

# Mechanisms of translocation of ER chaperones to the cell surface and immunomodulatory roles in cancer and autoimmunity

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Endoplasmic reticulum (ER) chaperones (e.g., calreticulin, heat shock proteins, and isomerases) perform a multitude of functions within the ER. However, many of these chaperones can translocate to the cytosol and eventually the surface of cells, particularly during ER stress induced by e.g., drugs, UV irradiation, and microbial stimuli. Once on the cell surface or in the extracellular space, the ER chaperones can take on immunogenic characteristics, as mostly described in the context of cancer, appearing as damage-associated molecular patterns recognized by the immune system. How ER chaperones relocate to the cell surface and interact with other intracellular proteins appears to influence whether a tumor cell is targeted for cell death. The relocation of ER proteins to the cell surface can be exploited to target cancer cells for elimination by immune mechanism. Here we evaluate the evidence for the different mechanisms of ER protein translocation and binding to the cell surface and how ER protein translocation can act as a signal for cancer cells to undergo killing by immunogenic cell death and other cell death pathways. The release of chaperones can also exacerbate underlying autoimmune conditions, such as rheumatoid arthritis and multiple sclerosis, and the immunomodulatory role of extracellular chaperones as potential cancer immunotherapies requires cautious monitoring, particularly in cancer patients with underlying autoimmune disease.

**Keywords:** calreticulin, damage associated molecular patterns, ER stress, immunogenic cell death, post-translational modification

## INTRODUCTION

The endoplasmic reticulum (ER) is one of many specialized organelles in the cell with diverse and apparently ever expanding functionality. When the ER was first observed in chick embryonic cells by electron microscopy, it was simply described by Porter, Claude and colleagues as one of many “submicroscopic cytoplasmic components” (1). The term “endoplasmic reticulum” was adopted by Porter and Palade because of its general morphology and intracellular location (2). Palade in his original *Science* article (3), described the ER as an “organ of complex geometry that endows it with a large surface for trapping proteins for export.” Once the subcellular fractionation of the ER organelle was possible (4), two of the major functions of the ER, namely calcium sequestration (5) and the correct assembly, folding and secretion of glycoproteins became established over the pursuing decades (6–8).

In particular, a number of proteins within the ER were discovered to be critical for the correct quality controlled folding and assembly of nascent glycoproteins – these proteins were termed chaperones and included a wide array of unrelated protein families. Chaperones are also involved in protein repair after episodes of cell stress, especially thermal shock, hence several proteins are termed “heat shock proteins (HSP)”. Some of the most plentiful luminal ER chaperones and folding enzymes in order of relative

abundance are HSP47, binding immunoglobulin protein (BiP), ERP57, protein disulfide isomerase (PDI), gp96 (GRP94; HSP90), and calreticulin (9), which all fulfill unique functions required for protein assembly. For instance, PDI, a folding enzyme, assists in the correct joining of cysteine residues to create reduced disulfide bonds in nascent proteins in order to form thermodynamically stable proteins. PDI is present in millimolar quantities in the lumen of the ER of secretory cells, reflecting its importance in disulfide bond formation (10). Other proteins within the ER work in unison with isomerases to help fold, glycosylate, and post-translationally modify the majority of the 18,000 proteins that are transported to other organelles, the cell surface or beyond (11). Chaperones and folding enzymes are also involved in a number of intracellular immune functions including the formation of MHC class I and II molecules and antigen peptide loading.

During chemical or physical cell stress, the expression of chaperones are rapidly increased. Likely reasons for this rise in chaperone production are: (a) an attempt to generate correctly folded proteins to help the cell survive or, (b) to assist in shutting down the protein manufacture and aiding degradation in preparation for cell death. Another consequence of this stress response may be the relocation of chaperones to the cell surface via a number of pathways and the eventual release of chaperones into the

extracellular space. On the surface, or in the extracellular space, some chaperones can signal the innate immune system to target “sick/abnormal” cells for engulfment and subsequent activation of adaptive immune responses. Indeed, the presence of chaperones on the cell surface or in the serum, is associated with disease, particularly cancers and autoimmune diseases (Table 1). Of note, chaperone proteins operating within the ER do so in an environment very different from that in other organelles or outside of cells. For example, the ER has a greater oxidizing environment with high  $\text{Ca}^{2+}$  (~1 mM) and the number and frequency of proteins is more abundant than in other organelles (12, 13). In this review, we describe the functions of ER chaperones in immunity, and discuss the different mechanisms of ER protein translocation and their possible roles in various disease pathologies.

### EXTRACELLULAR CHAPERONES CAN ACT AS DAMPs

The presence of so-called Pathogen-Associated Molecular Patterns (PAMPs) on e.g., microbes acts as a “danger signal” for the innate and adaptive immune system and helps the immune system to mount protective responses. Many intracellular host and “self” proteins that are not normally presented to the immune system similarly act as danger molecules or “alarmins” upon their release from (dying) cells. One of the most prominent of such so-called Damage Associated Molecular Patterns (DAMPs) is the high-mobility group box 1 (HMGB1) DNA binding protein. HMGB1 normally resides in the nucleus of cells, loosely bound

to chromatin, but is released into the extracellular space during necrosis. This in contrast to apoptosis, where the interaction between HMGB1 and chromatin is strengthened, thus preventing the release of HMGB1 (35). Once in the extra-cellular environment, HMGB1 acts as danger signal that leads to the maturation of dendritic cells by binding to the receptor for advanced glycation end products (RAGE) and via the Toll-like receptors, TLR2 and TLR4. This subsequently triggers clonal T-cell expansion and ultimately leads to the killing of targets cells. Of note, dendritic cells also release their own HMGB1 upon activation, which amplifies their activation and is required for clonal expansion, survival, and functional polarization of naive T-cells (36). Similarly, HMGB1 is actively secreted by monocytes and macrophages upon their activation, resulting in increased HMGB1 serum levels as shown in mice (37).

Although ER chaperones are actively retained in the ER and should normally not be immunogenic, many reports have highlighted their role as DAMPs in the extracellular space. ER chaperones like calreticulin, BiP, and gp96 can activate the immune system once secreted in the extracellular space. In this respect, calreticulin was found to be the major determinant in the process of immunogenic cell death (ICD), as described in detail below (see Calreticulin Exposure Determines ICD). Similarly, tumor-secreted BiP induced antigen-specific anti-tumor responses by activating CD8 T-cells in murine cancer models (38). In addition, extracellular gp96 can also elicit tumor-specific immunity (39). Thus,

