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REVIEW

Calreticulin in phagocytosis and cancer: opposite roles in immune response outcomes

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Abstract

Calreticulin (CRT) is a pleiotropic and highly conserved molecule that is mainly localized in the endoplasmic reticulum. Recently, CRT has gained special interest for its functions outside the endoplasmic reticulum where it has immunomodulatory properties. CRT translocation to the cell membrane serves as an "eat me" signal and promotes efferocytosis of apoptotic cells and cancer cell removal with completely opposite outcomes. Efferocytosis results in a silenced immune response and homeostasis, while removal of dying cancer cells brought about by anthracycline treatment, ionizing-irradiation or photodynamic therapy results in immunogenic cell death with activation of the innate and adaptive immune responses. In addition, CRT impacts phagocyte activation and cytokine production. The effects of CRT on cytokine production depend on its conformation, species specificity, degree of oligomerization and/or glycosylation, as well as its cellular localization and the molecular partners involved. The controversial roles of CRT in cancer progression and the possible role of the CALR gene mutations in myeloproliferative neoplasms are also addressed. The release of CRT and its influence on the different cells involved during efferocytosis and immunogenic cell death points to additional roles of CRT besides merely acting as an "eat me" signal during apoptosis. Understanding the contribution of CRT in physiological and pathological processes could give us some insight into the potential of CRT as a therapeutic target.

Keywords Calreticulin · Apoptosis · Phagocytosis · Cancer · Immunogenic cell death · Immune response

Introduction

Calreticulin (CRT) is a 46 kDa soluble protein that localizes primarily to the endoplasmic reticulum (ER) and is highly conserved in all species. Two of the major functions of the ER are Ca^{2+} sequestration and glycoprotein assembly and secretion; CRT has a crucial role in both. CRT is involved in Ca^{2+} homeostasis; it regulates diverse cellular functions and acts as a lectin-like chaperone with specificity for monoglucosylated oligosaccharide in asparagine-linked glycoproteins that contributes to their correct folding in the ER [1–3]. The structure of CRT comprises three domains: The N-terminal globular domain, a flexible proline-rich P intermediate arm-like domain and a C domain at the carboxyl terminal. The N terminal of the molecule is predicted to be a highly folded globular structure, with eight anti-parallel β sheets connected by loops, its amino acid sequence is extremely conserved and binds Zn^{2+} [4]. It contains three cysteine residues conserved from higher plants to humans that are responsible for the correct folding of the protein. This domain can interact with α integrins and with the DNA binding site of steroid receptors [5, 6]. The P domain consists of an extended region stabilized by three anti-parallel β strands [7] and includes a proline-rich sequence with two sets of three highly conserved repeats. This region binds Ca^{2+} with high affinity ($K_d = 1\ \mu M$), and together with the N domain, is essential for the lectin-like chaperone function

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[3]. The C domain binds Ca^{2+} with high capacity due to its acidic nature, with more than 35 aspartic and glutamic acid residues, which function as multiple low-affinity Ca^{2+} binding sites and terminates with the ER retrieval signal [8, 9] (Fig. 1).

With the discovery of CRT in compartments beyond the ER, immunogenic roles and additional functions have been revealed. Among the functions that have gained particular interest in the last years are its role in promoting efferocytosis, in the clearance of dying cells by macrophages, and in the immunogenic cell death (ICD) of cancer cells induced under specific treatments. In the present paper, the recent findings on the role of CRT during these crucial functions of myeloid-derived monocytic cells will be addressed.

