ENDOPLASMIC RETICULCUM AND ITS ROLE IN TUMOR IMMUNITY

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ENDOPLASMIC RETICULCUM AND ITS ROLE IN TUMOR IMMUNITY

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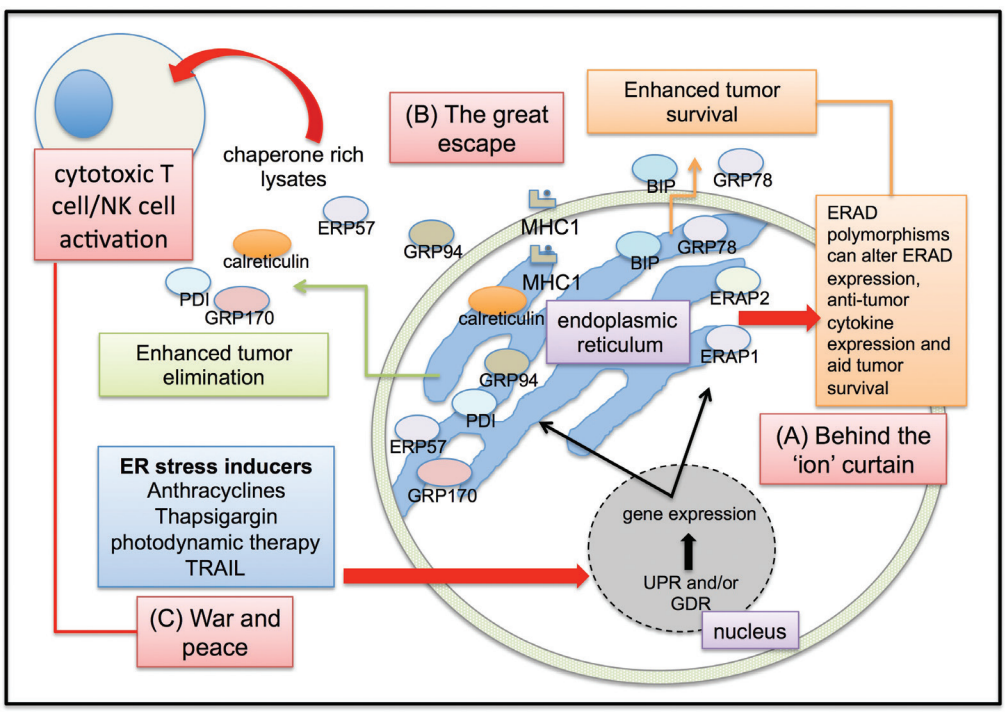

Endoplasmic reticulum and its role in tumor immunity. (A) The individual components of the endoplasmic reticulum are highly sensitive to environmental changes leading to activation of both UPR and GDR pathways. These pathways activate a complex series of transcription regulators that control the expression, activity and location of ER proteins. (B) The release of ER proteins can either enhance tumor survival or promote tumor elimination by triggering specific immunity. (C) The anti-tumor activities of multiple ER proteins are being exploited in vaccine development or individual molecules are being exploited that can activate specific arms of the immune response.

Image courtesy Paul Eggleton, Edwin Bremer and Marek Michalak

The endoplasmic reticulum (ER) is an organelle crucial to many cellular functions and processes, including the mounting of T-cell immune responses. Indeed, the ER has a well-established central role in anti-tumor immunity. Perhaps best characterized is the role of the ER in the processing of antigen peptides and the subsequent peptide assembly into MHC class I and II molecules. Such MHC/tumor-derived peptide complexes are pivotal for the correct recognition of altered self or viral peptides and the subsequent clonal expansion of tumor-reactive T-cells.

In line with the role of the ER in immunity, tumor-associated mutations in ER proteins, as well as ER protein content and localization can have both deleterious and advantageous effects on anti-tumor immune responses. For instance, loss of function of ER-aminopeptidases, that trim peptides to size for MHC, alter the MHC class I - peptide repertoire thereby critically and negatively affecting T-cell recognition. On the other hand, altered localization of ER proteins can have immune-promoting effects. Specifically, translocation of certain ER proteins to the cell surface has been shown to promote anti-tumor T-cell immunity by directing uptake of apoptotic tumor cells to professional antigen presenting cells, thereby facilitating anti-tumor T-cell immunity.