**Table 1 | Summary of abundant ER chaperones detected on the cell surface or in the extracellular environment and their association with various diseases.**

| Protein   | Localization outside ER                                     | Potential therapeutic   | Over/under expression in diseases   | Reference            |
|---|---|---|---|----------------------|
| HSP47/serpin peptidase inhibitor clade H, member 1 (SERPINH1) | Extracellular matrix and serum                              | microRNA-29a (miR-29a) down regulates HSP47 and inhibits cell migration and invasion in cervical squamous cell carcinoma          | HSP47 overexpressed in scirrhous carcinoma of the stomach, rheumatoid arthritis, systemic lupus erythematosus, and Sjögren's syndrome | (14–16)              |
| BiP/GRP78   | Cell surface, nucleus                                       | HKH40A, an 8-methoxy analog of WMC79, downregulates BiP, activates the UPR pathway and directly degrades the protein              | Many cancers, especially solid tumors and musculoskeletal diseases overexpress BiP  | (17–20)              |
| ERP57   | Cell surface, nucleus, cytosol, extracellular matrix, urine | Enhanced increase in cell surface ERP57 and calreticulin may enhance anthracycline-induced apoptosis                              | Under expression of ERP57 in breast and gastric cancer cells  | (21–24)              |
| PDI   | Cell surface  | Propionic acid carbamoyl methyl amides small molecules can act as PDI inhibitors to treat ovarian cancer                          | PDI is upregulated in CNS cancers, lymphoma's ovarian, lung and prostate cancer   | Reviewed by (25, 26) |
| GRP94/gp96  | Cell surface, transmembrane                                 | GRP94 siRNA may be useful in reducing resistance of human ovarian cancer cells to chemotherapy                                    | Upregulated in breast and ovarian cancer, lung and pancreatic cell lines  | (27–30)              |
| Calreticulin  | Cell surface, extracellular, cytosol                        | Photofrin- and hypericin-based photodynamic therapy increases cell surface calreticulin increasing anti-tumor host responsiveness | Calreticulin is upregulated in many cancers and musculoskeletal diseases  | Reviewed in (31–34)  |

*BiP, binding immunoglobulin protein; PDI, protein disulfide isomerase; UPR, unfolded protein response.*

ER chaperones released in the extracellular space induce (specific) immune responses. (17, 21, 40–44). The mechanism(s) by which such ER chaperones elicit immunity is not fully understood and may differ between respective chaperones. There is a substantial amount of evidence to suggest post-translational modifications of chaperones and peptide processing of chaperones changes the function and immunogenicity of at least some chaperones (see also below Retrotranslocation and Post-Translational Modification of Chaperones). For instance, in rheumatoid arthritis, citrullinated calreticulin is highly prevalent in the synovial tissue (45). This citrullinated calreticulin preferentially binds to the shared epitope (SE), a sequence motif in the  $\beta$ 1 domain of the HLA-DR molecule that is found in 90% of rheumatoid arthritis patients, and potentiates 10,000-fold greater SE-activated signaling in innate immune cells compared to non-citrullinated calreticulin (45, 46). Furthermore, signaling via the SE was blocked by anti-calreticulin antibodies, but also by antibodies against CD91. CD91 (alpha 2-macroglobulin receptor or the low density lipoprotein-related protein) is a receptor involved in endocytosis, and has also been described to regulate the immunogenicity of other ER chaperones like gp96, HSP90, and HSP70 (47).

Due to their protein folding function, extracellular chaperones are often present in complexes with antigenic peptides, which were generated in the cells from which they were released. In order to elicit an antigen specific immune response, these chaperoned peptides needs to be re-presented by antigen presenting cells. Indeed, gp96 can be re-presented by antigen presenting cells via cell surface receptor CD91, whereby the chaperone and its bound peptide are endocytosed. The chaperone-peptide complex then enters several trafficking and processing pathways, whereupon chaperone-derived peptides are re-presented on both MHC class I and II molecules to CD8<sup>+</sup> and CD4<sup>+</sup> T-cells, respectively. This process allows activation of both adaptive and indirectly innate immunity against Meth A fibrosarcoma (48). Similarly, gp96 release during virally induced lytic cell death induced activation of specific T-cells when tissue supernatant was pulsed onto antigen presenting cells (49). Besides, gp96 (47, 50), heat-shock treatment of Meth A fibrosarcoma induced HSP70 expression, which did not impair proliferation or cell viability. However, these cells failed to form a tumor mass when injected in mice (51). Further, heat-shocked murine leukemia cells elicited an anti-tumor immune response and protected against tumor formation upon re-challenge due to expression of HSP60 and HSP72 (52). This immune activating response depended on the maturation of dendritic cells and activation of cytotoxic T-cells (53). In addition, the co-injection of purified HSP70 with non-immunogenic apoptotic leukemia cells potentially generated anti-tumor immunity (54). Similarly, co-injection of non-immunogenic apoptotic colon or melanoma cells with calreticulin induced curative and protective T-cell immunity (55). However, extracellular calreticulin can also bind to C1q opsonized apoptotic cell debris and CD91 on monocyte/macrophages, leading to removal of apoptotic cells in a non-inflammatory manner (56). Of note, this pathway appears to be dysfunctional in some autoimmune diseases (57).

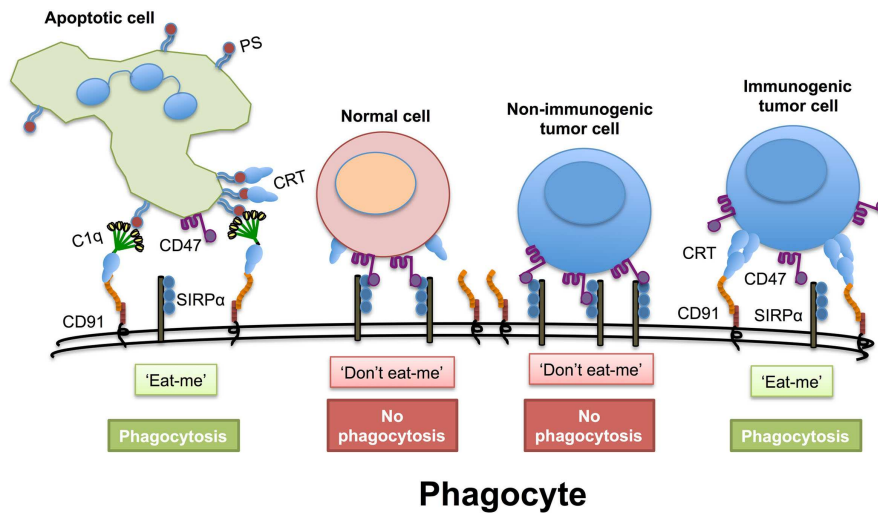
Taken together, despite their specialized functions in the ER, chaperones can be present in other cellular compartments, can be exposed on the cell surface, or may be released in the extracellular

space. Once outside the cell, chaperones can act as DAMPs and activate the immune system, which may promote the clearance of infections or induce an anti-tumor immune response, but may also result in autoimmunity. The exact mechanism of the immune promoting effects of chaperones is not yet fully understood and may differ from chaperone to chaperone, but is often associated with the receptor CD91.

Another intriguing function of ER chaperones in the extracellular space, in particular calreticulin, is their ability to counteract “don’t eat me” signals displayed on cells (**Figure 1**). Healthy cells and tumor cells display the “don’t eat me” CD47 molecule. However, many types of cancer cells express higher quantities of CD47 compared to normal cells. When cells express CD47 on their cell surface it helps them avoid phagocytosis, as CD47 engages with the anti-phagocytic receptor SIRP $\alpha$  on phagocytic cells (58). The administration of anti-CD47 blocking antibodies enhances phagocytic uptake of tumor cells, but surprisingly not healthy cells (59). The latter finding suggests that tumor cells possess an extra signaling molecule that promotes phagocyte activity against tumor cells that is absent on healthy cells. Several authors have suggested that this overriding “eat me” signal on tumor cells is calreticulin, which cannot be substituted by other chaperones (58, 60). However, this may not be the complete picture of tumor cell recognition, as calreticulin is also expressed to varying degrees on non-apoptotic cells. Therefore, the distribution of native or post-translationally modified isoforms of calreticulin on the cell surface and its association with other co-stimulants may be necessary for efficient targeting of cells for phagocytosis. A number of co-factors identified by ourselves and others aid in surface expression of calreticulin, including ATP, Lysyl tRNA, and ERP57 (50, 61, 62). Thus, in addition to the immune activating properties shared by calreticulin with other extracellular chaperones, calreticulin is also an important player in phagocytosis by counteracting the inhibitory signaling provided by CD47.