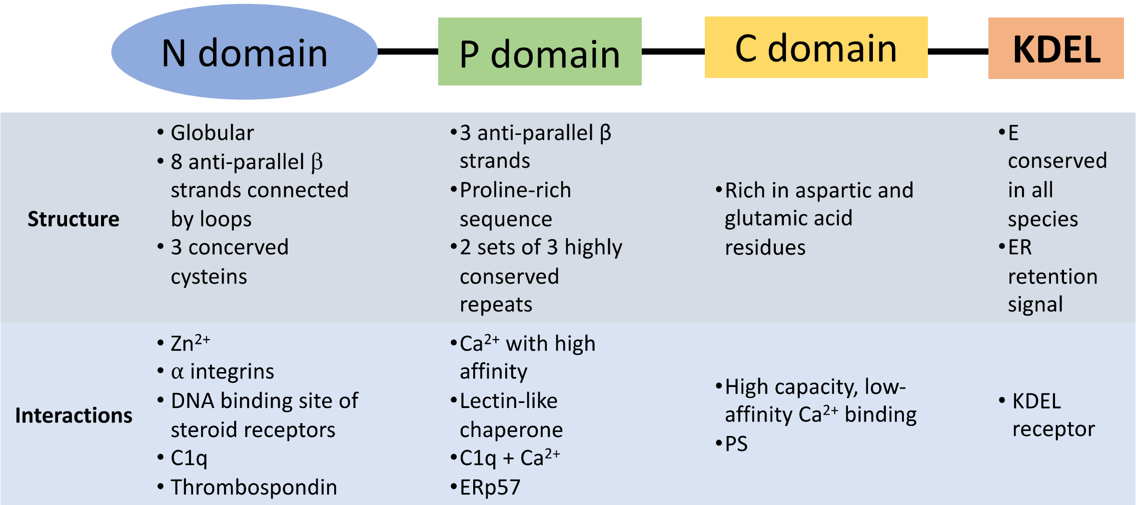
Calreticulin and efferocytosis

Phagocytosis is an essential process for a wide variety of entities, from unicellular organisms that use it as a means for nutrition, to multicellular organisms that depend on this complex process for correct function of the immune response through the activation of phagocytes. Moreover, phagocytosis is important for resolution of inflammation via the uptake of apoptotic cells, as well as for the maintenance of tissue homeostasis and protection against neoplastic cells. Failure in the clearance of apoptotic cells has been associated with autoimmunity, as it leads to pathologic inflammatory responses and induction of proinflammatory cytokine release [10].

Apoptosis, or programed cell death, is an ATP-dependent cellular process characterized by certain morphological changes, including membrane blebbing, nuclear condensation and biochemical alterations such as activation of proteases and fragmentation of DNA [11]. The uptake and removal of apoptotic cells by phagocytes, or efferocytic cells, is known as "efferocytosis", and is driven through several signals that flag the dying cell to be recognized and engulfed [12]. In fact, early in the apoptotic program, cells display molecular "eat me" signals on their surface to alert phagocytes that they must be removed [11]. Nowadays, CRT is recognized as one of such signals. The first report about CRT involvement in apoptosis was published by Ogden et al., who described that CRT has an important role in apoptotic Jurkat T cell clearance, driven by its ability to bind to the innate immune protein CD91 (also known as the low-density protein receptor-related protein, LRP-1), as well as to C1q and the mannose-binding lectin (MBL) [13]. Although C1q and MBL are involved in complement activation, they also attach to apoptotic cells by their globular domains to facilitate the ingestion by macrophages. After C1q or MBL attachment, CRT binds to the collagenous tails of both proteins, and to CD91 on the macrophage cell surface to promote the engagement of this receptor. These series of events initiate macropinocytosis that results in the engulfment of the apoptotic cell [13]. CRT functions at both sides of the phagocytic or efferocytic synapse, at the surface of the apoptotic cell ready to be engulfed, as well as on the phagocyte membrane and in the milieu surrounding the phagocytic synapse.

CRT on the apoptotic cell surface

Phosphatidylserine (PS) is one of the best characterized molecules that mediate phagocytosis, located in the inner leaflet of the cell membrane of living cells. Shortly upon induction



	N domain	P domain	C domain	KDEL
Structure	• Globular • 8 anti-parallel β strands connected by loops • 3 concerved cysteins	•3 anti-parallel β strands •Proline-rich sequence •2 sets of 3 highly conserved repeats	•Rich in aspartic and glutamic acid residues	•E conserved in all species •ER retention signal
Interactions	• Zn^{2+} • α integrins • DNA binding site of steroid receptors • C1q • Thrombospondin	•Ca^{2+} with high affinity •Lectin-like chaperone •C1q + Ca^{2+} •ERp57	•High capacity, low-affinity Ca^{2+} binding •PS	• KDEL receptor

Fig. 1 CRT structure and domain interactions. CRT has three structural domains, each one interacts with different molecular partners and has diverse functions

of apoptosis, PS translocates to the outer leaflet of the cell membrane and serves as an "eat me signal". CRT, normally localized in the ER lumen, also translocates to the outer cell membrane where, along with PS, promotes phagocytosis of apoptotic cells but not its adhesion to phagocytes. Recently, CRT on apoptotic cells was shown to bind to PS through its C-terminal acidic domain in a calcium-dependent manner, and serves as a PS-anchor factor that cooperates with other PS-binding molecules, such as C1q [14, 15]. Induction of apoptosis by UVB irradiation results in the mobilization of CRT to the outer cell membrane even before PS translocation [16]. CRT also interacts with C1q for the uptake of apoptotic cells [17–19]. The globular domain of CRT interacts with the globular and the collagen-like regions of C1q on the surface of PS-positive cells at the early stages of apoptosis [20]. In turn, C1q binds to the phosphoserine group of PS through its globular domain, acting as a bridging molecule [21]. CRT binds to PS with a ten-fold higher affinity as compared to C1q binding, interfering with the C1q-PS interaction [18, 19]. Thus, CRT, C1q, and PS are jointly implicated in the uptake of apoptotic cells, which strongly suggests that they act in conjunction during the initial recognition step, by promoting efferocytosis (Fig. 2). The differential interactions among these three molecules may modulate phagocytosis, as well as the phenotype induced in the phagocyte after apoptotic cell interaction and ingestion. The significance of CRT translocation to the cell surface is evidenced by experiments showing that CRT knockdown results in a less efficient phagocytosis and impairs C1q enhancement of the process [18].