These selected examples highlight a diverse and multi-faceted role of the ER in anti-tumor immunity. Molecular biological insights from the past decade have uncovered that ER components may affect tumor immunity and have invoked a variety of follow-up questions. For instance, how and why are ER proteins over-expressed in tumors? How do nucleotide and somatic mutations in ER chaperones/processing machinery affect the MHC/peptide complex and tumor cell immunogenicity? How do ER-proteins translocate to the cell surface? What if any is the potential role of extracellular ER protein in tumor immunotherapy/vaccines, and can they be delivered to the tumor cell surface by photodynamic therapy, anthracyclines or by other means? In this special research topics issue, we present basic and clinical research reports covering many aspects of ER proteins in cancer recognition by the immune system, therapy and drug development. We also present new insights into ER stress, signalling and homeostasis in immunogenic cell death in cancer, the effect of parasitic ER proteins on tumour growth, ER protein regulation of angiogenesis. A comprehensive series of articles highlight our understanding of an expanding avenue of tumour immunology and therapeutic development, which exploit a collection of proteins within the ER that are not obvious candidates for immunity against tumors.

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EDITORIAL
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Editorial: Endoplasmic Reticulum and Its Role in Tumor Immunity

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Keywords: aminopeptidases, autoimmunity, angiogenesis, cancer, genome damage, oxidoreductases, phage display, vaccines

Cancer cells express surface proteins and display antigens that differ from the "norm." These differences can be exploited to promote a therapeutic antitumor immune response. Specifically, components within the endoplasmic reticulum (ER) play a critical role in deciding which antigenic peptides are presented on the cancer cell surface to immune cells. Furthermore, under stress conditions, certain ER-resident proteins can exit the ER and translocate to the surface. The translocation of such ER proteins to the outside of the cell (1, 2) can lead to modulation of immune responses in cancer (3, 4), autoimmunity (5) and other diseases (6, 7). Normally, proteins undergo a number of ER stress checks for correct folding and, e.g., ability to resist inappropriate oxidation and reduction before secretion. Failing these quality controls leads to ER stress and triggers a series of unfolded protein responses (UPRs) to restore order. In cancer cells, these pathways can be dysregulated opening up the possibility of developing potential therapeutics to target cancer cells (8). In this topic, these various aspects of the ER in tumor immunity are explored in a series of focused review and research articles.

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BEHIND THE "ION" CURTAIN

Within the Ca^{2+}-ion rich confines of the ER, chaperones, oxidoreductases, aminopeptidases (ERAPs) work industriously for the benefit of the cellular state, regulating signaling to the "outside world." The calcium channels linking the ER lumen and cytosol act as ER stress gates and chaperones, such as GRP78, act as gate keepers deciding the fate of the cell by their ability to control Ca^{2+} release (9). Alterations in Ca^{2+} homeostasis in the ER can provoke cell stress and trigger one or more UPR coping mechanism pathways, which normally leads to either recovery of a stressed cell or non-inflammatory cell death. However, solid tumors typically thrive in a low oxygen and nutrient environment that usually triggers ER stress. Dicks and coworkers describe corrective UPR strategies that aid malignant cells to survive in this environment, with a focus on GRP78 (10). In brief, GRP78 transcription triggered by ER stress facilitates chromatin remodeling and DNA damage repair and in certain types of malignancies aids survival.

One of the best known immune-regulatory functions occurring within the ER is the assembly of the major histocompatibility complex (MHC)-I/antigen peptide complex. Stratikos and colleagues report on the role of the ER aminopeptidases (ERAP1 and 2) in generating mature antigenic epitopes for loading onto the MHC class I molecules, prior to their transport to the cell surface (11). The authors suggest that both ERAP 1/2 are required for natural killer and T cell-mediated immunity

against tumors. These highly polymorphic ERAPs contain many single nucleotide polymorphisms (SNPs) associated with diseases, including cancer (12). These SNPs can influence aminopeptidase expression, enzymatic activity, and antitumor cytokine expression. Such ERAP mutations may aid tumor cells to avoid immune surveillance and eradication (13).

THE GREAT ESCAPE

Endoplasmic reticulum chaperones and oxidoreductases can serve as "eat-me" signals on the surface of tumors cells, while promoting tumor growth on others. How ER chaperones escape retention from the ER and move to the plasma membrane remains contentious (14). Several articles within this e-book describe mechanisms to prevent and allow escape of chaperones from the ER and how this influences tumor recognition. Gutiérrez and Simmen describe the regulatory processes involved in retaining or recapturing ER proteins as they attempt to leave the ER (15). Gutiérrez and Simmen describe the conditions by which ER chaperones and oxidoreductases (calreticulin, ERp57, PDI, and GRP94) escape retention and enhance tumor elimination by the immune system. Conversely, other ER proteins (BiP/GRP78) are expressed on many cancer cell surfaces and enhance proliferation, angiogenesis, and therapeutic resistance (16). Undoubtedly, if the "escape" and retention of ER proteins to and from the cell surface can be controlled, the process could be exploited for specific cancer therapies. However, methods to trigger escape of potentially immunogenic regulatory proteins from the ER will have to be strictly regulated, given their ability to modulate tumor growth and induce unwanted adaptive immunity in other diseases. Wiersma and coworkers (5) highlight the fact that in autoimmune diseases, cell stress provokes extracellular release of some ER proteins, which can affect innate and adaptive immune systems and trigger inflammation (17–19).