## APPEARANCE OF EXTRACELLULAR ER CHAPERONES AND AUTOANTIBODIES IN DISEASE STATES AND INDUCTION OF IMMUNITY

During disease, cells are often exposed to high levels of stress that may eventually lead to cell death. Stress and cell death may trigger release of intracellular proteins like chaperones. In line with this, extracellular calreticulin is present in the synovial fluid surrounding the joints of patients with rheumatoid arthritis (43, 63). When proteins that normally reside intracellular become exposed to the immune system, this likely induces (auto)antibody responses. Indeed, early studies demonstrated that ER chaperones are target of autoimmunity in murine models (64) and patients (57), leading to the generation of autoantibodies against a number of chaperones in serum of patients with autoimmune diseases or malignancies (17, 21, 40–44) (**Table 2**). Thus, ER chaperones are being released and can trigger autoantibody formation. This release occurs most likely from dead, dying, or stressed cells and may be accompanied by their post-translational modification. For instance, a number of autoimmune diseases are known to have increased cell death in the form of dysfunctional apoptosis and increased necrosis (65), leading to an array of highly concentrated chaperone proteins in membrane bound ER “blebs.” Here, these



**FIGURE 1 | Disruption of the “don’t eat me” signal.** Cells express the “don’t eat me” signaling molecule CD47 on their cell surface that interacts with SIRP $\alpha$  on phagocytes. This must be overridden when cells are preparing to die. During apoptosis, normal cells express greater amounts of phosphatidylserine (PS), which both the first component of complement (C1q) and calreticulin (CRT) can bind to directly. Extracellular calreticulin can act as a bridging molecule between C1q and CD91 on phagocytes and

enhance the uptake of apoptotic cells. Even if normal cells have transient non-PS bound calreticulin on their cell surface this may not be sufficient to override the CD47–SIRP $\alpha$  “don’t eat me” signal. Non-immunogenic tumor cells have high levels of CD47 on their cell surface to avoid phagocytosis. However, immunogenic tumor cells have high levels expression of calreticulin on their cell surface that appears in punctate patches that can promote an “eat me” signal.

**Table 2 | The generation of anti-chaperone antibodies in autoimmune diseases and cancers.**

| Disease                       | Anti-chaperone  | Reference    |
|-------------------------------|---|--------------|
| <b>AUTOIMMUNE DISEASES</b>    |   |              |
| Autoimmune hepatitis          | Anti-ERp57 IgG  | (67)         |
| Inflammatory bowel disease    | Anti-calreticulin/BiP IgG                             | (44, 68)     |
| Juvenile idiopathic arthritis | Anti-BiP IgG  | (40)         |
| Myasthenia gravis             | Anti-GRP94 IgG  | (69)         |
| Primary biliary cirrhosis     | Anti-calreticulin IgA                                 | (70)         |
| Rheumatoid arthritis          | Anti-calreticulin/BiP/GRP94/calnexin IgG              | (43, 44, 71) |
| SLE                           | Anti-calreticulin IgG/anti-PDI IgG/BiP/GRP94/calnexin | (44, 72, 73) |
| Systemic sclerosis            | Anti-BiP/GRP94/calnexin IgG                           | (44)         |
| <b>CANCERS</b>                |   |              |
| Colorectal carcinoma          | Anti-BiP IgG  | (74)         |
| Refractory celiac disease     | Anti-calreticulin IgA                                 | (75)         |
| Pancreatic cancer             | Anti-calreticulin IgG                                 | (76)         |
| Melanoma                      | Anti-GRP94  | (77)         |
| Hepatoma                      | Anti-PDI IgG  | (73)         |

chaperones are susceptible to attack by reactive oxygen and nitrogen species, leading, e.g., to nitrosylation. Such post-translational modifications may make ER chaperones sufficiently “foreign” as to elicit an immune response. Whether the initiation of an immune response to ER chaperones is simply a reflection of a “normal” preventative autoimmune reaction that ensures removal of dying

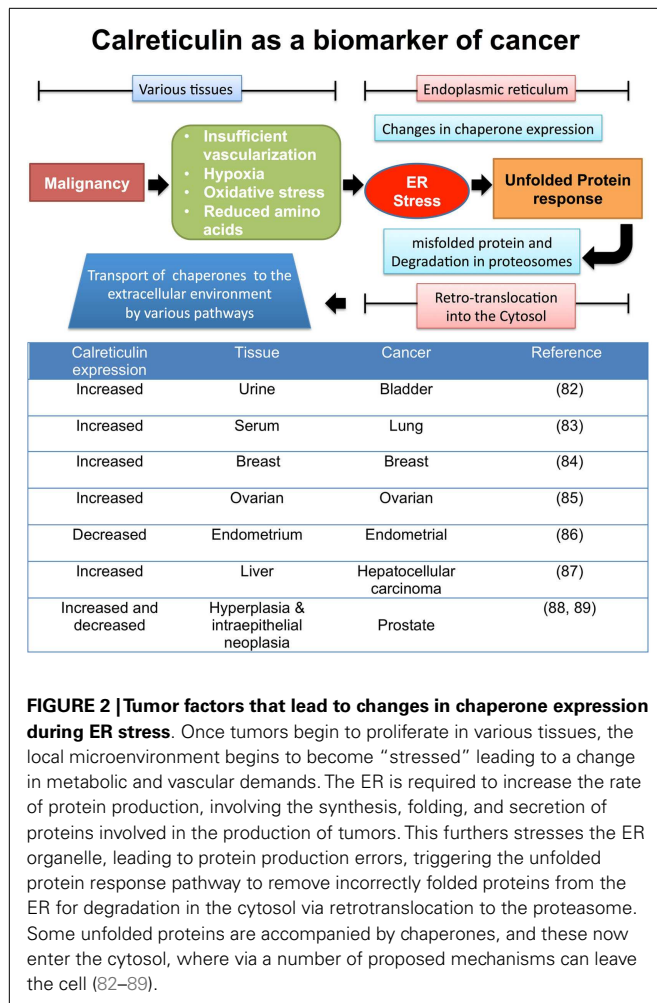
and/or damaged cells, or a precursor to autoimmune disease has been debated ever since the proposal of the “danger theory” model in 1994 (66).

Of note, the overexpression of chaperones has been considered as a sign of increased malignancy, with calreticulin in particular being over-expressed in numerous tumor tissues possibly to cope with increased ER stress (Figure 2). Whilst this may thus be simply a biomarker of increased ER stress due to malignancy, some studies have suggested chaperones are engaged directly in the spread of tumors by promoting cell proliferation (78), migration (79), and metastasis (80, 81).