Other proteins with collagen-like structures including MBL, surfactant proteins, and adiponectin are also involved in opsonizing apoptotic cells [22]. Adiponectin, a hormone produced by adipocytes, with anti-diabetic and anti-inflammatory properties, is structurally similar to C1q and was shown to promote apoptotic cell clearance by opsonizing dying cells and binding to CRT on the phagocyte through CD91-mediated efferocytosis. Adiponectin also mediates in vivo clearance of apoptotic cells, by preferentially opsonizing the apoptotic blebs and preventing autoimmunity. CRT on the macrophage surface interacts with adiponectin and mediates its anti-inflammatory properties [23]. CRT also interacts with the heparin-binding domain of thrombospondin-1 [13], which is secreted by apoptotic monocytes and promotes phagocytosis and dendritic cell (DC) immune suppression through CD91 [24].

CRT is also present on the surface of healthy cells, but it does not induce their uptake by phagocytes. Living cells are not phagocytized thanks to "don't eat me" signals, such as CD47 and CD31 [25], which balance the pro-phagocytic signals CRT and PS, and therefore suppress phagocyte function. During apoptosis, CRT clustering is coordinated with a decrease in CD47 [26], a molecule whose interaction with signal regulatory protein (SIRP)-α (also known as CD172a) in macrophages prevents phagocytosis of viable cells, through the activation of the immunoreceptor tyrosine-based inhibition motifs (ITIMs) [27]. In addition, engulfment of viable cells, in particular neutrophils, by a mechanism termed programmed cell phagocytosis (PrCP) has been reported. The authors showed that macrophages are the source of CRT on the surface of the living neutrophils to be engulfed, and that CRT binds to asialoglycans exposed on the neutrophil surface [25, 28]. These findings suggest that PrCP could be a more general phenomenon involving other cell types involved in tissue homeostasis.

CRT on the phagocyte surface

CRT on the surface of apoptotic or viable cells is able to interact with CD91 on the phagocyte surface, thus acting as a trans-activating signal for CD91 [26]. But CRT on the phagocyte surface also mediates removal of cells interacting with CD91 in a cis-activating manner, to promote the engagement of this receptor. CRT exposure on the macrophage cell surface is dependent on the Toll like receptor (TLR)-Bruton's tyrosine kinase (BTK) pathway that results in phosphorylation of CRT, as well as in secretion and binding to CD91, leading to an efficient apoptotic cell uptake [29]. Additionally, BTK phosphorylation of CRT in macrophages is a crucial effector of living cancer cell phagocytosis and removal. In fact, upregulation of CRT on macrophages enhances phagocytosis. Furthermore, experiments performed with anti-CRT antibodies suggest that phagocytosis is mediated by macrophage cell surface CRT, rather than cancer cell surface CRT. This mechanism may be important for phagocytosis of cells that do not express CRT, such as red blood cells [30].

There are additional receptors that trigger efferocytosis in addition to CD91. The scavenger receptor A (SR-A) is a known ligand of CRT [31]. However, the role of SR-A on activation of phagocytosis upon interaction with CRT, either secreted or on the apoptotic cell and/or phagocyte surface, has not been completely analyzed. SR-A has been shown to be responsible for 50% of apoptotic thymocyte clearance in vitro [32]. Patients with systemic lupus erythematosus (SLE) exhibit impaired apoptotic cell clearance by phagocytes that has been associated with self-tolerance breakdown and have anti-SR-A autoantibodies that inhibit apoptotic cell clearance in vitro [33–35]. This suggests that interaction of CRT with the SR-A on macrophages is important in the activation of efferocytosis in vivo and warrants further evaluation.

