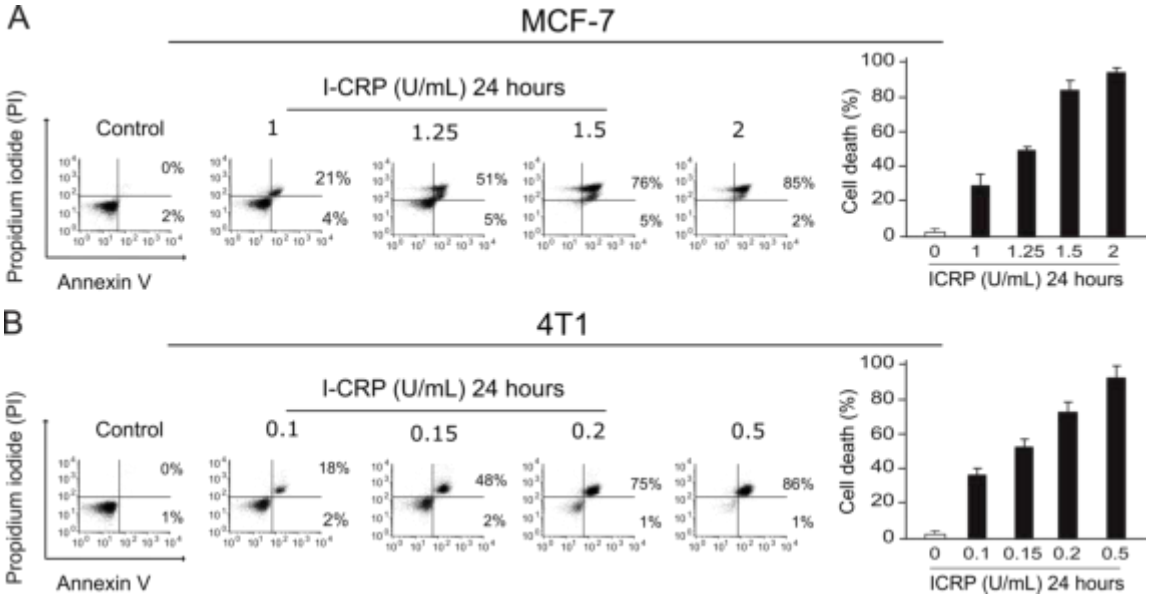

Figure 14. IMMUNEPOTENT CRP induces regulated cell death in a concentration-dependent manner in breast cancer cells. A-B. Representative dot plots (left) and quantifications (right) of cell death measured by flow cytometry through Annexin-V and PI staining in MCF-7 (**A**) and 4T1 cells (**B**) treated

with different concentrations of ICRP for 24 h. The means (± SD) of triplicates of at least three independent experiments were graphed.

2. IMMUNEPOTENT CRP increases cytoplasmic Ca^{2+} levels in breast cancer cells.

With the purpose of describing the implication of Ca^{2+} in the mechanism of cell death induced by ICRP, cytosolic Ca^{2+} levels in MCF-7 and 4T1 cells were first assessed by confocal microscopy. Results indicated that 18 h-treatment with CC_{50} of ICRP induced an augmentation of Ca^{2+} levels in the cytoplasm in comparison with untreated cells (control) (Fig. 15A and 15B). Thus, cytosolic level of Ca^{2+} was quantified by flow cytometry, data showed that ICRP CC_{50} treatment for 18 h induced an increment in Ca^{2+} concentration in the cytoplasm in 40% to 50% of breast cancer cells (Fig. 15C and 15D).

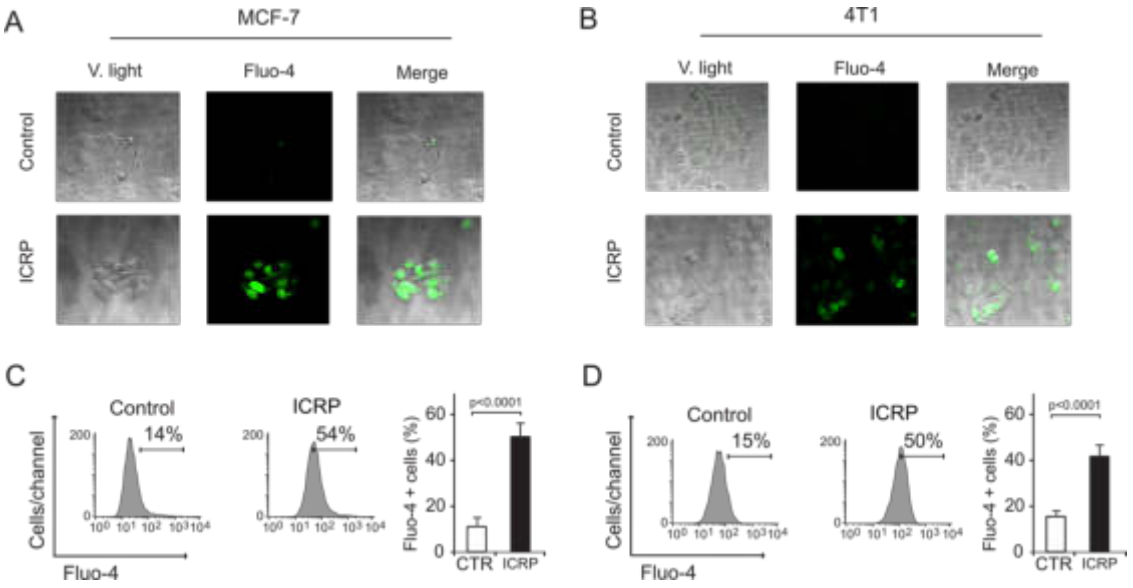

Figure 15. IMMUNEPOTENT CRP induces an increase in the cytoplasmic Ca^{2+} levels of MCF-7 and 4T1 cells. A-B. Confocal microscopy representation of Ca^{2+} cytoplasmic levels measured through Fluo-4 AM staining in MCF-7 (**A**) and 4T1 cells (**B**) in absence (control) or presence of ICRP CC_{50} for 18 h. **C-D.** Representative histograms (left) and quantifications (right) of Ca^{2+} cytoplasmic levels assessed through Fluo-4 AM staining by flow cytometry in absence (control) or presence of ICRP CC_{50} for 18 h in MCF-7

(**C**) and 4T1 (**D**) cells. Graphs represent the means (± SD) of triplicates of at least three independent experiments.

3. IMMUNEPOTENT CRP triggers Ca^{2+}-dependent ROS production, loss of Δψm, and CRT exposure, which led to cell death in breast cancer cells.

As an increase of cytosolic Ca^{2+} levels was observed in breast cancer cells after ICRP treatment, the implication of Ca^{2+} augmentation in cell death was analyzed by assessing ICRP-induced cell death in presence or absence of the extracellular Ca^{2+} chelator BAPTA. As observed in Figure 16, ICRP induced regulated cell death in up to 50% of cells after 24-h treatment, and this cytotoxicity was significantly inhibited in presence of BAPTA, in conclusion, ICRP generated a Ca^{2+}-dependent cell death in MCF-7 and 4T1 cells.

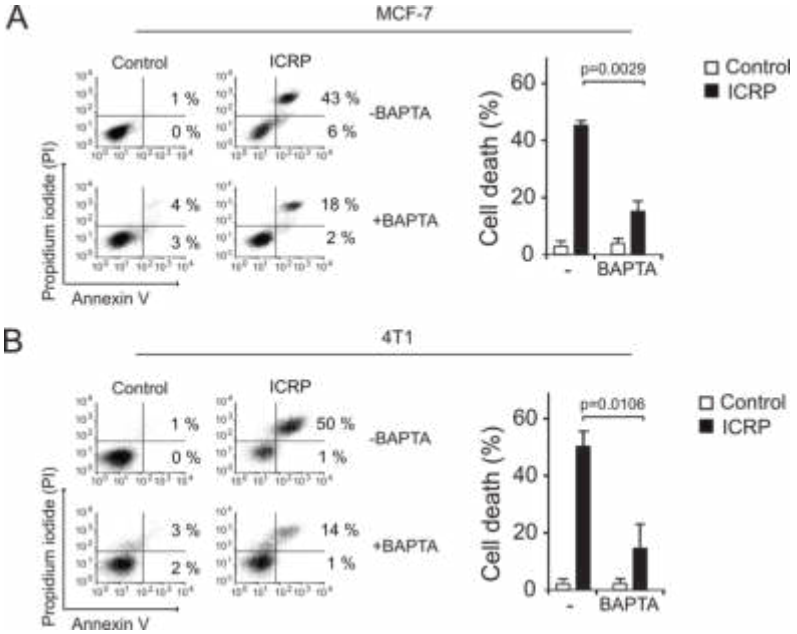

Figure 16. IMMUNEPOTENT CRP induces Ca^{2+}-dependent cell death in breast cancer cells. A-B. Representative dot plots (left) and quantifications (right) of cell death measured by flow cytometry through Annexin-V and PI staining in MCF-7 (**A**) and 4T1 cells (**B**) treated with ICRP CC_{50} for 24 h in presence or

absence of BAPTA. Graphs represent the means (± SD) of triplicates of at least three independent experiments.

Then, Δψm and ROS production were evaluated in presence or absence of BAPTA, as these processes have been observed in ICRP-induced cell death in cancer cells, and they are associated with Ca^{2+} deregulation. As observed in Figure 17, ICRP caused loss of Δψm in up to 60% of cells, and this cellular process was significantly inhibited in presence of BAPTA. In addition, ICRP triggered ROS production in up to 55% of cells, which was also inhibited in presence of BAPTA (Fig. 18). Thus, ICRP generated a Ca^{2+}-dependent loss of Δψm and ROS production in MCF-7 and 4T1 cells.

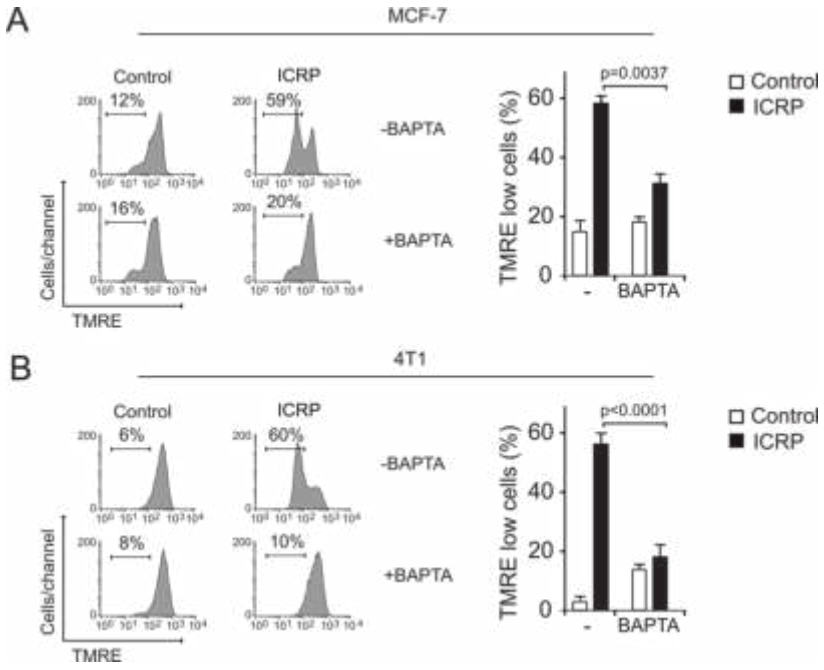

Figure 17. The loss of mitochondrial membrane potential mediated by IMMUNEPOTENT CRP requires Ca^{2+} influx from extracellular space in breast cancer cells. A-B. Representative histograms (left) and quantifications (right) of loss of mitochondrial membrane potential evaluated through TMRE staining by flow cytometry in MCF-7 (**A**) and 4T1 cells (**B**) treated with ICRP CC_{50} for 24 h in presence or

absence of BAPTA. Graphs represent the means (± SD) of triplicates of at least three independent experiments.