The production of anti-chaperone antibodies could possibly be a mechanism to suppress innate and adaptive immune responses in autoimmunity, while inadvertently neutralizing chaperone-dependent immune responses that help prevent cancer. It is known that patients with prior autoimmune disease are at a higher risk of subsequently developing certain forms of cancer (90–93). In contrast, some patients with parasitic diseases, for example, *Trypanosoma cruzi* are more resistant to developing some forms of cancer (94–96). In a number of forms of cancer anti-chaperone antibodies have been detected (see Table 2), but the clinical relevance of chaperone antibodies in the circulation of cancer patients have not been evaluated in depth. Whether anti-chaperone antibodies enhance tumor growth by blocking detection by immune cells, or are generated to protect against tumor formation are questions that remains to be addressed.

### MECHANISMS OF TRANSLOCATION OF ER CHAPERONES TO THE CELL SURFACE – KDEL MOTIFS AND RECEPTORS

Our own studies and those of independent researchers have focused on the release of ER-resident chaperones like calreticulin,



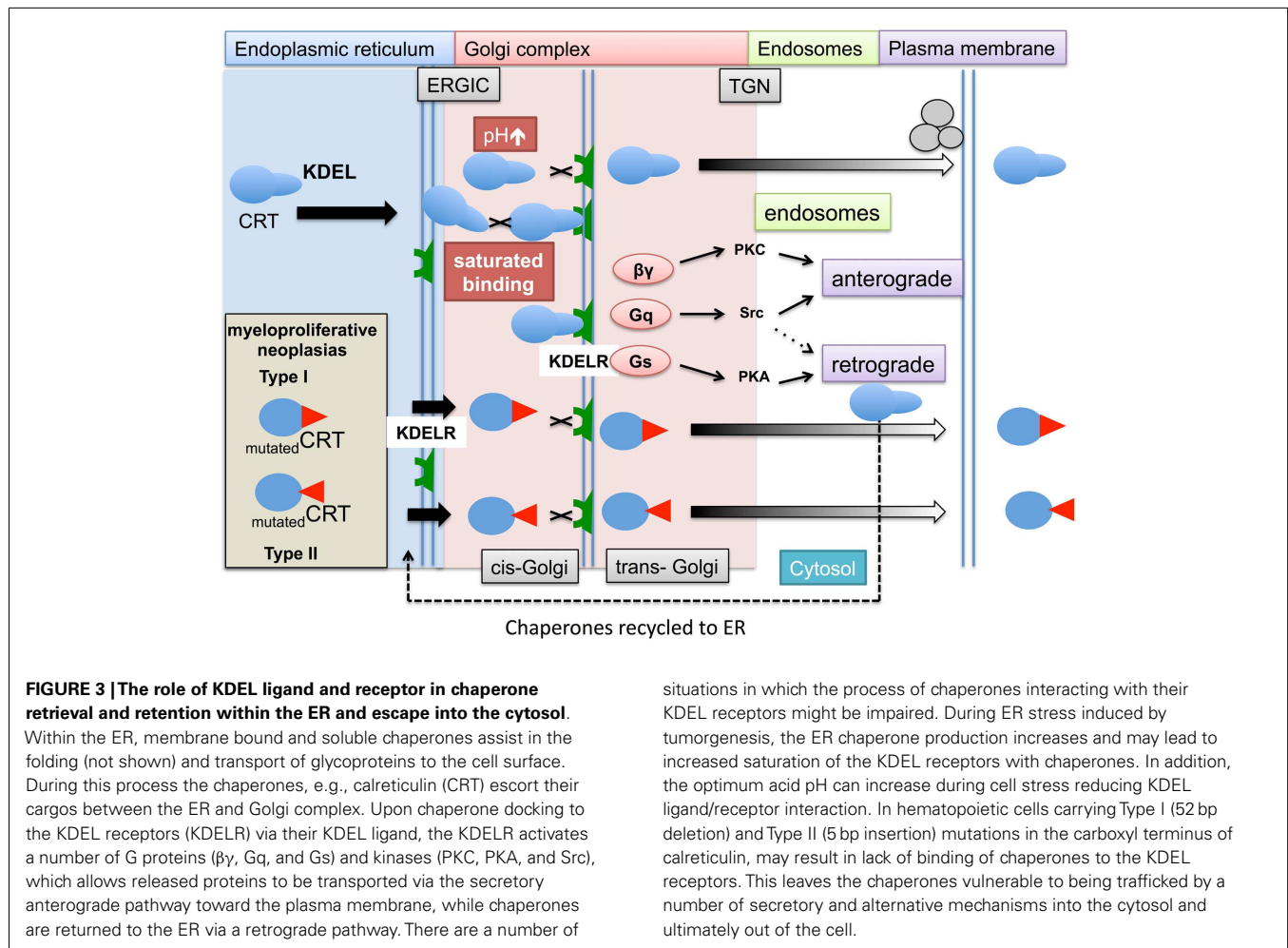
BiP, gp96 and PDI. The ER is an industrious place of protein production and transport therefore it was argued that the chaperone proteins must be distinguished from secretory proteins to be exported in order to prevent their release via the secretory pathway. Munro and Pelham (97) identified a carboxyl terminus sequence of Lys-Asp-Glu-Leu (KDEL) on three ER-resident proteins, namely BiP, gp96, and PDI. They showed that deletion of the KDEL sequence from BiP, led to its “secretion” from mammalian cells. Subsequently many other chaperones were found to have a KDEL carboxyl terminus or a related sequence (Figure 3), including calreticulin, ERP72, and others. Chaperones armed with a KDEL sequence can safely traffic protein cargos in vesicles between the ER, Golgi complex intermediate ER-Golgi (ERGIC) complex, and Trans Golgi Network (TGN). These secretory pathway organelles and intermediates possess docking stations or KDEL receptors, which can recapture chaperones and returns them to the ER.

KDEL containing chaperones are present on the cell surface of various animal and human cells. Two decades ago gp96 was observed on mouse sarcoma (98) and *Xenopus* lymphoid cells (99). Evidence is not restricted to the transport of ER luminal chaperones. The transmembrane ER chaperone calnexin has been detected on the surface of various immature thymocyte cell lines

complex with CD3 antigen, (100). At the time, it was speculated that the lack of retention of such the ER proteins was most likely during their initial formation, and that nascent ER proteins in immature hematopoietic cells may adopt a folding formation that masks their retention ligand, which is later corrected in mature thymocytes. A murine fibroblast cell line (3T6) when placed under various cell stress conditions including heat shock (43°C for 30 min), or lowering the intracellular pH with Na<sup>+</sup>/H<sup>+</sup> transporter inhibitors or alkalinizing the endosomal compartments with chloroquine, resulted in the cell surface expression of HSP47 (101). This study provided evidence that interaction of KDEL proteins binding to KDELRs is dependent upon a stable pH environment.