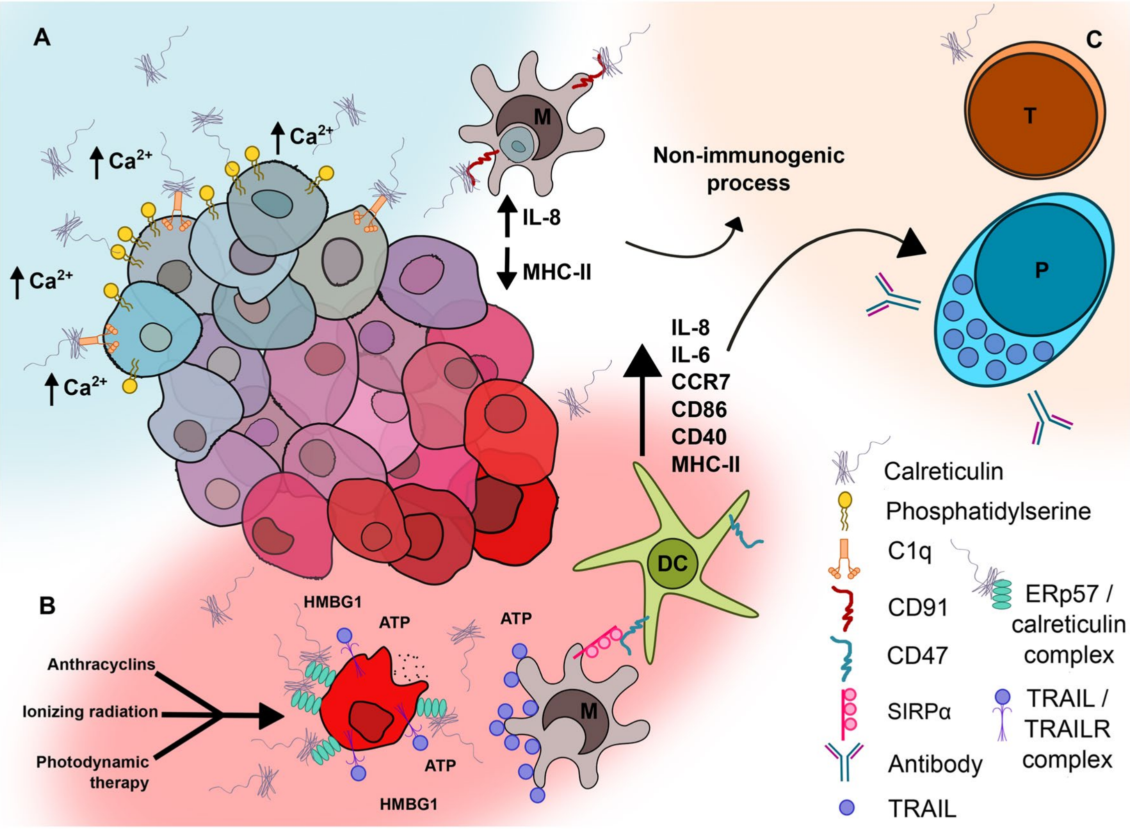


Fig. 2 CRT involvement in efferocytosis and cancer. **a** A possible model for the role of CRT in phagocytosis involves the strong binding of the C-terminal acidic domain of CRT, in a calcium-dependent manner, to patches enriched in PS in the outer membrane of the apoptotic cell, which act as a strong "eat-me" signal. Besides, CRT can also interact via its globular domain with C1q, adiponectin and other structurally related proteins on the apoptotic cell during early but not late apoptosis. On the other hand, the globular and P-domains of CRT (generic polypeptide binding site) may engage, through a calcium-independent manner, to macrophages or DC, attached to phagocytic receptors such as CD91 or SR-A. CRT could activate CD91, both in a trans- and cis-activating manner, as CRT is found on both the apoptotic cell as well as on the phagocyte membrane. CD91 activation results in enhanced phagocytosis with a non-immunogenic outcome. CD47 serves as a "don't eat me" signal and prevents living cells to be phagocytosed when it interacts with SIRP-α on the macrophage. **b** After receiving an immunogenic cell death (ICD)-stimulant such as ionizing radiation, photodynamic therapy or anthracyclines, dying cancer cells expose a complex formed by CRT and the ERp57 protein, which together stimulate their ingestion by phagocytes. Another form of ICD that is concomitantly expressed is that related with the TNF-related apoptosis inducing ligand (TRAIL), which binds to TRAIL receptors found on the surface of malignant cells to induce the extrinsic apoptosis pathway with concomitant translocation of CRT to the cell membrane. **c** CRT translocation on dying cancer cells leads to phagocytosis, processing of cancer cells, activation and subsequent regulated production of pro-inflammatory cytokines, such as TNF-α, IL-6, MCP-1/CCL-2 and IL-8, to secure an appropriate inflammatory environment while avoiding harm to the host. Besides, it promotes T cell activation and B cell differentiation into antibody-secreting plasma cells to elicit a specific response against malignant cells. Macrophage (M), T lymphocyte (T), plasma cell (P), dendritic cell (D). (Figure was created by ASC with Inkscape version 0.48.5)