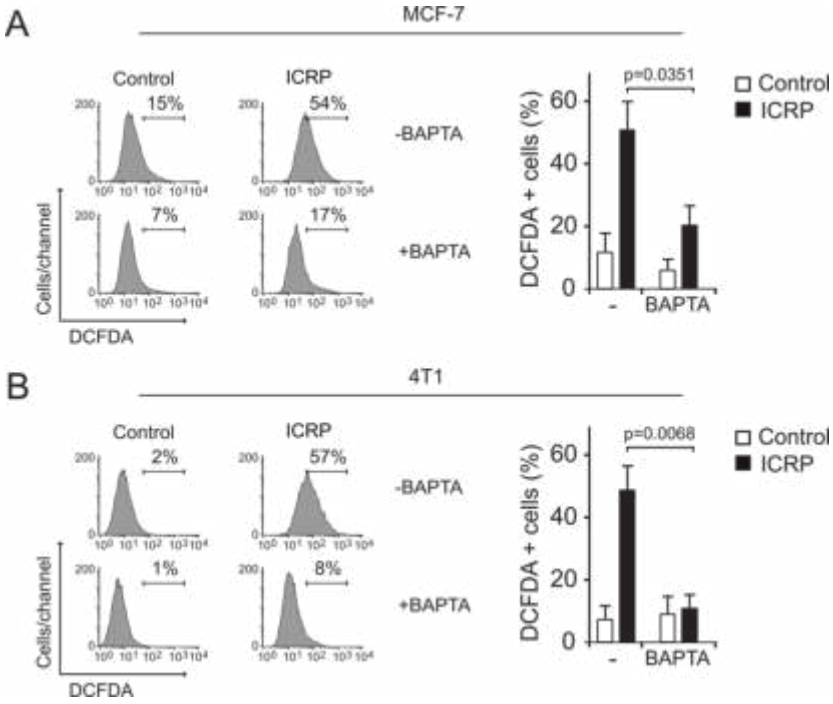

Figure 18. IMMUNEPOTENT CRP triggers Ca^{2+}-dependent ROS production in breast cancer cells. **A-B.** Representative histograms (left) and quantifications (right) of ROS production assessed through DCFDA staining by flow cytometry in MCF-7 (**A**) and 4T1 cells (**B**) treated with ICRP CC_{50} for 24 h in presence or absence of BAPTA. Graphs represent the means (± SD) of triplicates of at least three independent experiments**.**

Furthermore, CRT exposure was evaluated after ICRP treatment in presence or absence of BAPTA, as exposure of this protein is associated with ER stress, which is the major storage of Ca^{2+} within the cell. As observed in Figure 19, CRT exposure was observed after ICRP CC_{50} treatment for 24 h in up to 60% of cells, which was significantly inhibited in presence of BAPTA, thus, the influx of extracellular Ca^{2+} within the cell is required for CRT exposure.

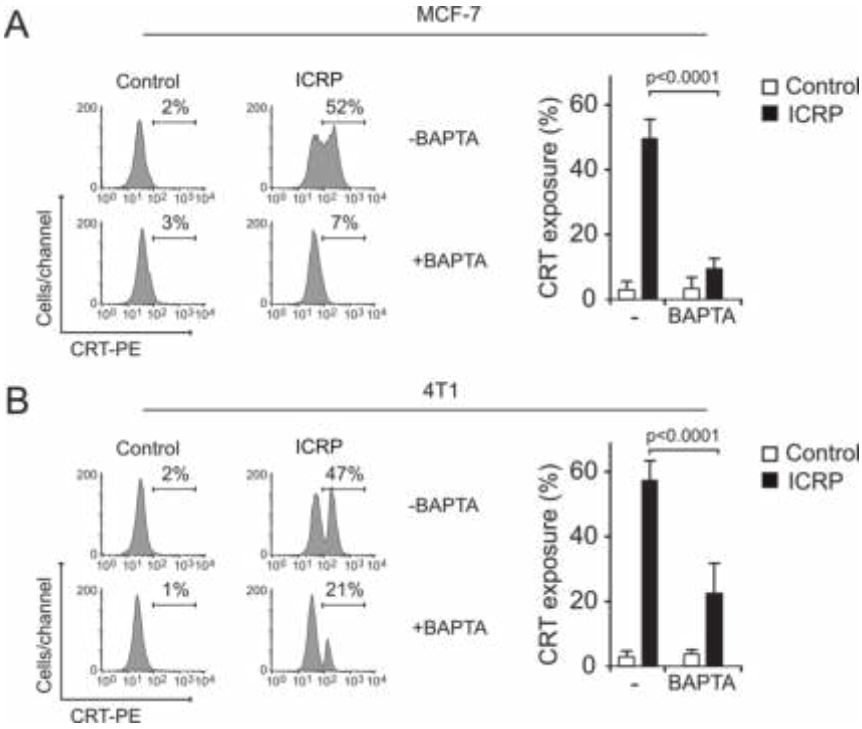

Figure 19. IMMUNEPOTENT CRP triggers Ca^{2+}-dependent CRT exposure in MCF-7 and 4T1 cells.

A-B. Representative histograms (left) and quantifications (right) of CRT exposure assessed through Calreticulin-PE antibody staining by flow cytometry in MCF-7 (**A**) and 4T1 cells (**B**) treated with ICRP CC_{50} for 24 h in presence or absence of BAPTA. Graphs represent the means (± SD) of triplicates of at least three independent experiments**.**

4. IMMUNEPOTENT CRP causes ER stress, autophagosome formation and DAMPs release in breast cancer cells.

P-eIF2α regularly antecedes CRT exposure in the course of ICD, nonetheless P-eIF2α is not necessarily followed by CRT exposure, particularly when the ER stress response reestablishes cell homeostasis, and thus, P-eIF2α was evaluated after ICRP treatment. ICRP induced P-eIF2α in 50% to 60% of breast cancer cells treated with ICRP CC_{50} for 18 h (Fig. 20A). In addition, autophagosome formation was induced by 24-h treatment

with ICRP CC_{50} in 40% to 55% of MCF-7 and 4T1 cells (Fig. 20B). CRT exposure, ER stress, and autophagosome formation are part of the principal biochemical processes of ICD. Thus, the release of ATP and HMGB1 (DAMPs associated with ICD) were assessed after ICRP exposure. As observed in Figure 20C and 20D, ICRP induced 276- and 11-fold ATP release, and 1.6-, and 1.3-fold HMBG1 release in MCF-7 and 4T1 cells, respectively, as compared with untreated control.

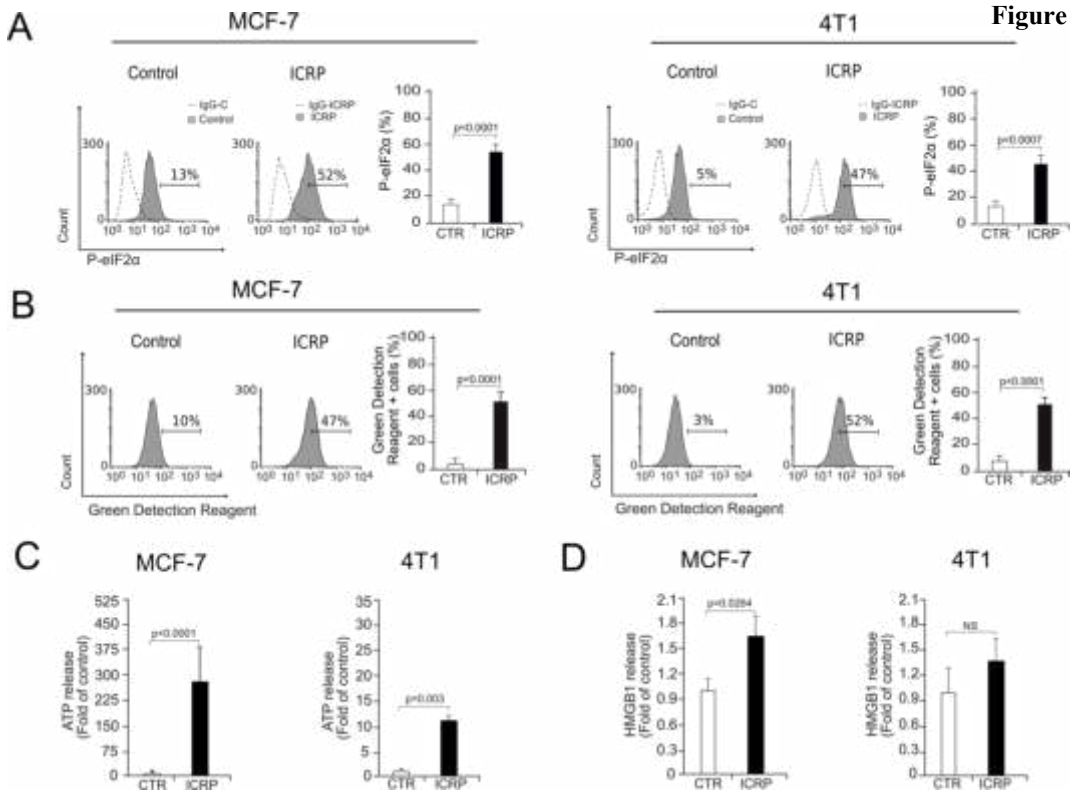

Figure 20. IMMUNEPOTENT CRP triggers ER stress, autophagosome formation, and DAMPs release in MCF-7 and 4T1 cells. A. Representative FACS histograms of P-eIF2α staining (in grey) and IgG isotype antibodies (dotted) of cancer cells left untreated (control) or treated with ICRP CC_{50} for 24 h. Charts are the quantification of P-eIF2α staining in controls and cancer cells treated with ICRP. **B.** Representative histograms (left) and quantifications (right) of autophagosome formation measured through Green Detection Reagent staining by flow cytometry in absence (control) or presence of ICRP CC_{50} for 24 h in MCF-7 and

4T1 cells. **C-D.** Quantification of ATP release through bioluminescence detection (**C**) and HMGB1 release assessed by ELISA (**D**) in supernatants of MCF-7 and 4T1 cells in absence or presence of ICRP CC_{50} for 24 h. Graphs represent the means (± SD) of triplicates of at least three independent experiments.

5. ICRP- treated tumor cell lysate induces maturation of BMDCs.

After determining that the principal biochemical characteristics of immunogenic cell death were evoked by ICRP, we evaluated the capacity of ICRP-TCL to maturate BMDCs. As observed in Figure 21A and 21B, the exposure of BMDCs to ICRP-TCL (BMDCs-ICRP-TCL) significantly increased the expression of CD80 (57%), and CD86 (65%); whereas maintaining CD11c expression (48%), in comparison with unstimulated BMDCs (C80: 44%, CD86: 45%, and CD11c: 49%). These results resemble the ones observed by our positive control, LPS, which significantly incremented CD80 (56%) and CD86 expression (70%); and also maintained CD11c expression (48%) in these cells.

Furthermore, a significant increase of TNF-α release was observed in BMDCs stimulated with ICRP-TCL (760 pg/mL) or LPS (7763 pg/mL), in comparison with unstimulated BMDCs (53 pg/mL) (Fig. 21C). Thus, ICRP-TCL induced phenotypic and functional BMDCs maturation.