In humans, there are three KDEL receptor genes (KDELR1, KDELR2, and KDELR3) that encode for three types of seven transmembrane spanning KDEL receptors. KDEL receptors have a high degree of amino acid homology ~65–85%, with the KDELR3 gene producing two isoforms with even higher homology to each other. These receptors are mostly concentrated in the Golgi complex, but are also found in all of the above-described secretory organelles whereas they are absent in endosomal vesicles. The binding of chaperones requires both the KDEL sequence on the chaperone and the KDEL chaperones to be unmutated. This is exemplified by the recent discovery that patients with myeloproliferative neoplasms (MPNs) that did not have a janus kinase 2 (JAK2) mutation (a mutation occurring in the vast majority of patients) are characterized by somatic mutations in their calreticulin gene (102). Such mutations lead to release of calreticulin by megakaryocytes, possibly into the bone marrow (103). Interestingly, many of the mutations are found in the carboxyl terminus of the protein leading to changes in peptide structure. This region of calreticulin has a low affinity binding site for calcium and contains the KDEL sequence that is believed to be important in retaining the protein within the ER (104). Mutated forms of calreticulin identified in MPN lack KDEL raising the possibility that some mutated calreticulin isoforms may not be retained in the lumen of the ER by KDEL receptors, whilst other are retain in the ER despite lacking a KDEL sequence (personal communication – Prof Tony Green).

The above may account for some of the extracellular calreticulin, but does not fully explain why extracellular and cell membrane bound calreticulin are observed in other forms of cancers or in autoimmune patients (see Figure 3 and Table 1). The notion that KDEL receptors “retain” chaperones has changed over a number of years and it is now believed KDEL receptors act more as retrieval systems shepherding chaperones between the ER and Golgi complex during cell stress via retrograde (104) and allowing their protein cargoes to move toward the plasma membrane via anterograde (105) transport pathways. If these pathways are impaired, chaperones could accumulate in the cytosol in endosomal vesicles. Moreover when KDEL receptors become saturated with chaperones, non-bound chaperones may escape the retrograde retrieval system and fail to return to the ER. Certain chaperones have additional retention mechanisms. The enzyme aminoacyl-tRNA synthetase (AIMP1) enhances the dimerization of gp96 and aids greater retention of gp96 by the KDEL-1 receptor; suggesting different ER chaperones rely on different regulatory retention mechanisms (106).



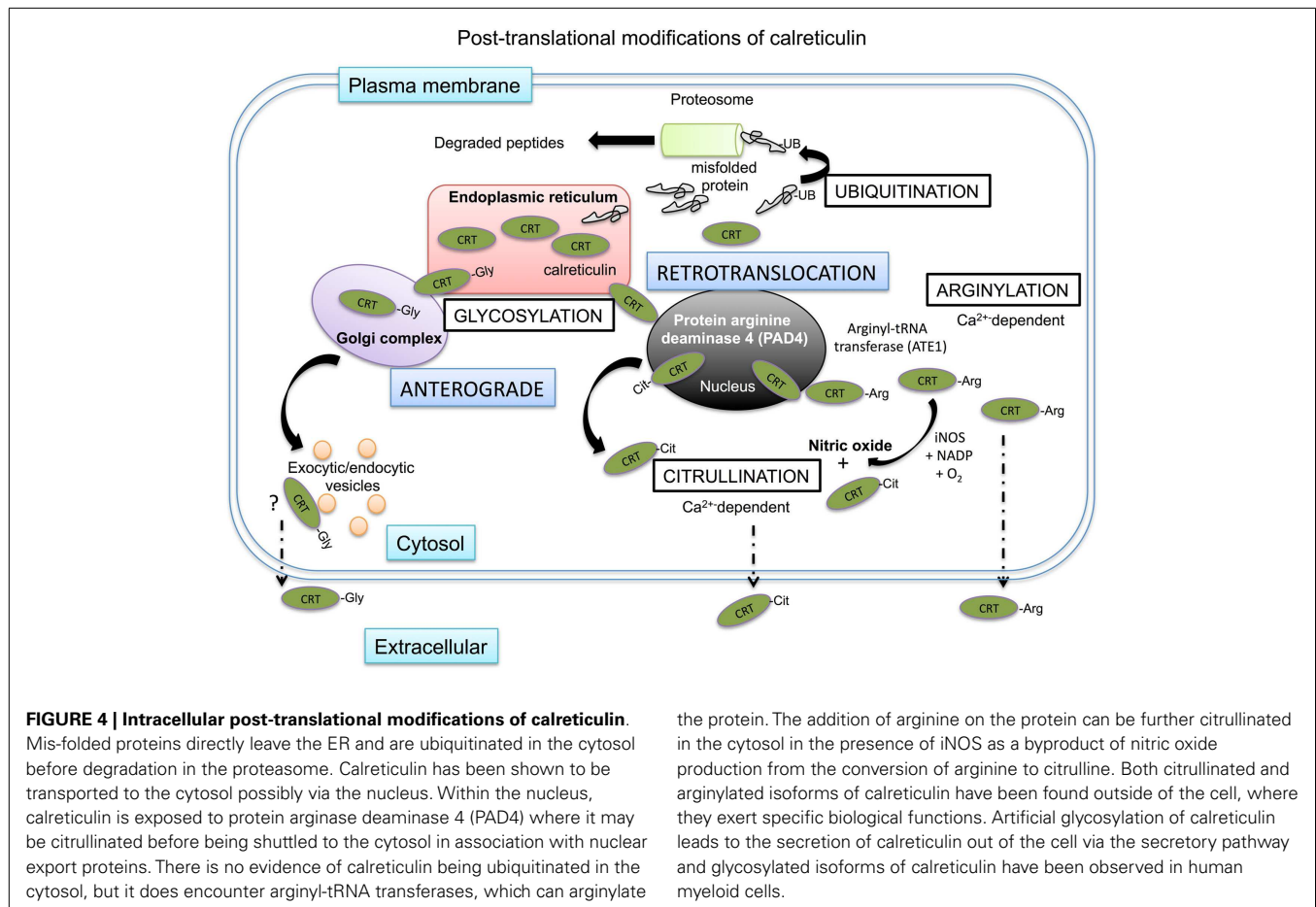
A large number of mutations have also been identified in the KDEL receptor but many of these do not affect the intracellular location or KDEL binding capacity of KDEL receptors. However, retrograde transport of the KDEL containing proteins is dependent on a presence of a single aspartic acid residue in the seventh membrane-spanning region, which may be important for conformational changes and intermolecular interaction in the membrane bilayer of KDEL receptor possessing vesicles (107). The binding of KDEL ligands to the KDEL receptors leads to activation of a number of specific kinase signaling pathways, specifically activation of G-proteins (108). This triggers a series of signaling pathways (109) that can aid the return of chaperones back to the Golgi complex and ER retrograde pathways or possibly transport them toward the plasma membrane by anterograde pathways in endosomal compartments (Figure 4).

### RETROTRANSLLOCATION AND POST-TRANSLATIONAL MODIFICATION OF CHAPERONES

Many of the chaperones of the heat shock protein family are normally resident in the cytosol (47). However, other chaperones such as calreticulin are typically retained in the ER, but have also been identified in the cytosol after having somehow escaped the retrograde retention pathway between the ER and Golgi complex

(Figure 4). The expression of ER chaperones on the cell surface or extracellular environment could be explained if chaperones can be demonstrated to reach the cell surface via the anterograde type secretory pathways. In the case of calreticulin, its normal physiological isoform cannot enter the secretory pathway as it is non-glycosylated. However, Panaretakis and colleagues created a glycosylated form of calreticulin that trafficked to the cell surface in an anterograde manner via the Golgi complex/actin mediated exocytic vesicle secretory pathway in murine colon cancer cell line CT26 (110). Further, a naturally glycosylated form of calreticulin has been observed in the myeloid tumor cell line HL60 (111). Therefore, in certain settings glycosylation of calreticulin may occur and may trigger secretion into the extracellular space. Such glycosylation may occur on surface exposed asparagine peptides in the P-domain of the protein that can, at least artificially, be N-glycosylated.