Effect of released CRT on macrophage phenotype

CRT is involved in the recognition step of phagocytosis and it influences the immune response elicited after apoptotic cell ingestion, through the stimulation of cytokines and membrane molecules on the phagocytes. CRT is rapidly exposed on the cell membrane and released to the environment after UVB irradiation-induced apoptosis and after being endocytosed by macrophages, it affects their polarization [16]. The interaction of CRT and its molecular partners with phagocytes is essential in determining the balance between tolerance and immunogenicity. However, the effect of CRT on cytokine production by phagocytes after efferocytosis is controversial. The different and sometimes inconsistent functions of CRT can be attributed to its interaction with multiple molecular partners and receptors, its localization, the glycosylation level or the species involved. The diverse settings seem to determine whether pro-inflammatory or anti-inflammatory cytokines are produced. For example, absence of CRT on the apoptotic cells that were phagocytized by THP-1 human monocyte-derived macrophages, results in higher production of IL-6 and monocyte chemotactic protein (MCP-1), suggesting that CRT has a modulatory effect. TNF-α and IL-10 production were not detected after incubation with apoptotic HeLa cells [18], while treatment with human recombinant CRT (rCRT) results in micropinocytosis of the recombinant protein, cell migration and stimulation of a modified pro-inflammatory phenotype, characterized by an increase in IL-8 production and expression of CD40 and PDL-1, while IL-6 secretion was not modified. Concomitantly, CD14 expression was up-regulated and was accompanied by a diminished apoptotic cell phagocytic capacity. However, a decrease in MHC II expression in contrast to classically-activated macrophages was observed, implying an impaired activation of $CD4^+$ T cells, suggesting a modulatory role for CRT on antigen presentation [16]. Thus, it seems that human CRT induces a mixed M1/M2 polarization of macrophages. Mouse rCRT, on the other hand, induces TNF-α and IL-6 expression and production, through interactions with the SR-A and activation of the nuclear factor kappa B (NF-kB) and mitogen-activated protein kinases (MAPK) pathways. Moreover, oligomerized rCRT has a greater effect [36]. Accordingly, mouse rCRT fragment 39–272 that contains the N and P domains of CRT was able to induce TNF-α through a TLR-4/CD14-dependent manner [37]. Formation of CRT oligomeric complexes is known to regulate protein activity and signaling pathways, and to modulate their cross-talk. CRT oligomers are better macrophage stimulators than the monomeric form. Huang et al. identified the immunological activating site of mouse CRT to residues 150–230 being able to induce TNF-α production by peritoneal macrophages and showed that this activity was dependent on dimerization [38]. Similarly, the status of CRT glycosylation interferes with pro-inflammatory cytokine production, since native murine CRT has weak macrophage stimulatory activity compared to rCRT expressed in a prokaryotic system [39]. In addition, CRT from human and mouse origin differ in their ability to induce maturation of DC. While human rCRT induces DC maturation with production of low levels of the proinflammatory cytokines TNF-α, IL-6, IL-1β and IL-12p70 and high levels of IL-4 and GM-CSF, mouse rCRT does not stimulate production of TNF-α, IL-12, IL-1β, IL-6, IL-13, GM-CSF, CD86 or MHC II up-regulation, and does not induce DC maturation [40, 41] (Table 1). It is clear that exogenous CRT acting through different receptor pathways is able to activate cytokine production by macrophages or DC. However, more research is needed to elucidate the factors affecting the phenotype induced, such as species specificity, degree of oligomerization and/or glycosylation, as well as cell localization. The release of CRT from apoptotic

Table 1 Effect of CRT on macrophages and DC

Species	Stimulus	Cell type	Cytokines analyzed	Effect Cytokines	Surface markers	Effect Surface Markers	Reference
Human	Apoptotic HeLa Cells	THP-1	IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α, MCP-1/CCL2	Diminished production of IL-6, IL-8, MCP-1	N/A	N/A	Verneret et al. [18]
Human	rCRT	THP-1	IL-6, IL-8	Induces production of IL-8 not IL-6	CD11b, CD11c, CD14, CD40, CD206, PD-L1, MHC II	Up-regulation of CD14, CD40, PD-L1 down regulation of MHCDR and CD74	Osman et al. [16]
Human	rCRT	MDDC	GM-CSF, IFN-γ, IL-10, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12p70, TNF-α	Low levels of TNF-α, IL-6, IL-1b, IL-12p70 High levels of IL-4, GM-CSF	CD83, CD86, MHC II, CD14, CD40, CCR5, CCR7	Up-regulation of CD83, C86, CD40, CCR7, MHC II Downregulation CD14	Bajor et al. [40]
Mouse	rCRT	MDDC	TNF-α, IL-12, IL-1α, IL-6, IL-13, GM-CSF	None	CD86, MHCII	None	Bak et al. [41]
Mouse	rCRT (oligomers and monomers)	Peritoneal macrophages	TNF-α, IL-6	Augmented production	N/A	N/A	Duo et al. [36]
Mouse	r39-272 (N and P domains)	Peritoneal macrophages	TNF-α	Augmented production	N/A	N/A	Hong et al. [37]

N/A not available, *MDDC* monocyte derived dendritic cells, *rCRT* recombinant calreticulin cells, endocytosis, the effect on macrophage cytokine production and cell surface antigen expression points to additional roles of CRT besides merely acting as an "eat me" signal during apoptosis.