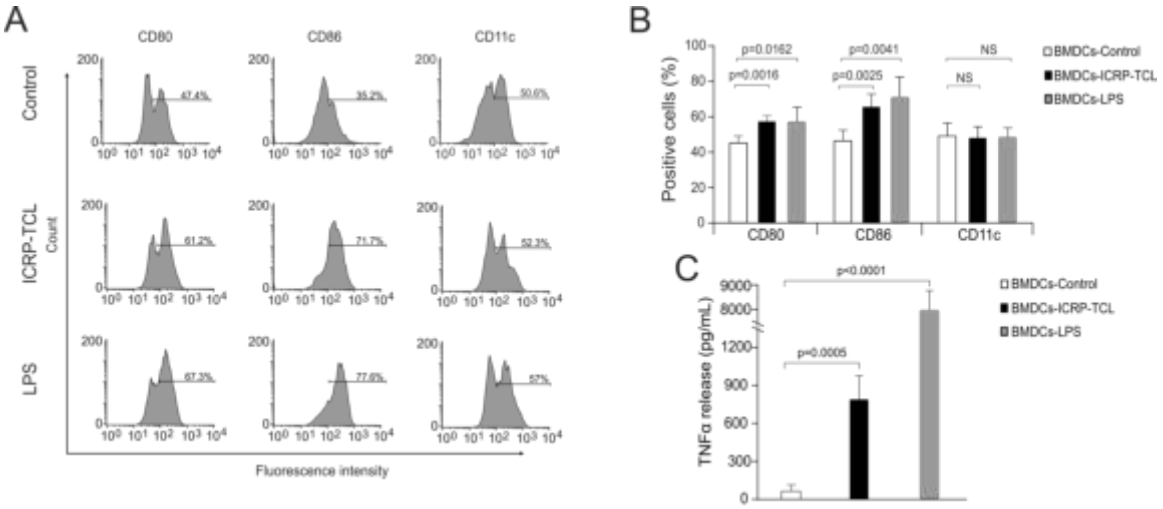

Figure 21. ICRP-TCL induces BMDCs maturation. A. Representative flow cytometry histograms showing the percentage of CD80+, CD86+, and CD11c+ BMDCs unstimulated (negative control), in co-culture ratio 1:3 with ICRP-treated 4T1 cells (ICRP-TCL), or stimulated with 1 μg/mL of LPS (positive control) during 24 h. **B.** BMDCs were treated as in A and the means (± SD) obtained of five independent experiments were graphed. **C.** Quantification of TNF-α concentration in supernatants of BMDCs treated as in A, expressed as the means (± SD) of five independent experiments. n= 5 mice per group.

6. Mature BMDCs exposed to ICRP-tumor cell lysate triggers anticancer immune responses.

After the evaluation of ICRP-TCL-mediated BMDCs maturation, the next step was to investigate if these mature cells could induce T cell priming. Figure 22A and 22B show a significant increase of TNF-α (380 pg/mL) and IFN-γ (650 pg/mL) release in co-culture of BMDCs-ICRP-TCL and T cells, in contrast BMDCs-Control and T cells co-culture (TNF-α: 95 pg/mL and IFN-γ: 185 pg/mL).

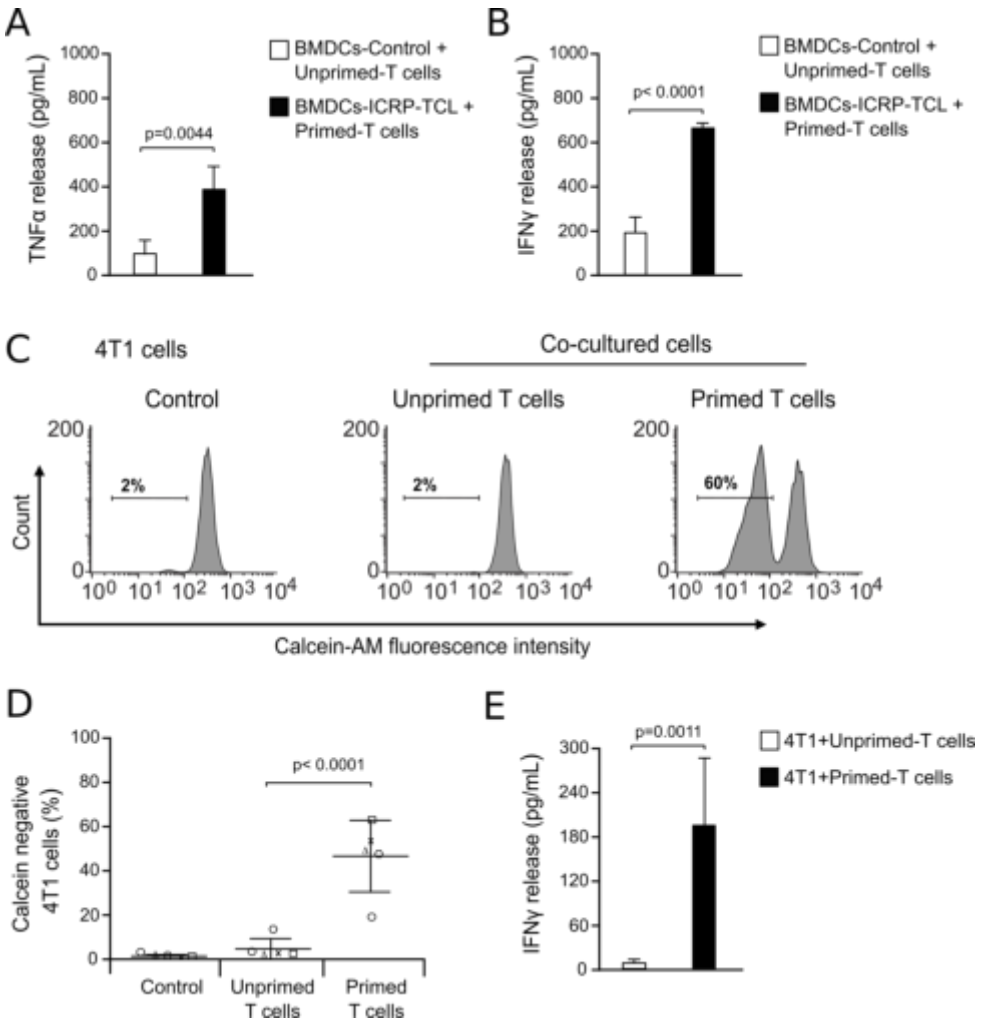

Figure 22. BMDCs exposed to ICRP-TCL triggers anticancer immune response. A-B. Concentration of TNF-α (**A**) and IFN-γ (**B**) in supernatants of unstimulated BMDCs (BMDCs-Control) or stimulated with ICRP-TCL (BMDCs-ICRP-TCL), in co-culture ratio 1:3 with T cells for 96 h, expressed as the means (± SD) of five independent experiments. **C.** Representative flow cytometry histograms showing the percentage of calcein negative 4T1 cells left alone (Control), or in co-culture ratio 1:5 with unprimed T-lymphocytes (previously co-cultured with unstimulated BMDCs) or primed T-lymphocytes (previously co-cultured with BMDCs-ICRP-TCL) for 24 h**. D.** Percentage of calcein negative 4T1 cells treated as in C. **E.** Quantification of IFN-γ concentration in supernatants of 4T1 cells treated as in C, expressed as the means (± SD) of three independent experiments. n= 5 mice per group.

Primed T cells obtained after co-culture with BMDCs-ICRP-TCL caused a cytotoxic effect in up to 70% of 4T1 cells, whereas the cytotoxicity induced by unprimed T cells was up to 19% of cancer cells; no cytotoxicity was detected in 4T1 cells without T cells (Fig. 22C and Fig. 22D). In addition, a significant increase of IFN-γ release was observed in the co-culture of primed T cells with 4T1 cancer cells (192 pg/mL), in comparison with the co-culture of unprimed t cells with 4T1 cells (8 pg/mL) (Fig. 22E). These data confirm the efficient antigen presentation by BMDCs-ICRP-TCL to T cells and the immunocompetence of these T cells against 4T1 cells.

7. Prophylactic vaccination with ICRP-TCL prevents tumor establishment in BALB/c mice.

In order to test the ability of ICRP-TCL to activate adaptive immune system *in vivo*, we performed a well-established prophylactic tumor vaccination model in immunocompetent BALB/c mice (Fig. 23A). Immunization of mice with ICRP-TCL prevented tumor growth at the challenge site in nine out of ten mice, moreover, the tumors growing on the challenge site of the unvaccinated (PBS) mice reached up to 1 200 mm^3 in all six mice (Fig. 23B), confirming that ICRP-TCL induced a potent immune response in vivo, reflected in 90% (9/10) of 60-day survival rates of mice in ICRP-TCL group, whereas all control mice were euthanized by day 20 (6/6) (Fig. 23C).

8. Prophylactic vaccination with ICRP-TCL induces long-term antitumor memory in BALB/c mice.

To investigate the effect of ICRP-TCL on the memory response *in vivo*, tumor-free mice that survived 150 days from a previous prophylactic vaccination with ICRP-TCL were re-challenged with living 4T1 cancer cells, naïve mice challenged with living 4T1 cancer cells were used as control (Fig. 24A). Results show that tumor growth was prevented in re-challenged mice, while continuous tumor growth was observed in naïve mice, challenged for the first time (Fig. 24B). This reflected in 100% (9/9) of survival in ICRP-TCL re-challenged mice, while naïve mice perished by day 15 (5/5) (Fig. 24C). These results strongly suggest the stimulation of long-term antitumor immune memory by ICRP-TCL prophylactic vaccination.

To better characterize this immune response we assessed tumor establishment, DCs maturation, T cell distribution, and splenocytes-tumor specific cytotoxicity after three days of tumor challenge (naïve mice) / re-challenge (ICRP-TCL) (Fig. 25A).

Results reveal that naïve mice presented tumor establishment with an extensive infiltration of neoplastic cells in the striated muscle tissue; furthermore, these cells demonstrated an intense mitotic activity (Fig. 25B left). On the other hand, ICRP-TCL mice showed a discrete infiltration of neoplastic cells in the striated muscle tissue, observing a strong infiltration of lymphocytes and polymorphonuclear cells (Fig. 25B right).

Moreover, histopathological analyses from tumor-draining lymph nodes showed a diffuse lymphoid hyperplasia in naïve mice, whereas ICRP-TCL mice present a follicular

lymphoid hyperplasia, indicating a modulated response associated with immunological memory (Fig. 25C).

Due to these differences, we next evaluated the proportion of mature dendritic cells in tumor draining lymph nodes. Results indicated that re-challenged ICRP-TCL group did not show significant difference in the percentage of DCs, but a higher proportion of mature DCs (CD11c+CD86+) were present when compared with challenged naïve mice (Fig. 25D).

Then, we assessed the proportion of T cells in peripheral blood, and no differences were detected in total CD3+ cells, while a significant decrease of CD4+ T cells and an increase of CD8+ T cells was observed in ICRP-TCL mice, when compared with naïve mice (Fig. 25E).

Furthermore, splenocytes from ICRP-TCL mice mediated a cytotoxic effect in 4T1 cells, inducing loss of cell viability in up to 50% of cancer cells, whereas no significant cytotoxic effect was detected in 4T1 cells co-cultured with splenocytes from naïve mice (Fig. 25F). Finally, a significant increase of IFN-γ release was observed in the co-culture of 4T1 cells with splenocytes from ICRP-TCL mice (95 pg/mL), in comparison with the co-culture of 4T1 cells with splenocytes from naïve mice (27 pg/mL) (Fig. 25G), indicating tumor specific cytotoxicity.