There is also evidence to suggest that ER chaperones can leave the ER via a retrotranslocation pathway, particularly under stress conditions (112). Mis-folded proteins retrotranslocate into the cytosol and are commonly post-translationally modified by a process of ubiquitylation. In brief, ubiquitin binds to lysines on the protein, which act as a proteasomal degradation signal for the protein (113). Afshar and colleagues, used digitonin to specifically



permeabilize only the outer cell membrane of mammalian cells, while leaving the membranes of intracellular organelles intact (114). Using this strategy they recovered ~14% of total calreticulin, whilst other chaperones such as PDI and gp96 were retained in the ER. Of note, the recovered calreticulin was not ubiquitinated, suggesting that calreticulin passed into the cytosol through an ubiquitin- and proteasome-independent retrotranslocation pathway. In a series of deletion experiments, they showed that the C domain of calreticulin mediated this retrotranslocation. Reversely, insertion of the C-domain of calreticulin in PDI allowed this chaperone to retrotranslocate to the cytosol. There is some evidence to suggest that such retrotranslocation of calreticulin from the ER to the cytosol occurs via the nucleus, where it may interact with proteins with nuclear export signals and exit the nucleus in complex with nuclear proteins (115).

Whether this cytosolic calreticulin is the source of plasma-membrane calreticulin is not known for certain. However, calreticulin on the cell membrane has been found to be arginylated (116). Protein arginylation is catalyzed by a cytosolic-based enzyme, arginyl-tRNA protein transferase (ATE1). Under cell stress conditions, ATE1 can promote the linkage of arginine to N-terminal amino groups, but also to mid-chain side groups of aspartate and glutamic acid (117, 118). Such arginylated isoforms of calreticulin have been found in the cytosol associated with stress granules, but are not found in the ER (119). Once on the cell surface, arginylated

calreticulin can influence cell survival, with exogenously applied arginylated calreticulin increasing cellular apoptosis and overcoming resistance to apoptosis (116). Of note, this may not be the case for other isoforms of calreticulin detected outside the cell. Interestingly in cells lacking ATE1, no calreticulin could be detected on the cell surface, suggesting that arginylation of calreticulin is a requisite for surface exposure. As mentioned earlier, another isoform of calreticulin exists in the form of citrullinated calreticulin, which was found to modulate immune function in rheumatoid arthritis patients (120, 121).

### CALRETICULIN, NITRIC OXIDE, AND INHIBITION OF FLIPASES

Many chaperones and HSP in the cytosol of cells are detected on the cell surface, but very little is known as how these get out of cells via non-ERGIC pathways. Despite this, for many years some of these proteins, especially the HSP70 and HSP90 families of proteins have been known to play a number of extracellular roles in infections, autoimmune disease, and tumor-specific recognition (122). Some chaperones present in the cytosol may associate with the phospholipids facing the lumen of the cell. Heat shock protein chaperones are known to be in close proximity to the plasma membranes and assist in the translocation of proteins across the membrane for export out of the cell. In artificial lipid bilayers, HSP have been demonstrated to create ATP-dependent transmembrane ion channels (123). We showed

calreticulin binds in a  $\text{Ca}^{2+}$ -dependent manner directly to phosphatidylserine (PS) (112). Normally 80% of PS is located on the inner leaflet with only 20% of PS on the outer surface of healthy cells. The polar head of PS was shown to bind to CRT with high affinity ( $K_D = 1.5 \times 10^{-5}$  M) (124). We observed the interaction of calreticulin occurred in punctate regions of the membranes and in a further study demonstrated the calreticulin was associated with lipid rafts (as demonstrated by incorporation of cholera toxin B) in association with ERp57 (61). Whether these chaperones associated with lipid rafts can leave the cells through dimerizing and clustering in rafts that bud from the cell is unknown.

As discussed above, citrullinated calreticulin binding to the SE on the surface of effector cells can lead to NO production in opposite cells. NO production is a cell stress signal that can deplete ER  $\text{Ca}^{2+}$  and lead to overexpression of calreticulin (125). The overexpression of calreticulin may result in the protein leaving the ER by the mechanisms discussed above. The KDEL retention receptors may also become saturated preventing its retention in the ERGIC complex. The increased calreticulin further promotes intracellular NO production (126). Cytosolic calreticulin has the ability to bind to PS on the inner leaflet in a  $\text{Ca}^{2+}$ -dependent manner in close proximity to the flipase, aminophospholipid translocase (APLT). In an environment of increase NO activity, the SH groups of APLT are susceptible to transnitrosylation/oxidation, this leads to the inhibition of APLT to retain PS on the inner leaflet of the plasma membrane. Our own experiments demonstrated that Jurkat T-cells exposed to S-nitroso-L-cysteine-ethyl-ester, an intracellular NO donor and inhibitor of APLT results in PS and calreticulin externalization together in an S-nitrosothiol-dependent and caspase-independent manner (112). Other forms of cell stress also appear to promote surface expression of chaperones that can be exploited to tumor eradication as discussed below.

### CALRETICULIN EXPOSURE DETERMINES IMMUNOGENIC CELL DEATH

The potential pro-immunogenic role of chaperones gained prominence by the discovery that cell surface exposure of calreticulin determines the immunogenicity of cancer cell death. This so-called ICD is induced by certain chemotherapeutics, e.g., anthracyclins, or irradiation, and hinges on the rapid pre-apoptotic translocation of calreticulin to the cell surface (55). Such surface-exposed calreticulin induces the uptake of dying cancer cells by CD11c-positive myeloid dendritic cells, leading to tumor antigen presentation to T-cells and concomitant clonal T-cell expansion. Injection of calreticulin-exposing dying tumor cells prevented tumor growth upon re-challenge with viable tumor cells. Selective knock-down of calreticulin reduced the phagocytic uptake of anthracyclin treated cells by dendritic cells and abolished T-cell-mediated elimination of the tumor. Analogously, apoptotic human bladder cancer cells and murine colon cancer cells treated with the photodynamic therapeutic hypericin exposed calreticulin on their membrane. Again, surface calreticulin induced maturation of human immature dendritic cells, and elicited an anti-tumor immune response in mice (50). Of note, non-immunogenic cytotoxic treatment of cancer cells was converted to immunogenic by co-treatment with recombinant calreticulin (55), highlighting the pivotal role of calreticulin in ICD.

In addition to calreticulin exposure, late apoptotic or necrotic release of HMGB-1 from dying cells, and subsequent binding to TLR-4 on dendritic cells was necessary to obtain optimal antigen presentation of chemotherapy or radiotherapy treated cancer cells (127). Indeed, dendritic cells lacking TLR-4 or its downstream adaptor molecule Myd88 could not present antigen from dying tumor cells and did not elicit a T-cell mediated anti-cancer immune response in mice (127). Further, knock-down of HMGB-1 inhibited the potential of irradiated tumor cells to stimulate dendritic cells. In addition to the role of HMGB1 in ICD, it was found that upon hypericin treatment of bladder cancer cells or upon oxaliplatin or doxyrubicin treatment high levels of ATP were secreted, which like calreticulin also preceded apoptotic PS exposure (50). Inhibition of ATP abolished the inflammatory response (128).