CRT in immunogenic cell death (ICD) to promote clearance of cancer cells

During recent years, several studies have revealed that CRT possesses roles beyond the maintenance of physiological homeostasis in healthy subjects. This fact is particularly true in cancer. Ramsamooj et al. did one of the first studies regarding the involvement of CRT in cancer; the authors showed that in the irradiated human head and neck squamous carcinoma cell line SQ-20B, CRT expression is induced [42]. By that time the precise role of CRT in cancer development and maintenance was not elucidated.

Phagocytes are crucial in surveillance against cancer and CRT is involved in the process. Currently, CRT translocation to the cell surface has been linked with sensitization to the induction of apoptosis of cancer cells after treatment with specific chemotherapeutics, i.e. doxorubicin, mitoxantrone, oxaliplatin, bortezumib, as well as with either ionizing-irradiation or photodynamic therapy [43–45].

CRT exposure under these conditions stimulates the adaptive immune response known as "immunogenic cell death" (ICD), an apoptotic process that induces tumor-specific immune responses and is associated with secretion of several other damage-associated molecular patterns (DAMPs), including ATP, the nuclear non-histone chromatin-binding protein high mobility group box 1 (HMGB1), annexin A1 (ANXA1), and type I interferon (IFN-I) [43, 44]. After chemotherapy or radiation, CRT gets translocated to the pre-apoptotic tumor cell surface, which can then be recognized by antigen presenting cells such as DC, which in turn activate specific T cells and subsequent differentiation of B cells into antibody-secreting plasma cells [46].This statement clearly suggests that DAMPs can act as linkers between the innate and adaptive immune systems to engulf and elicit efficient immune responses against malignant cells and that CRT exposure by chemotherapeutics such as anthracyclines could be employed to induce ICD that potentiates the immune system's capacity to kill cancer cells.

Mass spectroscopy of membrane proteins of cells that receive ICD inducers showed that CRT interacts with the disulphide isomerase ERp57 to form the CRT/ERp57 complex, which works as an "eat-me" signal for phagocytic cells such as DC [47]. Both CRT and ERp57 are ER proteins, and are mobilized to the cell surface even before the translocation of PS molecules [47]. Besides, it is known that TNF-related apoptosis inducing ligand (TRAIL), commonly found in natural killer (NK), T-cells, and natural killer T-cells (NKT), is a CRT exposure inductor. TRAIL binds to the five TRAIL receptors (TRAILR) on tumor cells to promote the assembly of death-inducing signaling complex (DISC), which is bound to the Fas-associated protein with death domain (FADD) and the pro-form of caspase-8, to start the extrinsic apoptosis pathway [48, 49] (Fig. 2). The relevance of CRT for the development of the ICD process has further been demonstrated by blocking CRT expression with specific siRNAs, resulting in inhibition of ICD [47].

In the specific case of murine neoplastic cells, it has been demonstrated that depletion of CRT abolishes their phagocytosis by DC and the immunogenicity of cell death, but the injection of dying cells with rCRT adsorbed to the cellular surface in syngeneic murine models promotes both phagocytosis and rejection of the tumor [11, 43]. Particularly, the ability of anthracyclines such as doxorubicin to stimulate translocation of CRT has been related with its capacity to inhibit the protein phosphatase 1/GADD34 complex, which triggers exposure of CRT [11, 50]. The exposure of CRT is the result of phosphorylation of the eukaryotic translation initiation factor (eIF)-2α, related with ER stress [45]. Pre-apoptotic CRT exposure determines whether dying cancer cells will be phagocytized and together with DAMPs, elicit an antitumor immunogenic response. Furthermore, CRT is required for the stability and nuclear localization of the p53 transcription factor, which is important for the induction of apoptosis. One report demonstrated that in the *CRT* $^{-/-}$ mouse embryonic fibroblast (MEF) cell line, there is a nuclear accumulation of the p53 regulator murine double minute gene (*Mdm2*), which leads to p53 degradation via the proteasome [51]. These results imply that CRT regulates p53 function by affecting its rate of degradation and nuclear localization.

Both spontaneous or therapy-driven CRT exposure in neoplastic cells correlates with a better prognosis in cohorts of acute myeloid leukemia (AML) and in non-small cell lung carcinoma (NSCLC) subjects [43]. In fact, one study done with non-surgical NSCLC patients demonstrated by immunohistochemistry that exposure of CRT is a potential tool to stratify patients in low- and high-risk groups, and it may be used as a prognostic factor for overall survival. This study showed that a low CRT expression was associated with a median overall survival of 11–28 months, being such patients mostly in pathologic stages III-IV, against a high CRT expression related with a median overall survival of 100–101 months in pathological stage I. Interestingly, they also found that high expression of CRT was associated with an increase in the number of both mature and myeloid DC, as well as with more infiltration by effector memory CD4$^+$ and CD8$^+$ T cells in the tumor microenvironment [44]. In line with these findings, CRT is present in the granules of cytotoxic T lymphocytes (CTL), where it interacts with perforin and potentially aids CTL-induced lysis, as CTL from

CALR$^{-/-}$ mice are unable to exhibit a normal interaction with target cells and develop diminished lysis abilities [52].