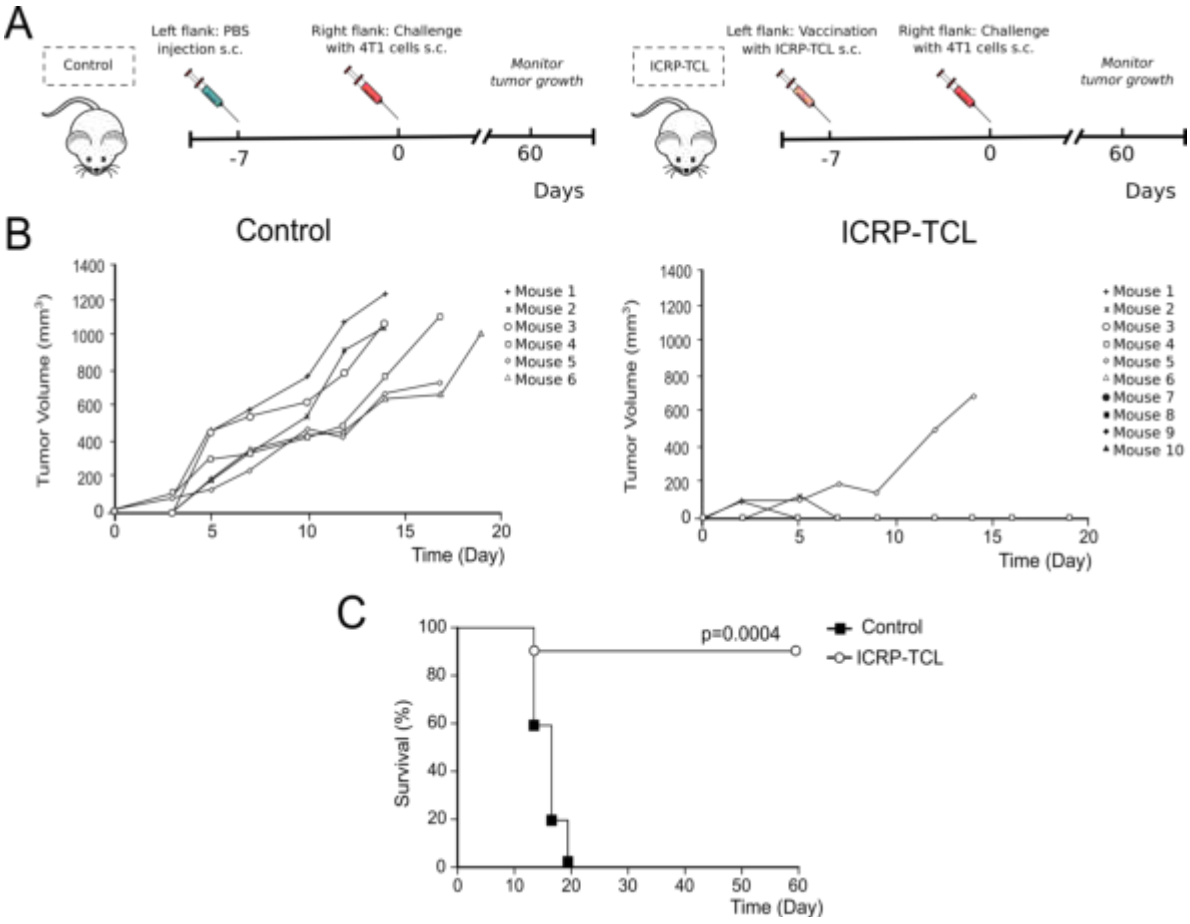

Figure 23. Prophylactic vaccination with ICRP-TCL prevented tumor establishment in BALB/c mice.

A. Mice were inoculated s.c. with $1.5x10^6$ dying 4T1 cells or with PBS on the left flank side. On day 7 after vaccination, mice were challenged s.c. on the opposite flank with $5x10^5$ living 4T1 cells. Tumor growth on the challenge site was evaluated for up to 60 days after the challenge. **B.** Tumor volume on the challenge site of unvaccinated mice (Control, n= 6 mice) or vaccinated with $1.5x10^6$ ICRP-treated 4T1 cells (ICRP-TCL, n= 10 mice). Each line represents one mouse. **C.** Kaplan Meier graph with the percentage of survival in mice treated as in B.

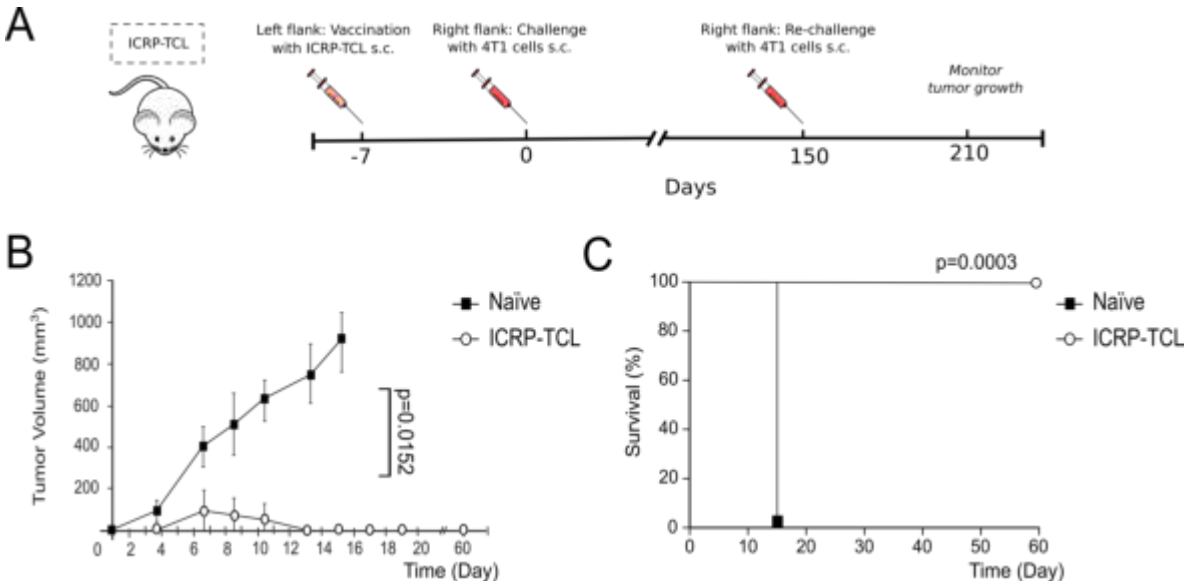

Figure 24. Prophylactic vaccination with ICRP-TCL induces long-term antitumor memory in BALB/c mice. A. Mice in remission after ICRP-TCL prophylactic vaccination were re-challenged s.c. with $5x10^5$ living 4T1 cells after 150 days of prophylactic vaccination. Tumor growth on the challenge site was evaluated for up to 60 days after re-challenge. **B.** Tumor volume on the challenge site of naïve mice (black square, n= 5 mice) or mice in remission after a previous $1.5x10^6$ ICRP-TCL vaccination (white circle, n= 9 mice). **C.** Kaplan Meier graph with the percentage of survival of mice treated as in B.

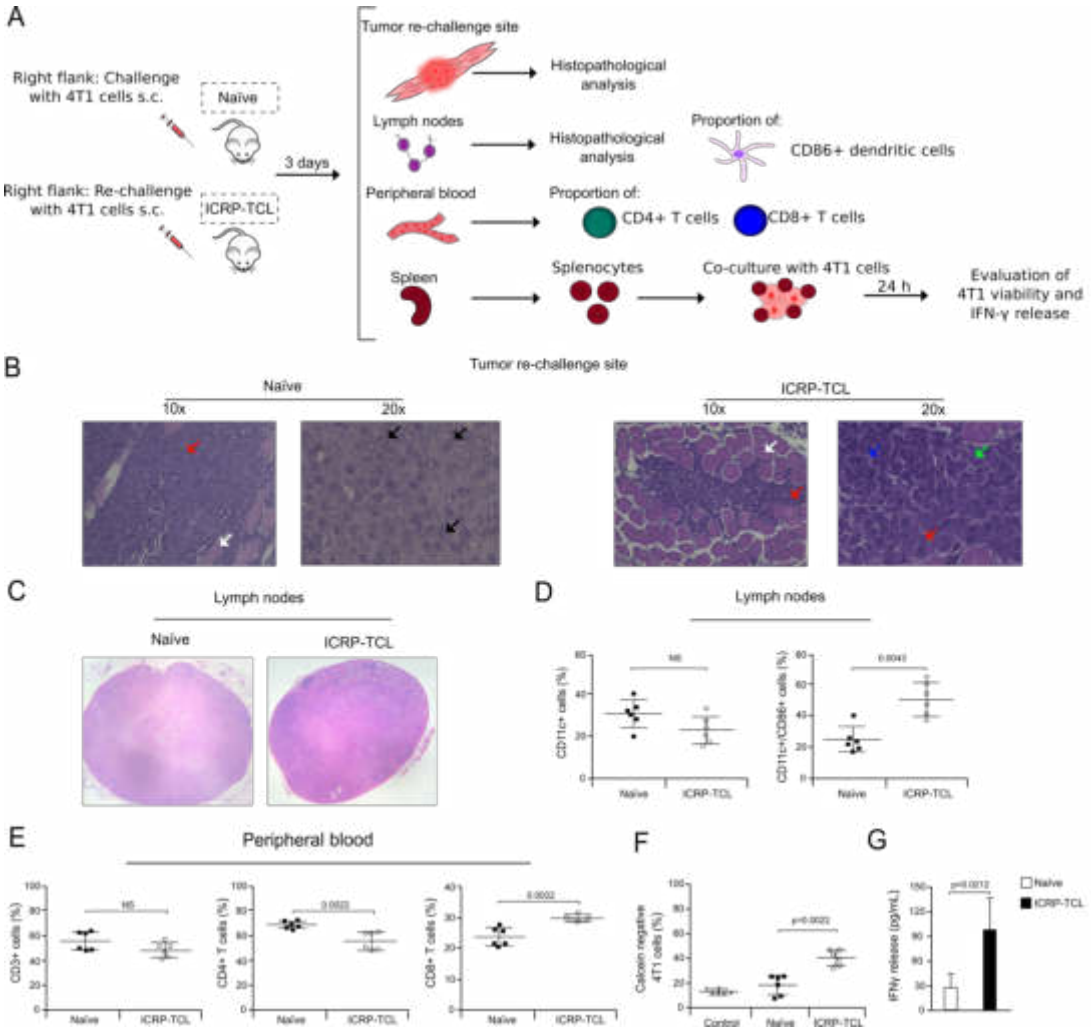

Figure 25. ICRP-TCL prophylactic vaccination modulates tumor establishment, DCs maturation, T cell distribution, and splenocytes-tumor specific cytotoxicity after tumor re-challenge. A. Naïve mice (n=6) and mice in remission after ICRP-TCL prophylactic vaccination (n=6) were challenged / re-challenged s.c. with $5x10^5$ living 4T1 cells. Three days later, the tumor re-challenge site, tumor-draining lymph nodes, peripheral blood, and spleen were obtained. **B-C.** Histology from tumor re-challenge sites (**B**) and lymph nodes (**C**) of naïve and ICRP-TCL mice stained with H&E. Normal tissue (white arrows), tumor cells (red arrows), mitotic cells (black arrows), lymphocytes (blue arrows), polymorphonuclear cells (green arrows). **D.** Percentage of CD11c and CD86 positive cells in tumor draining lymph nodes of naïve and ICRP-TCL mice **E.** Proportion of CD3, CD4, and CD8 positive cells in peripheral blood of naïve and ICRP-

TCL mice. **F.** Percentage of calcein negative 4T1 cells left alone (Control), or co-cultured with splenocytes from naïve or ICRP-TCL mice for 24 h (co-culture ratio 1:40). **G.** Quantification of IFN-γ concentration in supernatants of co-cultures obtained as in F, expressed as the means (± SD) of three independent experiments (n= 6 mice per group).

9. Therapeutic vaccinations with ICRP-TCL induce tumor regression in tumor-bearing mice, and long-term antitumor memory.

After the evaluation of the immunogenicity of ICRP-induced cell death, the therapeutic potential of ICRP-TCL *per se* was tested in tumor-bearing mice. For this, BALB/c mice were inoculated with $5x10^5$ living 4T1 cells, and when tumors reached 100 mm^3, therapeutic vaccinations were performed every three days during two weeks with s.c. inoculation of $1.5x10^6$ dying 4T1 cells (ICRP-TCL) or PBS (control) on the opposite flank (Fig. 26A).