Based on the above, there is a cascade of events that determines the immunogenicity of cell death. Here, calreticulin is translocated to the cell membrane during early (pre-apoptotic) stages of dying tumor cells, which facilitates efficient uptake by dendritic cells. In addition, the release of ATP during early apoptotic stages is essential to mount an immune response. Further, HMGB-1 release at late apoptotic stages is required for efficient antigen presentation by dendritic cells to T-cells. Of note, whereas capsaicin treatment induced pre-apoptotic calreticulin exposure and ATP release, HSP90 and HSP70 release occurred (129). Similarly, hypericin treated cancer cells actively exposed calreticulin, with no detectable levels of HSP90, calnexin, or BiP. However, at later (late apoptotic) stages, certain levels of extracellular calreticulin, HSP70 and HSP90 were detected, as a result of passive extracellular release (31). Thus, calreticulin exposure is required to induce ICD, although several additional stimuli contribute to an efficient immune response.

### TRANSLOCATION OF CALRETICULIN TO THE CELL SURFACE DURING CANCER THERAPY

The exact translocation pathway of calreticulin during ICD is not known. In certain cases, the chaperone ERp57 was found to steer calreticulin translocation, specifically upon anthracyclin treatment (21, 62). ERp57 and calreticulin extracellular expression levels correlated and also co-translocated to the surface of mitoxantrone treated tumor cells. Further, calreticulin and ERp57 were needed for each others translocation in mitoxantrone and radiation treated cells, as calreticulin knock-outs failed to expose both calreticulin and ERp57 to the cell surface and *vice versa* (21, 62). In contrast, the interaction between ERp57 and calreticulin was not required to induce calreticulin cell surface exposure in thapsigargin treated cells (130). Here, mouse embryonic fibroblasts (MEFs) that expressed a mutated form of calreticulin that was unable to bind ERp57, had equal amounts of cell surface calreticulin compared to wildtype MEFs during thapsigargin treatment. Similarly, the translocation of calreticulin upon hypericin photodynamic therapy was not accompanied by co-translocation of ERp57 (31, 50). However, both mitoxantrone and hypericin mediated translocation of calreticulin was blocked by Brefeldin A, an inhibitor of anterograde protein transport from the ER to the Golgi apparatus (50, 110). In addition, extracellular calreticulin but not ERp57 was required to induce phagocytosis and subsequent induction of



anti-tumor immune responses (31, 62). Thus, the mechanism and routing of calreticulin to the cell surface seems to be dependent on the ICD-inducing compound, and likely also cell type dependent.

### TRANSLOCATION OF ER CHAPERONES REQUIRES ACTIVATION OF THE ER STRESS RESPONSE

The exposure of tumor cells to anthracycline antibiotics such as doxorubicin, mitoxantrone (55) or physical treatments such as photodynamic therapy with hypericin (32) commonly induce ER cell stress. This ER stress response appears to be an obligatory step in inducing extracellular expression of ER chaperones. In contrast, nuclear damage or signaling is not a requisite, as enucleated cells exposed calreticulin on their surface to a similar degree as observed for normal cells upon anthracycline therapy (21).

The ER stress response via PERK and eIF2 $\alpha$  was found to be involved in the translocation of calreticulin to the cell surface during ICD. When PERK phosphorylates eIF2 $\alpha$ , translation initiation is halted, resulting in reduced protein synthesis. In mitoxantrone treated CT26 colon cancer cells, the translocation of calreticulin and ERp57 was accompanied by phosphorylation of PERK and its substrate eIF2 $\alpha$  (21). Similarly, hypericin mediated photodynamic therapy induced eIF2 $\alpha$  phosphorylation and PERK activation (50). When CT26 cells were depleted for PERK or when a non-phosphorylatable form of eIF2 $\alpha$  was expressed, this completely abolished calreticulin/ERp57 exposure, whereas it did not affect the sensitivity toward anthracycline induced cell death. In contrast, eIF2 $\alpha$  was not required for hypericin induced calreticulin exposure, but solely relied on PERK activation (50). This discrepancy might rely on the pronounced localization into the ER of hypericin, whereby sufficient ER stress might already be induced upon photodynamic disruption of the organelle. In line with this, the photodynamic therapeutic photofrin, which has a less pronounced ER localization, was not able to induce calreticulin exposure (50). However, also spontaneous release of calreticulin from acute myeloid leukemia (AML) blast was associated with eIF2 $\alpha$  hyperphosphorylation (131). Furthermore, the disruption of the PP1/GADD34 complex, a complex that is involved in the dephosphorylation of eIF2 $\alpha$  was already sufficient to induce calreticulin exposure (55, 132). Thus, the induction of an ER stress response is required to induce extracellular calreticulin exposure, which might be induced via various pathways, depending on the therapeutic.

In addition to ER stress, the formation of reactive oxygen species (ROS) and reduction of ER Ca<sup>2+</sup> levels may favor cell membrane surface exposure of calreticulin. Indeed, most therapies that can induce ICD also induce ROS formation. When CT26 cells, treated with anthracyclines or radiation therapy, were incubated with ROS scavengers (N-Acetyl cysteine, glutathion ethyl ester) this prevented apoptosis as well as calreticulin exposure (110). Similarly, the presence of the <sup>1</sup>O<sub>2</sub> quencher L-histidine decreased calreticulin translocation in hypericin treated bladder cancer cells (50). However, the presence of redox stress alone does not suffice to translocate calreticulin, as cisplatin treated osteosarcoma cells were unable to expose calreticulin, although significant levels of apoptosis, mitochondrial damage, and ATP release were induced (133). This lack in calreticulin exposure was associated with inefficient induction of the ER stress response as eIF2 $\alpha$  was only minimally

phosphorylated upon cisplatin treatment. Of note, thapsigargin treatment alone was also inefficient for induction of calreticulin exposure, although it did phosphorylate eIF2 $\alpha$  (110, 133). Interestingly, when cisplatin and thapsigargin therapy were combined, this restored the ER stress response and induced calreticulin exposure, which was sufficient to induce an immune response in mice (133). Of note, thapsigargin is an inhibitor of SERCA pumps, whereby the ER Ca<sup>2+</sup> levels decrease, which might also contribute to ER stress. Indeed, levels of cell membrane expressed calreticulin were enhanced in thapsigargin treated neuroblastoma cells, which were genetically manipulated to have reduced Ca<sup>2+</sup> levels in the ER (134). Of note, in addition to the ER stress response, a specific apoptotic response is also required in some cases, whereas it is not necessary in others. In this respect, caspase-8 activation was needed to induce calreticulin/ERp57 translocation in mitoxantrone treated CT26 cells or MEFs, as cells depleted for caspase-8 lost their ability to translocate calreticulin/ERp57 (110). In contrast, inhibition of caspase-8 activity did not affect hypericin induced calreticulin exposure (50). Thus, ER stress and ROS production are both required for calreticulin translocation, whereas additional stimuli, i.e., caspase activation or ER Ca<sup>2+</sup> depletion, are essential depending on therapeutic strategy of cell type.