Other functions of CRT in cancer biology

Besides the aforementioned beneficial functions of CRT in oncological conditions, there are certain controversial roles of CRT related with cancer progression. Certainly, it has been revealed that CRT has pro-angiogenic functions due to its ability to promote the expression of vascular endothelial growth factor (VEGF) and of the phosphatidylinositol-glycan biosynthesis class F protein (PIG-F) in gastric cancer cells, which leads to cell proliferation, migration and poor prognosis [50, 53]. However, the increase of VEGF in neuroblastoma (NB) is not related with cancer progression [54]. Indeed, Weng et al. reported that CRT positively upregulates VEGF both at the mRNA and protein levels in three different NB cell lines due to the induction of hypoxia-inducible factor (HIF)-1α expression, which in turn upregulates VEGF [55]. Interestingly, they also found that cellular proliferation rate is lower in CRT-overexpressing NB cells, which suggests that increased CRT expression in NB cells predicts a favorable outcome [55]. On the other hand, it has been reported in vitro and in vivo in Burkitt tumor models that both exogenous CRT and the CRT fragment vasostatin, are inhibitors of angiogenesis via targeting endothelial cells that in turn suppress tumor growth. Although the exact mechanism of inhibition is still unknown, it is reported that CRT lacking the N-terminal 1-120 amino acids obstructs endothelial cell proliferation in a way comparable to vasostatin [56].

Interestingly, while in NB the augmented CRT expression is correlated with better survival rates, higher CRT levels are associated with obscure prognosis in pancreatic cancer and esophageal squamous cell carcinoma patients [50]. Currently, cancer invasion and metastasis in hepatocellular carcinoma, pancreatic, esophageal, gastric and colon cancers, as well as in melanoma and leukemia, have been reported to potentially be correlated with CRT overexpression [57]. Previous studies have also found that increased CRT expression is associated with cell migration and attachment of bladder cancer cells. *CALR* gene knockdown is related with the suppression of cell migration and proliferation in the J28 and T24 bladder cancer cell lines in an in vivo model. In fact, it is known that there is a decrease in the phosphorylation status of the adherent protein focal adhesion kinase (FAK) in *CALR* knockdown bladder cancer cells, which diminishes cell adhesion to extracellular matrix, particularly to type I collagen [58]. The relationship between CRT and cellular mobilization is reinforced by one study that revealed that CRT suppression on rhabdomyosarcoma cells inhibits the activation of matrix metalloproteinase-2 which, in turn, enhances endothelial cell migration and the angiogenesis process [59]. Thus, the role of CRT in cancer cell progression and metastasis seems to be dependent on the cancer phenotype.

CALR gene mutations in cancer

Recurrent somatic mutations in the *CALR* gene, encoding CRT, were reported in 2013 in the majority of patients with the most common Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) that are not mutated in the intracellular cytokine-signaling molecule Janus kinase 2 gene (JAK2) or the myeloproliferative leukemia protein gene (MPL), i.e., essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF). Such alterations in *CALR* are described as frameshift mutations in exon 9, and they may be either deletions (type 1, with a deletion of 52 base pairs) or insertions (type 2, with insertion of 5 base pairs) in > 80% of patients with *CALR* mutations [60]. These mutations are found without the Janus kinase 2 V617F (JAK2 V617F) point mutation, and they activate the JAK/STAT signaling pathway in megakaryocytic and granulocytic progenitor and precursor cells by interacting with the thrombopoietin receptor [61] and by increasing the MAPK activity [62], conferring autonomy of the cell from interleukin (IL)-3-related cellular growth [62–64]. The mutant CRT protein lacks the C-terminal ER retention signal (KDEL), which suggests that CRT may be present in an altered subcellular localization resulting in impaired CRT-associated Ca^{2+} binding function [61, 62]. Indeed, higher levels of circulating CRT have been reported in patients with myelofibrosis [65]. Additionally, compared with ET or PMF patients with *JAK2* mutations, those with *CALR* mutations present lower granulocytes and hemoglobin levels, with higher numbers of platelets [63]. However, the *CALR* mutation does not affect the sensitivity to phagocytosis by macrophages in ET and PMF patients, suggesting that myeloproliferation is caused by the activation of the JAK-STAT pathway not by inhibiting phagocytosis [66]. In addition, *CALR*-mutated patients show defective plasma cytokine levels, reduced levels of IL-4, -5 and IFN-γ in plasma with increased levels of IL-1β, -6, -10, -17, and TNF-α, as well as DC maturation. Freshly isolated monocytes from these patients fail to differentiate into immature DC, and mature DC are defective in CD40 and CD80 expression as compared to healthy donors. Moreover, T cells from the Th1 compartment are reduced, suggesting that lack of DC maturation impacts T cell differentiation [67, 68].