Therapeutic vaccinations with ICRP-TCL induced tumor regression in eight out of nine mice, whereas, the tumors growing on control group reached up to 600 mm^3 in all five mice (Fig 26B), reflected in 88.88% (8/9) of 60-day survival rates of mice in ICRP-TCL group, whereas all control mice were euthanized by day 19 (5/5) (Fig. 26C). Tumor-free mice that survived 150 days from previous therapeutic vaccinations with ICRP-TCL were then re-challenged with living 4T1 cancer cells. Naïve mice challenged with living 4T1 cancer cells were used as control (Fig. 27A). Results demonstrated that tumor growth was prevented in re-challenged mice, while continuous tumor growth was observed in naïve mice, challenged for the first time (Fig. 27B). This was reflected in 100% (8/8) of survival in ICRP-TCL re-challenged mice, while naïve mice were euthanized by day 15 (5/5) (Fig.

27C). These results strongly suggest the stimulation of long-term antitumor immune memory by ICRP-TCL therapeutic vaccinations.

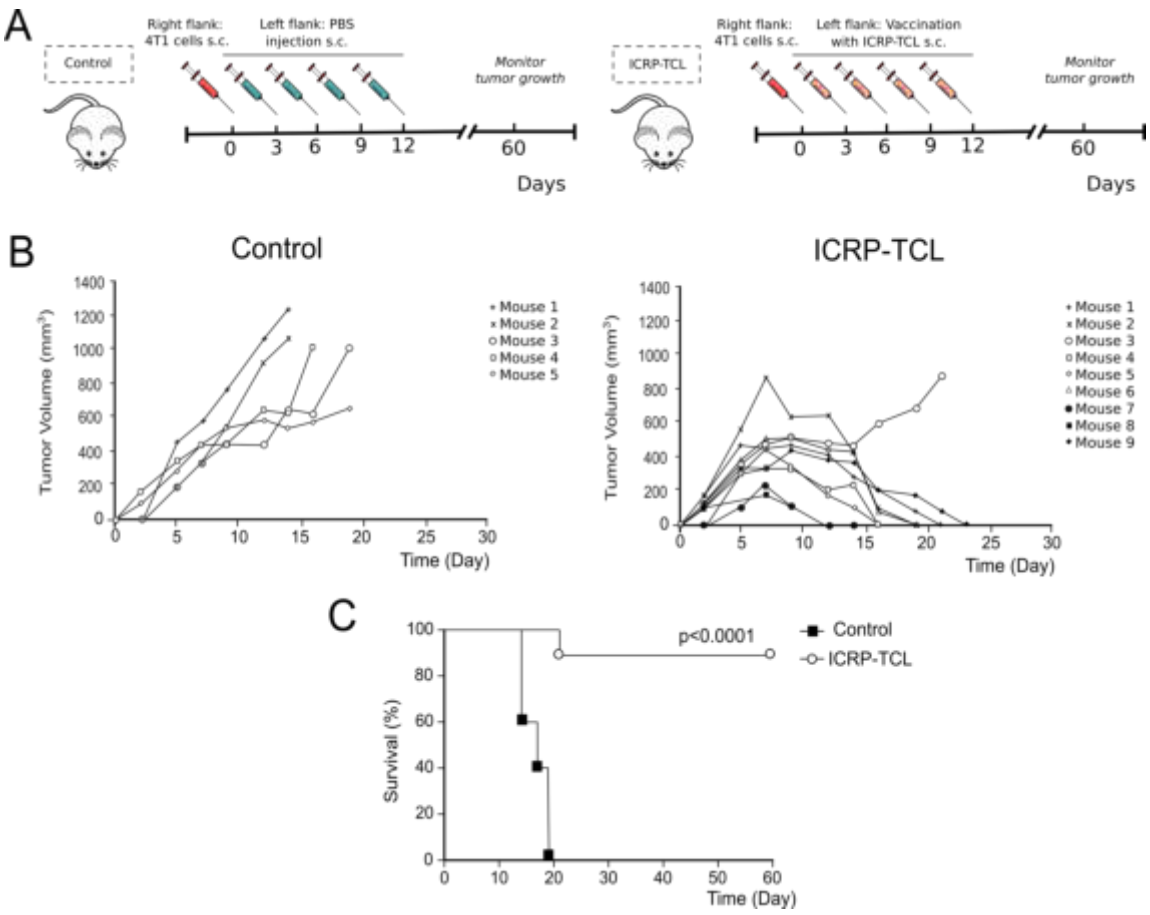

Figure 26. Therapeutic vaccinations with ICRP-TCL induce tumor regression in tumor-bearing BALB/c mice. A. Mice were inoculated s.c. with $5x10^5$ living 4T1 cells on the left flank side. When tumors reached 100 mm^3, therapeutic vaccinations were performed every three days during two weeks with s.c. inoculation of $1.5x10^6$ dying 4T1 cells (ICRP-TCL) on the opposite flank. PBS injections served as control. Tumor growth on the challenge site was evaluated for up to 60 days after the challenge. **B.** Tumor volume on the challenge site of control mice (n= 5 mice) or mice treated with $1.5x10^6$ ICRP-treated 4T1 cells (ICRP-TCL, n= 9 mice). Each line represents one mouse. **C.** Kaplan Meier graph with the percentage of survival in mice treated as in B.

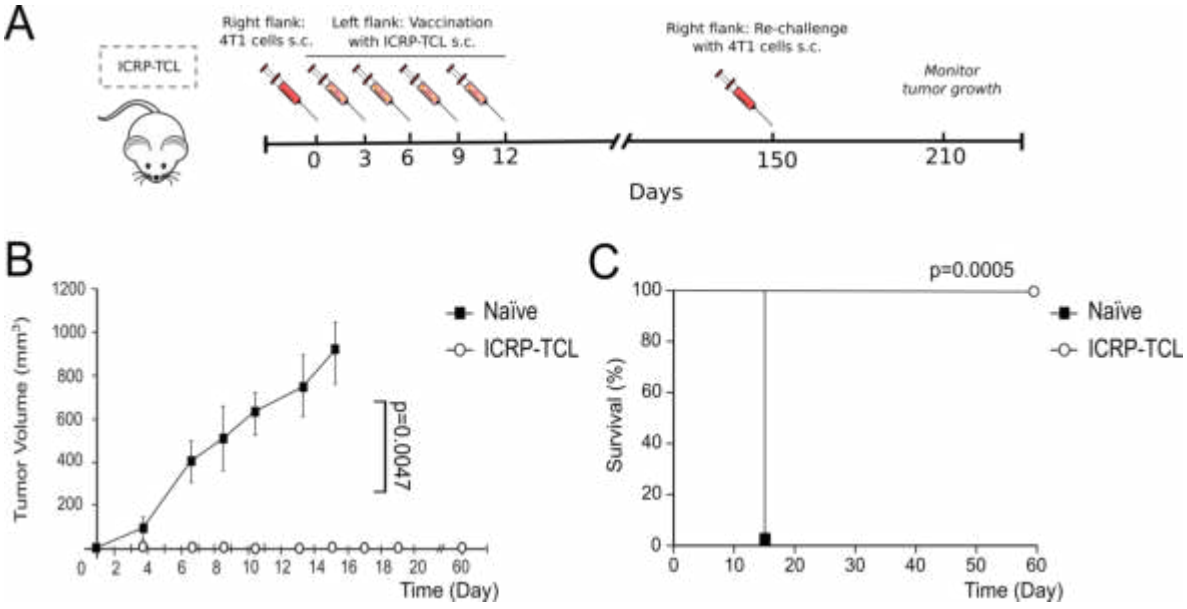

Figure 27. Therapeutic vaccinations with ICRP-TCL induce long-term antitumor memory in BALB/c mice. A. Mice in remission after ICRP-TCL therapeutic vaccinations were re-challenged s.c. with $5x10^5$ living 4T1 cells after 150 days of the first inoculation. Tumor growth on the challenge site was evaluated for up to 60 days after re-challenge. **B.** Tumor volume on the challenge site of naïve mice (black square, n= 5 mice) or mice in remission after a previous $1.5x10^6$ ICRP-TCL vaccinations (white circle, n= 8 mice). **C.** Kaplan Meier graph with the percentage of survival of mice treated as in B.

VIII. DISCUSSION

The results obtained in this work demonstrated the Ca^{2+}-dependence in ICRP-induced cell death in MCF-7 and 4T1 cells and indicate that IMMUNEPOTENT CRP may have the capacity to turn breast cancer cells into potential vaccines *in vivo*, through immunogenic cell death induction.

In this section I considered important to discuss the following core aspects of the cytotoxic mechanism of IMMUNEPOTENT CRP in cancer cells, and its application on cancer-therapy:

- The role of Ca^{2+} in the duality of IMMUNEPOTENT CRP
- Biochemical characteristics of IMMUNEPOTENT CRP-mediated cell death in cancer
- ICRP-mediated cell death can return cancer cells to their immunity-cycle
- IMMUNEPOTENT CRP is a bona fide immunogenic cell death inducer
- Therapeutic potential of tumor cell lysates obtained by ICRP treatment
- IMMUNEPOTENT CRP in the landscape of cancer therapy

The role of Ca^{2+} in the duality of IMMUNEPOTENT CRP

IMMUNEPOTENT CRP is a promising immunotherapy with antitumoral and immunomodulatory effects. Cancer cells exposed to ICRP treatment succumb to regulated cell death (Martinez-Torres et al. 2019; Martínez-Torres et al. 2018; Rodríguez-Salazar et

al. 2017), nevertheless non-malignant cells, such as PBMCs and monocytes, did not exert cell death when are treated with ICRP (Franco-Molina et al. 2006; Sierra-Rivera et al. 2016), hence ICRP induce a selective cytotoxicity on malignant cells.

Here, it was proved that ICRP induced cell death in breast cancer cells via Ca^{2+} overload, this work demonstrated that the disruption of Ca^{2+} signaling mediated by ICRP is the first event described so far in the cytotoxic pathway of ICRP in cancer cells. It is important to mention that profound changes in the expression of Ca^{2+} channels and pumps can occur in some cancers, for instance, ORAI1 and TRPV6 expression is increased in the basal subtype of breast cancer, whereas ER+ breast cancer cells overexpress ORAI3 (Monteith, Prevarskaya, and Roberts-Thomson 2017). ORAI3 is capable of inducing a store-operated conductance, but its magnitude is considerably smaller than that seen with ORAI1 (Shuttleworth 2012), this could be one of the main reasons of the sensibility for ICRP-induced cell death observed in 4T1 cells in comparison with MCF7 cells.

Alterations at the ER-mitochondria interface in breast cancer have also been reported, for instance, the expression of Sig1R is higher in metastatic potential breast cancer cells than in normal tissues, Sig1R binds to IP_3R, thereby stabilizing IP_3R at the MAMs and enhancing IP_3R-mediated Ca^{2+} fluxes to the mitochondria. Also, high expression of IP_3R3 has been observed in human malignant tissues, this is important because compared with IP_3R isoforms 1 and 2, which are located at the ER membranes, IP_3R3 is highly enriched at the MAMs (and is considered a MAM marker) (Kerkhofs et al. 2017; Morciano et al. 2018). This phenotype in cancer cells allows them to proliferate, as key cell cycle events rely on Ca^{2+} signaling, also, specific oncogene and pro-oncogene pathways involve Ca^{2+}

signaling, including those bestowing resistance to apoptosis. In addition, tumor cell migration and invasion are modulated by specific Ca^{2+}-permeable ion channels, and signaling leading to epithelial to mesenchymal transition (EMT) phenotype is Ca^{2+}-dependent (Monteith, Prevarskaya, and Roberts-Thomson 2017). Hence, there is an opportunity to target the Ca^{2+} signaling in cancer, as these cells are more sensitive to the disruption of Ca^{2+} signaling, and this may promote or induce cancer cell death.