### POTENTIAL ROLE OF EXTRACELLULAR ER CHAPERONES AS THERAPEUTICS IN CANCER THERAPY: EVIDENCE FOR ICD IN CLINICAL SETTINGS

Most of the work on ICD has been performed in animal studies or *in vitro*. However, there are some studies on the existence of ICD in the clinic. For instance, a combination of heat shock/ $\gamma$ -ray/UV-radiation therapy was used to induce cell death in primary indolent non-Hodgkin's lymphoma cells, which were *ex vivo* loaded on autologous dendritic cells, for vaccination strategies (135). Here, 6 out of 18 patients showed clinical and immunologic responses. Of note, the levels of calreticulin and HSP90 exposure were significantly higher in heat shock/ $\gamma$ -ray/UV-radiation treated tumor cells from responders compared to non-responders. In line with this, clinical responders showed higher amounts of circulating antibodies against HSP90 and calreticulin after vaccination. In contrast, there was no difference in the amount of cell death or HSP70 or HMGB-1 release between tumor cells from responders and non-responders. Similarly, there was no difference in the expression of HLA class I and II. As a consequence, NK-cell maturation was increased, which directly correlated with the levels of calreticulin and HSP90 expression. In another study, the expression of cell surface calreticulin was found on AML blasts, although this was regardless of chemotherapy (131). In addition, the *in vivo* treatment of patients with anthracyclines did not enhance calreticulin exposure on malignant blasts and did not alter the serum calreticulin levels. However, the presence of calreticulin on the cell surface of malignant AML blasts did associate with enhanced immune responses, since T-cells from calreticulin-positive patients produced IFN $\gamma$  upon interaction with autologous dendritic cells, whereas T-cells from calreticulin-negative patients failed to respond upon this trigger. However, the overall survival of these AML patients did not correlate with calreticulin levels. The capacity of clinical drugs to induce ICD was also tested on primary patient derived ovarian and prostate cancer cells. Exposure

to anthracyclines was sufficient to induce translocation of calreticulin, HSP70 and HSP90 to the cell surface, and HMGB-1 release at later time point (136), but the clinical implications of ICD in these cancer types warrants further analysis. In addition to the well known ICD inducers (i.e., anthracyclins and radiation), cardiac glycosides were recently also recognized as inducers of ICD, also eliciting anti-cancer immune responses in mice (137). Using retrospective clinical analysis of human carcinoma patients, it was found that the administration of the cardiac glycoside digoxin during chemotherapy improved overall survival of patients with colorectal, breast or head, and neck cancer. However, it should be noticed that this positive effect was only observed in patients treated with chemotherapeutics considered as non-immunogenic. Indeed, the addition of digoxin failed to affect overall survival of patients that received anthracyclin therapy.

Taken together, in clinical settings, calreticulin and associated chaperones can be exposed on tumor cells or in serum from patients. However, the induction of immune responses and benefit in terms of survival are not as straightforward as postulated in animal studies. Thus, many challenges remain in terms of identifying the essential set of signal requisites for induction of ICD in order to achieve efficient immune responses upon ICD in patients.

### CHALLENGES FOR THE THERAPEUTIC IMPLICATION OF ICD

From the above, it appears that the induction of ICD and accompanied calreticulin exposure on tumor cells is a promising strategy to obtain curative cancer therapies in patients. However, there are several challenges that remain to be addressed. First, the induction of calreticulin exposure by anthracycline therapy seems to be hampered *in vivo* and shows a high variability between patients (131, 138). Although, calreticulin was found on malignant blast from AML patients, this was independent of therapy and caused by spontaneous release (131). Similarly, apoptotic AML cells, which died spontaneously or as a result of cytotoxic drugs *in vivo* assays, showed calreticulin exposure and release of HSP70 and HSP90. However, there was a wide variation in the levels between different patients, which depended on individual patient characteristics, rather than the cell death inducing therapeutics (138). Thus, ways of reliably and uniformly inducing calreticulin exposure in cancer patients will have to be identified.

Secondly, induction of ICD by a certain chemotherapeutic appears to be cell type and perhaps context-dependent. For instance, thapsigargin was found to induce an ER stress response in CT26 colon cancer cells, but failed to stimulate cellular calreticulin/ERp57 exposure (110). In contrast, thapsigargin induced both ER stress and calreticulin release in neuroblastoma and MEFs (139). In the case of the former, surface exposure of calreticulin was strongly enhanced when  $Ca^{2+}$  levels in the ER lumen were depleted (134). Also in primary cells isolated from ovarian and prostate cancer patients, anthracyclines were able to induce calreticulin exposure and release of HSP70 and HSP90, whereas there was completely no induction of ICD upon UV-radiation (136). Therefore, optimal treatment strategies need to be evaluated for each cancer type with special focus on combining different therapies to optimize induction of key immunogenic molecules. Indeed, in non-Hodgkin lymphoma cell lines (NHL), the combination of heat shock,  $\gamma$ -ray, and UVC-ray therapy induced higher amounts

of calreticulin and HSP90 exposure, and HMGB-1 and ATP release than each single treatment (135).

Of note, many of the cytotoxic agents that in pre-clinical models of ICD elicit pre-apoptotic calreticulin exposure, such as doxorubicin, can induce severe myelosuppression and leukopenia. This toxicity may negatively affect the pro-immunogenic effect of extracellular calreticulin in patients by deleting requisite immune components of the ICD pathway. Indeed, although calreticulin-dependent ICD has been described for various cytotoxic agents in pre-clinical settings these typically have not translated into reports on effective anti-cancer immunity upon treatment of patients. In this respect, the identification of optimally immunogenic treatments with minimum toxicity toward critical immune cells seems warranted, e.g., in further combination with therapeutics that selectively target negative immunoregulatory cells such as myeloid-derived suppressor cells and regulatory T-cells.

Finally, as already discussed above, the calreticulin “eat me” signaling is counterbalanced by the “don’t eat me” signaling via CD47. For high CD47-expressing cancer it may therefore be beneficial to include CD47-blocking therapeutics in order to optimize therapeutic efficacy. In this respect, it is interesting to mention that the only study in which clinical responses to tumor expressed calreticulin was found, has been described in NHL patients (135). These NHL patients typically also show strong overexpression of CD47 (59).

### CONCLUSION

Chaperone molecules play a number of specific roles related to protein processing within the cell. However, new knowledge indicates that a select number of chaperones in the extracellular environment can play a role in both innate and adaptive immunity that may be useful in the treatment of tumors. In contrast, the release of potent immunogenic-stimulating molecules may have a detrimental role in some autoimmune diseases. Therefore, it is crucial to understand how various post-translational modified forms of chaperones are released from cells under resting and stressed conditions and how the released chaperones exert their immune-promoting responses. Clearly, there are several ways in which these chaperone proteins can be released from cells other than through the process of passive necrosis. Their complex interactions with the immune system, especially chaperone-immune cell signaling pathways and receptors interactions requires further studies to help understand their role of potential therapeutics to treat cancers and in their ability to induce inflammation in autoimmune disease.

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