Post-translational modifications of CRT in inflammatory diseases and cancer

CRT suffers other post-translational modifications at its N-terminal domain, through protein kinase C, which ultimately modifies CRT interactions and functions, and induces signaling pathways that result in glycosylation of the protein and sub-cellular re-localization [69]. As mentioned above, CRT phosphorylation is needed for CRT exposure on the macrophage cell surface and secretion, necessary for efficient cell uptake of both apoptotic and living cancer cells [29, 30]. Among other relevant post-translational modifications of CRT, the generation of citrullinated CRT (C-CRT) by deimination of arginine can be found in rheumatoid arthritis (RA) synovial fluid and tissues. C-CRT, in turn, strongly binds to RA shared epitopes on the RA-associated MHC allele HLA-DRB1 to start an inflammatory cascade, through the activation of reactive oxygen species and nitric oxide synthase in adjacent cells [70]. Moreover, a study performed by Clarke et al., identified the presence of circulating C-CRT and of anti-C-CRT IgG antibodies in RA patients, and even in bronchiectasis patients before the development of RA [71]. Thus, CRT citrullination may contribute to the disease process.

Another post-translational modification reported in CRT is arginylation, a process promoted under low Ca^{2+} conditions in which arginine molecules are added to glutamate, aspartate or cysteine residues at the N-terminus of cytosolic CRT. Arginylated CRT (R-CRT) was found in the cytosol in contrast to non-R-CRT that was present in the ER [72]. R-CRT has a longer half-life than that of non-R-CRT, suggesting that arginylation promotes CRT stability [73]. Arginylation is not necessary for cell surface of CRT exposure but R-CRT is a more efficient “eat me” signal during apoptosis of bortezomib-treated glioma cells [74, 75]. Although R-CRT is related with apoptosis when presented on the cell surface, it is also associated to the innate immunity-promoter stress granules [72, 73]. Sequestration of R-CRT to stress granules has been proposed as a cell death resistance mechanism of cancer cells to certain drugs [75], suggesting that subcellular localization results in opposing functions of R-CRT. Indeed, stress granules have been reported during tumor progression as elements that increase the resistance to apoptosis through the inhibition of the MAPK cascade [76] and as a response to treatment with the tyrosine kinase inhibitor sorafenib [77], a chemotherapy compound that blocks the vascular endothelial growth factor receptor and which is employed for advanced hepatocellular carcinoma patients [78]. Therefore, although not investigated yet, it may be expected that R-CRT could have certain roles in the promotion and/or persistence of apoptosis-resistant cancer as well. Elucidating the contribution of post-translational modifications on CRT may contribute to fully understand the different roles this protein has in the different immune-mediated diseases and cancer.

Concluding remarks

The importance of CRT in cell removal is underscored by its presence in lower organisms. CRT plays an important role in particle uptake and phagocytic cup formation by soil-living and parasitic amoeba. Besides, it is essential for phagosome biogenesis [79, 80]. As it turns out, the process of cell removal in higher organisms is highly dependent on CRT surface translocation, which impacts both immunogenic and non-immunogenic apoptosis. This, in turn, plays a crucial role in the initiation and resolution of inflammation. On the other hand, the prevalence of inflammatory-driven pathologies and cancer has increased in the last years and are major causes of morbidity and mortality in industrialized countries. The interaction between genes and environmental factors may explain the worldwide increase in these diseases. CRT is a common denominator in the cell removal processes that determine the immunological outcome of immunopathologies and cancer. The presence of CRT on the cell surface or in the extracellular milieu has functions beyond acting as an “eat me signal”, i.e., contributing to the silencing the immune response as a result of apoptotic cell removal and, on the other hand, facilitating immunogenicity after elimination of cancer cells by certain chemotherapeutics. Failure to efficiently control the immune response may result in autoimmunity, tumor growth and metastasis. The apparent contradictory roles that CRT has on the immune system depend on the molecular partners CRT interacts with, the regions of the protein involved, the CRT mutations and polymorphisms, the subcellular localization of CRT, as well as the post-translational and conformational modifications, which finally impact the receptor interactions and the signaling pathways stimulated in the phagocytes. Elucidating the CRT-induced pathways may give information on how to intervene to promote the correct type of apoptosis: ICD for the eradication of cancer cells, or non-immunogenic apoptosis for the prevention of autoimmunity. Further work must be performed to completely understand the different roles that CRT has in the induction, promotion and resolution of cancer and autoimmune diseases.

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