It is not irrational to think that the selectivity of ICRP-induced cell death to cancer cells is because of differences between Ca^{2+} signaling in malignant and non-malignant cells. This has been already studied with other treatments, for instance, a CD47-agonist peptide triggered a transient increase of intracellular Ca^{2+} levels that then returned to baseline without inducing cell death in normal B cells, but in chronic lymphocytic leukemia cells, the treatment induced a strong and sustained Ca^{2+} mobilization that did not return to basal levels, leading to cell death (Martinez-Torres et al. 2015). Hence, it is important to evaluate the differences between Ca^{2+} signaling triggered by ICRP in normal and cancer cells.

It is also interesting that ICRP treatments increased the expression of the activation marker CD69 in PBMCs. Whereas in NK cells, ICRP induced the activation of these cells and increased the degranulation marker CD107a (Manuscript in preparation). The activation of immune cells and the degranulation process in NK cells are two processes that have been widely associated with Ca^{2+} mobilization (Maul-Pavicic et al. 2011; Vig and Kinet 2009). These findings suggest that ICRP may be able to trigger different Ca^{2+} signaling, one leading to the activation of immune cells and another one leading to the death of

cancer cells. However, Ca^{2+} signaling has not been demonstrated in immune cells treated with ICRP, thus, this opens a perspective of Ca^{2+} analysis on these cells.

Biochemical characteristics of IMMUNEPOTENT CRP-mediated cell death in cancer

ICRP induces a caspase-independent ROS-dependent regulated cell death in cervical and lung cancer cell lines, involving loss of Δψm, cell cycle arrest and DNA degradation (Martinez-Torres et al. 2019; Martínez-Torres et al. 2018). Moreover, in leukemic cell lines ICRP induced apoptosis through ROS production, mitochondrial damage and nuclear alterations (Lorenzo-Anota et al. 2020), hence, ICRP triggers different cell death modalities depending on cancer cell lineage. In this work, it was demonstrated that ICRP induces regulated cell death, ROS production and Δψm loss in breast cancer cells, thus, the conserved mechanisms of action between all cancer cell lines evaluated to this day include the overgeneration of ROS and loss of Δψm.

ROS production and loss of Δψm are associated with deregulations in Ca^{2+} homeostasis (Orrenius, Zhivotovsky, and Nicotera 2003), furthermore, it has been demonstrated that ICRP induces calpain (Ca^{2+}-dependent proteases) activation in HeLa cells (Robles-Reyes 2011). Nevertheless, the implication of Ca^{2+} in ICRP-mediated cell death was unknown. Here, it was shown that ICRP induces cytoplasmic Ca^{2+} augmentation and ER stress, suggesting ER Ca^{2+} release. Additionally, it was demonstrated that the overgeneration of ROS, loss of Δψm, and cell death mediated by ICRP in breast cancer cells require the influx of extracellular Ca^{2+}. 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_3Rs decreased calcium release from the ER and inhibited cisplatin-induced cell death (Shen et al. 2016).

There are different pathways that can lead to the biochemical characteristics observed after ICRP treatment, for instance ICRP may induce Ca^{2+} depletion in ER, triggering ER stress that lead to CRT exposure 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 MAMs, generating Ca^{2+} overload, resulting in loss of $\Delta\psi m$ 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 such as PS exposure and DNA degradation (Orrenius, Zhivotovsky, and Nicotera 2003), two processes that have been observed in ICRP-generated cell death (Martinez-Torres et al. 2019; Martínez-Torres et al. 2018; Lorenzo-Anota et al. 2020). However, there are missing pieces in the puzzle of ICRP-mediated cell death, it is still unknown if ICRP generates Ca^{2+} depletion in ER-Ca^{2+} pool, and if so, how does ICRP generates it? As I mentioned before, this depletion could be modulated through IP_3 generation by PLC or by calcium-induced calcium release process,

thus, it would be important to elucidate how is the initial pulse of Ca^{2+} induced, in the pathway triggered after ICRP treatment.

In this work it was also demonstrated that CRT exposure triggered by ICRP treatment was inhibited when extracellular Ca^{2+} was chelated, this indicates that the Ca^{2+} influx from the extracellular space is required for an ER stress induction. These data suggest that the first pulse of Ca^{2+} might come from the extracellular space, stimulating calcium-induced calcium release process in ER, leading to Ca^{2+} depletion in ER, ER stress, and subsequent CRT exposure. However, this hypothesis must be verified with more studies. Dependence of Ca^{2+} fluxes for CRT exposure has been observed with other treatments, for instance, mitoxanthrone induced ER Ca^{2+} depletion that favors the exposure of CRT (Tufi et al. 2008).

According to these data, I hold the belief that the molecules that are present in the ICRP (such as cyclic nucleotides) might stimulate second messenger-operated channels (SMOCs), or ICRP might cause alterations in the membrane potential meaning a depolarization (which activates voltage-operated channels-VOCs) or hyperpolarization (which activates the transient receptor potential (TRP) ion-channel family), any of these events will allow the entry of Ca^{2+} into the cells, then, the calcium-induced calcium release process (CICR), whereby Ca^{2+} promotes its own release from ER, might amplifies these microscopic initiation pulses into Ca^{2+} propagating signals. CICR could cause depletion of Ca^{2+} from ER, leading to CRT exposure and the opening of store-operated channel (SOC), a plasma-membrane ion channel, that opens in response to the depletion of internal Ca^{2+} stores, which will increase dramatically the levels of Ca^{2+} in the cells leading to DNA

degradation after the activation of Ca^{2+}-dependent enzymes and also the activation of proteases. In addition, Ca^{2+} could be transported across the OMM and IMM of the mitochondrion, leading to a mitochondrial Ca^{2+}overload, ROS production, and loss of mitochondrial membrane potential. In conjunction, all these processes will trigger regulated cell death in cancer cells. However, it would be a rash to propose a detailed mechanism of action at this point, and further analysis and information is required.

The results presented in this project demonstrated that ICRP treatment increased autophagosome formation in breast cancer cells, it has been observed that autophagy plays different roles in the immunogenicity of cell death. In 2011, Michaud and colleagues showed that autophagy is dispensable for chemotherapy-induced cell death but required for its immunogenicity, as autophagy-deficient cancer cells fail to attract DCs and T cells into the tumor bed after treatment with chemotherapy, this was supported with the observation of a decrease in DAMPs release from dying tumor cells when autophagy was inhibited (Michaud et al. 2011). Moreover, inhibition of autophagy diminishes HMGB1 release (Tang et al. 2010; Thorburn et al. 2009). However, in 2013, Garg and colleagues showed that in contrast to expectations, ATG5 knockdown in cancer cells did not alter ATP secretion after Hyp-PDT. Furthermore, in response to Hyp-PDT, autophagy-attenuated cancer cells displayed enhanced CRT exposure, increasing their ability to induce DCs maturation and T cell proliferation (Garg et al. 2013). Hence, although it has been demonstrated that ICRP induces pro-survival autophagosome formation (Alvarez-Valdez, 2018), the role of autophagy in the immunogenicity of ICRP-induced cell death should be better described in further analysis.

In spite of the fact that the exact role of autophagy in ICRP-induced cell death immunogenicity has not been demonstrated, here it was shown that ICRP could trigger significative ATP secretion in MCF-7 and 4T1 cells, corresponding with the previous observation that autophagic vesicles loaded with ATP can fusion with the plasma membrane to release this nucleotide into the extracellular medium upon autophagy stimulation in a V-SNARE-dependent manner (Fader, Aguilera, and Colombo 2012). Additionally, higher levels of ATP release were observed in MCF-7 in comparison with 4T1 cells. 4T1 is a p53 deficient cell line (Sang et al. 2005), and previous studies demonstrated that ATP is produced at higher levels using oxidative phosphorylation in HCT116 cells expressing p53 versus similar cells lacking p53 (Puzio-Kuter 2011). Furthermore, ICRP treatment triggered HMGB1 release in MCF-7 but partial HMGB1 release in 4T1 cells. This low ICRP-mediated HMGB1 release could be due to the exposure of cells to ICRP CC_{50} treatment, which was not sufficient for a significant release of HMGB1, as some agents induce the release of HMGB1 at CC_{100} but not CC_{50} (Uscanga-Palomeque et al. 2019). Also, it has been demonstrated that the release/exposure of all DAMPs is not determinant for immunogenic cell death (Garg et al. 2013), thus the immunogenicity of the cell death induced by ICRP was further evaluated *ex vivo* and *in vivo*.

ICRP-mediated cell death can return cancer cells to their immunity-cycle

Cancer-Immunity Cycle concept implies that tumor-associated antigens, accompanied by danger signals, are released by cancer cells and then captured by dendritic cells (DCs) for processing, promoting priming and activation of effector T cell responses against the

cancer-specific antigens, however, cancer cells develop strategies to escape from this cycle, and it has been proposed that an alternative to make cancer cells immunogenic, and return them to the cancer-immunity cycle is through the induction of ICD (Chen and Mellman 2013).

Cancer dying cells by ICD inducers promote DCs maturation, which strongly activate anticancer immunity. According to current models, only few treatments induce intracellular signaling pathways that lead to a cancer cell death able to stimulate fully mature DCs, including γ-irradiation (Kim, Yun, and Han 2013), doxorubicin, oxaliplatin (Ghiringhelli et al. 2009), bortezomib (Cirone et al. 2012), and a CD47 agonist peptide (Martínez-Torres et al. 2019). Other therapies are only speculated to induce complete DCs maturation, or use LPS, IFN type 1, or others stimulants in combination with TCL to promote DCs' maturation (Chen et al. 2012; Schiavoni et al. 2011). In addition, some cancer treatments may cause semi-mature DCs, namely DCs that lack of phenotypic maturation markers or cytokine release, and thereby are unable to efficiently prime T cells (Dudek, Martin, et al. 2013). Here, it was demonstrated that the tumor cell lysate (TCL) obtained from ICRP-treated 4T1 cells (ICRP-TCL) induced phenotypic and functional maturation of BMDCs.

It has been observed that in the course of ICD, DAMPs exposure/release elicits the production of pro-inflammatory cytokines, such as TNF-α from DCs (Garg et al. 2016), here, ICRP-TCL triggered TNF-α release from BMDCs after 24 h of stimulation. Moreover, APCs that performed immunogenic phagocytosis can then prime CD4+ and CD8+ T cells and thereby trigger immunogenic T helper 1 (Th1) cell and cytotoxic T

lymphocyte (CTL) responses facilitating CTL-elicited cancer cell elimination through IFN-γ, FasL-CD95 interaction, and perforin-granzyme action (Green et al. 2009). In this study, primed T cells obtained after co-culture with BMDCs-ICRP-TCL caused a cytotoxic effect in 4T1 cells, whereas a significant increase of IFN-γ release was observed in the co-culture of primed T cells with 4T1 cancer cells, indicating an immune response against 4T1 cancer cells, which involves Th1-type cytokines. Hence, the results in this work strongly suggest that ICRP-mediated cell death can turn cancer cells immunogenic and this may promote that the cancer-immunity cycle succeed.

IMMUNEPOTENT CRP is a *bona fide* immunogenic cell death inducer

After *in vitro* and *ex vivo* evaluation of the strategies conceived to detect markers of ICD, the next step was to perform the gold-standard approach to detect ICD inducers which relies on vaccination experiments involving immunocompetent murine models and syngeneic cancer cells (Kepp et al. 2014). With this prophylactic tumor vaccination model, it was demonstrated that ICRP-TCL activates the adaptive immune system in 90% BALB/c mice. Usually, ICD inducers protect from 50 % to 90 % of individuals when used alone, without any type of adjuvants, such is the case of Hypericin-based photodynamic therapy (87%) (Garg et al. 2012), mitoxantrone (80%) (Menger et al. 2012), oxaliplatin (80%) (Tesniere et al. 2010), and nanosecond pulsed electric fields (50%) (Rossi et al. 2019). Other treatments need two previous vaccinations to induce slower tumor growth in vaccinated mice (Qin et al. 2017) or the use of combinational therapy to reach protection in 80% of the cases (Liu et al. 2019). Moreover, agents classified as ICD inducers have

been studied using TCL-loaded dendritic cell vaccines, but not TCL, and after several vaccines boosting they reach up to 70% of survival (Chen et al. 2012).

The results in this work are supported by the recent demonstration that breast tumor-bearing mice treated with ICRP present a decrease in tumor volume and increase in survival in comparison with untreated mice. Also, within the tumor, ICRP treatment decreased PD-L1, IDO and Gal-3 expression, IL-6, IL-10, and MCP-1 levels, and increased IFN-γ, and IL-12 levels. Moreover, ICRP treatment increase CD8+ T cells, memory T cells, and innate effector cells in peripheral blood, where it was also observed an increase in IFN-γ, and IL-12 levels (Santana-Krímskaya et al. 2020), indicating that these findings could be due to ICD induction in the tumor of these mice.

Recently, a list of properties of an ideal ICD inducer was proposed (Krysko et al. 2012), which I will discuss in regard to IMMUNEPOTENT CRP.

An ideal ICD inducer:

- *Should be an efficient instigator of regulated cell death (at doses that can be used preclinically or clinically without substantial toxicities or side effects).* ICRP induces regulated cell death in leukemic (Lorenzo-Anota et al. 2020), cervical cancer (Martínez-Torres et al. 2018), lung cancer (Martinez-Torres et al. 2019), and melanoma cell lines without affecting the viability of human monocytes and PBMCs, and murine peritoneal macrophages (Lorenzo-Anota et al. 2020; Franco-Molina et al. 2006; Sierra-Rivera et al. 2016). *In vivo*, ICRP-treatment decreased tumor weight and improved the survival of tumor-bearing mice in a murine

melanoma model (Franco-Molina et al. 2010), additionally, breast tumor-bearing mice treated with ICRP showed a decrease in tumor volume, and increase in survival in comparison with untreated mice, ICRP administration decreased Ki-67 and increased caspase-3 expression in tumor tissue, suggesting the induction of ICRP-mediated cancer cell death, moreover, ICRP treatment did not affect tissue histology of spleen, liver, kidney, brain, lung and heart of tumor-bearing mice (Santana-Krímskaya et al. 2020). Furthermore, in lung and breast cancer patients undergoing chemotherapy or/and radiation therapy, ICRP administration resulted in improved life quality and immunomodulatory activity (Franco-Molina et al. 2008; Lara et al. 2010). Thus, these results demonstrated that ICRP can be used in cancer patients without substantial toxicities or side effects, however it would be important to corroborate that ICD is taking place in cancer cells at the doses used clinically, it could be evaluated through the analysis of DAMPs levels in serum, as well as TILs, Tregs and MDSCs proportion, and CRT, RAGE, HMGB1, LC3 and STQSM1/p62 expression within tumor microenvironment in cancer patients undergoing ICRP administration.

- *Should be capable of inducing emission of multiple types of DAMPs, TLR agonists and immunogenic signals.* In this study it was demonstrated that ICRP treatment induce CRT exposure, which is an immunogenic "eat me" signal, as well as release of the immunogenic soluble factors ATP and HMGB1 in human and murine breast

cancer cells, also, in murine melanoma cells ICRP triggered CRT exposure and release of ATP, HSP70, HSP90 and HMGB1 (Rodríguez-Salazar et al. 2017).

- *Should not be subject to drug-efflux pathways.* As ICRP is not a drug, is a mixture of substances with biological activity, it may not be subject to drug-efflux pathways, also, it has not been described until today mechanisms of resistance to ICRP-induced cell death, however, ICRP needs an overgeneration of ROS to exert its cytotoxic effect (Martinez-Torres et al. 2019; Martínez-Torres et al. 2018), thereby, antioxidants could be able to diminish its cytotoxic effects in cancer cells.

- *Should be capable of inducing ER stress, which would make it possible to improve DAMP trafficking and to increase their emission.* In this work it was proved for the first time that ICRP induced eIF2α phosphorylation in human and murine breast cancer cells, which is a marker of ER stress (Kepp et al. 2015), also, in these cells ICRP triggers CRT exposure, that is another cellular process associated with ER stress (Grootjans et al. 2016). Thus, ICRP treatment induces ER stress that may facilitate the trafficking and emission of the DAMPs observed in this work.

- *Should be capable of overcoming loss-of-function mutations that cripple danger signaling during cancer microevolution.* It has been demonstrated that ICRP induces apoptosis in leukemia cell lines as inhibition of caspase-3, -8 and -9 led to significantly cell death diminish (Lorenzo-Anota et al. 2020). However, in this work it was proved that ICRP treatment can overcome mutations in apoptosis pathway, hence, is able to induce cell death and emission of DAMPs in MCF-7

cells, which are caspase-3 deficient (Wang et al. 2016), and 4T1 cells, a p53 deficient cell line (Sang et al. 2005). Moreover, ICRP induced caspase-independent cell death in cervical (Martínez-Torres et al. 2018) and lung cancer cell lines (Martinez-Torres et al. 2019).

- *Should be capable of downregulating cancer-based induction of pro-inflammatory transcription factors.* Activation of pro-inflammatory transcription factors such as NF-κB in cancer cells often correlates with increased tumor growth and a negative prognosis (Krysko et al. 2012). In LPS-stimulated human macrophages ICRP decrease IκB phosphorylation, NF-κB p50 and p65 subunit DNA binding activity (Franco-Molina et al. 2011), suggesting that ICRP may downregulate this pro-inflammatory transcription factor in cancer context, however it should be evaluated in further studies.

- *Should have negligible suppressive or inhibitory effects on immune cells such as mature dendritic cells, natural killer cells, CD3+CD4+ T cells (mainly TH1 phenotype), cytotoxic CD3+CD8+ T cells, memory CD3+CD4+ or CD3+CD8+ CD45RO+ T cells and B cells that infiltrate a tumor site following treatment, as they are likely to be required for immune reactions that are centered on the tumor.* ICRP does not affect the viability of human monocytes and PBMCs, and murine peritoneal macrophages (Franco-Molina et al. 2006; Sierra-Rivera et al. 2016). Furthermore, in a recent study ICRP treatment increased the expression of the activation marker CD69 in PBMCs and NK cells. Also, it was observed that ICRP modulates NK cells, by increasing the proportion of $CD56^{low}/CD16^{low}$ cells and

the degranulation marker CD107a, suggesting a cytotoxic phenotype in these cells. Moreover, ICRP potentiated T cell activation in anti-CD3-pre-activated T cells, and decreased CD4 and CD8 expression in T cells. Also, ICRP decreased expression of naïve markers, and did not induce senescence or exhaustion in T cells (unpublished observations of our research group). In breast tumor-bearing mice ICRP treatment increase CD8+ T cells, memory T cells, and innate effector cells in peripheral blood (Santana-Krímskaya et al. 2020). Additionally, ICRP administration in cancer patients resulted in an increase of the total leukocytes and T cells subpopulations (Franco-Molina et al. 2008; Lara et al. 2010). These results not only indicate that ICRP has negligible suppressive or inhibitory effects on immune cells, but also demonstrated its favorable immunomodulatory functions.

- *Should be able to inhibit immune-suppressive responses, such as those mediated by tumor-associated macrophages (TAMs; mainly M2 phenotype), myeloid derived suppressor cells (MDSCs), regulatory T cells and CD3+ CD4+ T cells (mainly TH2 phenotype).* It has been demonstrated that ICRP administration in breast tumor-bearing mice decreased the expression of the suppressor molecules PD-L1, IDO and Gal-3, however ICRP did not decrease either MDSCs nor Treg cells in the tumor tissue (Santana-Krímskaya et al. 2020).

- *Should be capable of directly targeting not only the primary tumor but also metastases.* It is not demonstrated that ICRP directly targets metastases, however, in murine melanoma, ICRP decreased VEGF production *in vitro* and *in vivo* and prevented metastasis in tumor-bearing mice (Franco-Molina et al. 2010). In

addition, in a murine breast cancer model ICRP significantly decreased VEGF and α-SMA expression in tumor tissue as compared with untreated mice (Santana-Krímskaya et al. 2020).

Hence, IMMUNEPOTENT CRP is not only a *bona fide* ICD inducer, but also possess most of the properties of an ideal ICD inducer. It has been proposed that the induction of ICD eventually results in long-lasting protective antitumor immunity due to the "anticancer vaccine effect" (Zhou et al. 2019). Thus, I will further discuss the memory responses that were observed in immunized mice after prophylactic vaccinations with ICRP-TCL.

It is known that T cell responses generally peaks ~1-4 days after a second antigen stimulation (Pennock et al. 2013; Punt et al. 2019). Here, tumor-draining lymph nodes after three days of tumor re-challenge were analyzed. The results demonstrated a follicular lymphoid hyperplasia in ICRP-TCL mice, which is associated with immunological memory, moreover an increase of mature DCs in ICRP-TCL mice was observed in comparison to the naïve group. Several studies have demonstrated that effector memory T cells potentiate the maturation of DCs, and in addition to T cells, BCR signaling is sufficient for memory B cells to induce complete activation of DCs (Maddur, Kaveri, and Bayry 2018). Additionally, in infection diseases it has been observed that DCs isolated from protectively immunized mice express a memory-like behavior different from that of DCs isolated from non-protectively immunized mice (Hole et al. 2019), thereby, memory-

like responses in DCs could be generated in a cancer context as well, however, it would be necessary to address this in future investigations.

An increase of CD8+ T cells over CD4+ T cells was also demonstrated in the peripheral blood of immunized mice, these results correspond with the observations that in the same host, memory assessments result in robust CD8+ T cell responses, but poor boosting of CD4+ T cell recall responses (Ravkov and Williams 2009), which is correlated with the demonstration that CD4+ memory cells proliferated for a shorter period of time than CD4+ naïve cells because of their cytokine profile (MacLeod et al. 2008).

Finally, tumor specific cytotoxicity by splenocytes from immunized mice was demonstrated, whereas no cytotoxicity was observed in the co-culture of 4T1 with splenocytes from naïve mice, indicating the activation of a rapid immune response triggered by the antitumor memory establishment. These evaluations correspond with the *ex vivo* assessment performed in this investigation, where it was observed an increase in Th1-type cytokines, which are associated with cytotoxic responses generation (Berger 2000).

Therapeutic potential of tumor cell lysates obtained by ICRP treatment

As a confirmatory assay, ICRP-TCL was assessed for their ability to mediate immune system-dependent therapeutic effects against established neoplastic lesions, in this scenario tumor cell lysates obtained by *bona fide* ICD inducers treatment mediate optimal therapeutic effects in immunocompetent mice. The results presented in this work

demonstrate that ICRP-TCL induced tumor regression and increase survival in 4T1-tumor bearing mice, that confirms the immune response triggered by ICRP-TCL against 4T1 cells, leading to tumor eradication.

Moreover, it is important to mention that whole tumor cell lysates have been proposed as a therapeutic approach in cancer therapy. There are several clinical trials using tumor cell lysates as immunogenic sources for cancer vaccine design, for instance, there is a study in phase I/II for subjects with recurrent ovarian, fallopian tube or primary peritoneal cancer to determine the immunogenicity of an autologous Oxidized tumor Cell Lysate (OC-L) administered in combination with a Toll-like receptor 3 agonist (NCT01312389), also, a phase II trial studies the effectiveness of autologous tumor cell vaccination plus immunologic adjuvant (GM-CSF) in treating patients who have metastatic cancer (NCT00002505), and another study uses resected tumor cells irradiated and mixed with CpG to create a vaccine (NCT00780988). In other clinical trials tumor cell lysates are used to pulse DCs, for example, a study in early phase I investigates the safety and efficacy of dendritic cells vaccines pulsed with autologous whole tumor cell lysate for treating advanced solid tumor patients with high tumor mutation burden (NCT03671720). The majority of these studies are based on models performed in mice, where most of them use tumor cell lysates in combination with adjuvants to reach a therapeutic success (Kawahara and Takaku 2015; Pyo et al. 2016; Si et al. 2017). Here, tumor regression in 88% of mice treated with ICRP-TCL was obtained without any type of adjuvants.

The four common whole tumor lysate preparations are obtained by the treatment of cancer cells with hypochlorous acid (HOCl), ultra-violet B (UVB)-irradiation, repeated freeze-

thaw, and hyperthermia. These treatments can induce the release of tolerogenic and immunogenic signals from cancer cells that could promote a tolerogenic transformation of DCs. Therefore, a whole tumor lysate preparation that simultaneously induces an immunogenic cell death and suppress the release immunosuppressive signals from the tumor cells would be highly desirable (Chiang, Coukos, and Kandalaft 2015). The findings in this work allow to propose the evaluation of tumor cell lysates obtained by ICRP treatment as an immunotherapy approach in cancer treatment, also these results strongly suggest that dendritic cells vaccines pulsed with ICRP-TCL may be a promising strategy for cancer therapy.

IMMUNEPOTENT CRP in the landscape of cancer therapy

In the past 25 years, research efforts in cancer therapy has largely addressed two lines of inquiries, one focused on the development of targeted agents that result in profound, but often not durable, tumor responses in genetically defined patient populations, and another approach looking for protected anticancer immunity to accomplish durable clinical responses. In this regard, immunity is influenced by a complex set of host, environment, and tumor factors that dictates the strength and timing of the anticancer response (Gotwals et al. 2017). These investigations led to the definition of the cancer–immune set point that can be understood as a balance between the stimulatory factors minus the inhibitory factors, which together must be equal to or greater than 1, over the summation of all T-cell antigen receptor (TCR) signals for tumor antigens (Chen and Mellman 2017). In this aspect, direct stimulation of immune cell or the induction of immunogenic cell death in cancer cells could alter the set point, for example, by propagating the cancer-immunity

cycle, which enhances the cancer-specific T-cell response. Thereby, IMMUNEPOTENT CRP, is a promising therapy, as it can induce immunomodulatory effects and eject ICD on breast cancer cells.

To this day, only a few therapies induce the immunogenic demise of cancer cells, and several studies indicate that ICD-inducing chemotherapy can initiate robust anticancer immunity increasing the efficacy of the treatment (Garg et al. 2017). Withal, as a negative aspect, the widespread use of chemotherapy has been implemented mostly on empirical (rather than immunological) grounds, thus, possible scenarios of chemotherapeutic action on the cancer–host relationship are immunosuppressive adverse effects and selection of therapy-resistant and immune-resistant cancer cells, thereby, one of the goals in cancer therapy is to identify doses and administration schedules that mediate maximal immunostimulatory effects (Zitvogel, Kepp, and Kroemer 2011).

In this regard, in 2016, Coronado-Cerda et al. observed that ICRP has a chemo-protective effect in bone marrow cells exposed to 5-FU (Coronado-Cerda et al. 2016). In addition, Rodríguez-Salazar et al. demonstrated that ICRP in combination with oxaliplatin (OXP) increased the exposure and release of DAMPs, moreover, administration of TCL derived from B16F10 cells treated with ICRP + OXP prevented melanoma growth in mice; whereas, TCL obtained from B16F10 cells treated with OXP did not protect from tumor growth (Rodríguez-Salazar et al. 2017). Recently, Santana-Krímskaya et al. observed that ICRP administration in 4T1-tumor bearing mice treated with doxorubicin and cyclophosphamide potentiates the anti-tumor effect of Dox/Cyclo chemotherapy, and modifies the tumor microenvironment decreasing the immune-suppression consequences

triggered by the administration of these chemotherapeutic agents (Santana-Krímskaya et al. 2020). Furthermore, in 2010 Lara et al. carried out a study in breast cancer patients receiving chemotherapy and ICRP as adjuvant. The results showed that 60% of the patients in the group receiving ICRP as adjuvant displayed a complete response, 32% showed a partial response and 8% did not respond. By contrast, in the group without the adjuvant, 39% showed a complete response, 50% displayed a partial response and 11% were non-responders. In addition, ICRP treatment in combination with chemotherapy resulted in quality of life enhancement during chemotherapy (Lara et al. 2010). Overall, observations in this study and previous data discussed above, propose the use of ICRP to optimize chemotherapy schedule.

Furthermore, along with the hurdle mentioned above, cancer is an evolving disease, and some cancer cells display alterations that prevent the activation of tumor-targeting immunity triggered by malignant cells undergoing ICD. This constitutes a major obstacle to the efficacy of ICD-inducing therapies, thereby is urgent the development of efficacious combinatorial regimens (Wu and Waxman 2018), thus, application of IMMUNEPOTENT CRP could have a pivotal effect by stimulating immune cells, and inducing ICD in cancer cells, however its effectives in combinatorial regimens must be evaluated in further studies.

IX. CONCLUSIONS

Overall, there results demostrate that IMMUNEPOTENT-CRP triggers an endoplasmic stress accompanied by the increase of cytoplasmic Ca^{2+} levels. Ca^{2+} entry to cancer cells leads to mitochondrial damage, ROS production, and CRT exposure. This intracellular signaling pathway promotes autophagosome formation and the release of ATP and HMGB1, which along with CRT exposure stimulate DCs maturation, priming of T cells, promoting an antitumor immune response *ex vivo*. Prophylactic vaccination with ICRP-TCL prevents tumor establishment in BALB/c mice leading to a long-term antitumor memory that involves DCs maturation in lymph nodes, CD8+ T-cells augmentation in peripheral blood, and *ex vivo* tumor-specific cytotoxicity by splenocytes. Moreover, therapeutic vaccinations with ICRP-TCL generate tumor regression in 4T1-tumor bearing mice, triggering long-term antitumor memory in treated mice. Hence, ICRP may have the capacity to turn breast cancer cells into potential vaccines *in vivo* through the induction of immunogenic cell death.

X. PERSPECTIVES

- 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.
- Investigate the nexus between Ca^{2+} deregulation and immunogenicity of ICRP-induced cell death in cancer cells.
- Evaluate Ca^{2+} levels in subcellular organelles (such as mitochondria and endoplasmic reticulum) in cancer cells treated with ICRP.
- Elucidate the role of mitochondrion-associated membranes of endoplasmic reticulum in ICRP-mediated cell death.
- Study the implication of autophagosome formation in the immunogenicity of ICRP-induced cell death.
- Evidence the mechanism of DAMPs release triggered by ICRP.
- Determinate the anti-tumor potential of ICRP treatment in immunodeficient mice, as ICD-eliciting agents must exhibit superior therapeutic efficacy when employed against tumors growing in immunocompetent mice as compared to immunodeficient hosts.
- Investigate the therapeutic potential of ICRP-TCL-loaded dendritic cell vaccines, with murine and human dendritic cells.
- Evaluate if ICRP enhance the immunogenic potential of chemotherapy-induced cell death.

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XII. BIOGRAPHICAL ABSTRACT

Alejandra Reyes Ruiz

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

Thesis: Study of Ca^{2+}-implication and immunogenicity of the regulated cell death induced by IMMUNEPOTENT CRP on breast cancer cells.

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Title of research work "Cytotoxic mechanism induced by IMMUNEPOTENT CRP on HeLa cells".

Awards:

- Special distinction for the quality and originality of the thesis work. UANL, Nuevo León, México, 2016.
- Best Poster of Microbiology/Immunology at Forum of Presentation and Advances in Research. UANL, Nuevo León, México, 2019.

Professional experience:

Instructor of Biology, Chemistry I and II, Biochemistry, Microbiology, and Pharmacology. Linda Vista Nursing School, Nuevo León México.

List of Scientific Communications

Scientific publications on Indexed Journals JCR:

Martínez-Torres, A. C., **Reyes-Ruiz, A**., Benítez-Londoño, M., Franco-Molina, M. A., & Rodríguez-Padilla, C. (2018). IMMUNEPOTENT CRP induces cell cycle arrest and caspase-independent regulated cell death in HeLa cells through reactive oxygen species production. BMC cancer, 18(1), 13.

Martínez-Torres, A. C., **Reyes-Ruiz, A.**, Calvillo-Rodríguez, K.M., Álvarez-Valdez, K.M., Uscanga-Palomeque, A.C., Tamez-Guerra R.S., Rodríguez-Padilla, C. (2020). IMMUNEPOTENT CRP induces DAMPS release and ROS-dependent autophagosome formation in HeLa and MCF-7 cells. BMC Cancer, 20(1):647.

Reyes-Ruiz, A., Calvillo-Rodríguez K.M., Martínez-Torres, A.C., Rodríguez-Padilla C. The bovine dialyzable leukocyte extract IMMUNEPOTENT CRP induces immunogenic cell death in breast cancer cells leading to long-term antitumor memory. Under review in British Journal of Cancer, submitted in February 2020.

Scientific publications in Arbitrated Journals:

Reyes-Ruiz, A., Benitez-Londoño M., Martínez-Torres, A.C., Rodríguez-Padilla C. (2017). Análisis del mecanismo citotóxico inducido por el IMMUNEPOTENT CRP sobre células HELA. Revista de Ciencias Farmaceúticas y Biomedicina (ISSN: 2448-8380), 16.

Londoño, M. B., **Reyes-Ruiz, A**., Martínez-Torres, A.C., Rodríguez-Padilla C. (2017). El IMMUNEPOTENT-CRP induce arresto en el ciclo celular y muerte celular regulada independiente de caspasas, pero dependiente de la producción de especies reactivas de oxígeno en células HeLa. Revista de Ciencias Farmaceúticas y Biomedicina (ISSN: 2448-8380), 3.

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la formación de autofagosomas en células HeLa y MCF-7. Revista de Ciencias Farmaceúticas y Biomedicina (ISSN: 2448-8380), 7.

Oral and poster presentations:

- "Análisis del mecanismo citotóxico inducido por el IMMUNEPOTENT CRP sobre células HeLa" **A. Reyes**, M. Benítez, A.C. Martínez, C. Rodríguez. Simposio Nacional de Ciencias Farmacéuticas y Biomedicina. FCQ, UANL, Nuevo León, México. August 2015. (**Poster**).
- "Análisis del mecanismo citotóxico inducido por el IMMUNEPOTENT-CRP sobre células HeLa". **A. Reyes**, A.C. Martínez, C. Rodríguez. III Encuentro de Jóvenes Investigadores. UANL y CONACYT, Nuevo León, México. October 2015. (**Oral**).
- "Análisis del mecanismo citotóxico inducido por el IMMUNEPOTENT-CRP sobre células HeLa". **A. Reyes**, A.C. Martínez, C. Rodríguez. 8º Simposio Internacional en Inmunopatogénesis e Inmunoterapia en Cáncer y Enfermedades Infecciosas. FCB, UANL, Nuevo León, México. November 2015. (**Poster**).
- "El IMMUNEPOTENT-CRP induce arresto en el ciclo celular y muerte celular regulada independiente de caspasas pero dependiente de la producción de especies reactivas de oxígeno en células HeLa". M. Benítez, **A. Reyes**, A.C. Martínez, C. Rodríguez. III Simposio Nacional de Ciencias Farmacéuticas y Biomedicina y I

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