The idiom "That which hath been is now; and that which is to be hath already been" (*King James Bible, Ecclesiastes 3:15*) is no better illustrated by the fact that parasites have been secreting chaperones for thousands of years as a defense mechanism against the human immune system (20, 21). Ramirez-Toloza et al. (22) describe how surface calreticulin on the Chagas disease causing parasite *Trypanosoma cruzi* blocks activation of complement and aids immune escape of the parasite. Moreover, people with Chagas disease appear less susceptible to certain malignancies (23), and Ramirez-Toloza et al. identify segments of calreticulin that can inhibit tumor angiogenesis.

WAR AND PEACE

Several papers in this e-book describe immune properties of ER proteins capable of raging "war" against tumors. Wang and colleagues describe the adjuvant properties of the stress inducible glucose-regulated protein 170 (GRP170). Previously, they showed an isoform of GRP170 was secreted in melanoma, prostate, and colorectal cancer cells (24–26). GRP170 associates with tumor antigens both intracellularly and extracellularly, acting like a double agent, inducing potent anticancer immunity when outside the cells, but aiding the survival of cancer cells when within the ER. The authors have exploited GRP170 to develop an immune adjuvant for cancer vaccines to trigger a number of adaptive immune processes. An alternative means of delivering antitumor chaperones to the cell surface is by inducing cell stress using photodynamic therapy (PDT) to generate localized production of reactive oxygen species by transfer of light energy from the photosensitizer chlorin C6. This strategy induces surface exposure of calreticulin within minutes of treatment in squamous carcinoma cells (27). Tumoricidal activity is enhanced when PDT treated cells are supplemented with additional recombinant calreticulin. In a similar manner, de Bruyn and coworkers describe that tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) recruits CRT to its TRAIL-receptor 2 DISC complex and dissociate CRT from CD47 on the cell surface of cancer cells (28), whereby it may or may not facilitate phagocytic uptake by dendritic cells.

A major aspect of ER protein stimulation of anticancer immunity is to activate specific cytotoxic T cells to provide long lasting immunity against developing tumors. Løset et al. illustrate how tumor-specific T cells armed with specific T-cell receptors (TCRs) could eradicate tumors by interacting with MHC class I containing tumor and/or chaperone peptides (29). Løset and coworkers highlight an alternative therapeutic approach that exploits soluble TCRs that engage peptide/MHC (pMHC) complexes, some of which are now in clinical trials. As an alternative to the stealth-like cancer eradication by TCR-transduced T cells, Graner and colleagues have proposed a more "blanket-bombing" approach. They describe the development of a vaccination rationale comprising of chaperone-rich cell lysates (CRCL) purified from solid tumors designed to induce a plethora of immune responses (30).

SUMMARY

The ER and its specialized proteins do play a major role in tumor immunity both indirectly and directly. Clearly, there is much more to understand but the potential role and therapeutic options of ER proteins, as described herein, will aid further research into this fascinating topic.

AUTHOR CONTRIBUTIONS

Dr. MM, Dr. PE, and Dr. EB have discussed/written the editorial content and approved it.

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PERSPECTIVE ARTICLE
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Endoplasmic reticulum stress, genome damage, and cancer

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Endoplasmic reticulum (ER) stress has been linked to many diseases, including cancer. A large body of work has focused on the activation of the ER stress response in cancer cells to facilitate their survival and tumor growth; however, there are some studies suggesting that the ER stress response can also mitigate cancer progression. Despite these contradictions, it is clear that the ER stress response is closely associated with cancer biology. The ER stress response classically encompasses activation of three separate pathways, which are collectively categorized the unfolded protein response (UPR). The UPR has been extensively studied in various cancers and appears to confer a selective advantage to tumor cells to facilitate their enhanced growth and resistance to anti-cancer agents. It has also been shown that ER stress induces chromatin changes, which can also facilitate cell survival. Chromatin remodeling has been linked with many cancers through repression of tumor suppressor and apoptosis genes. Interplay between the classic UPR and genome damage repair mechanisms may have important implications in the transformation process of normal cells into cancer cells.

Keywords: carcinogenesis, cell death, chromatin damage, coping responses, DNA breaks, endoplasmic reticulum, unfolded proteins

INTRODUCTION

Cells in the body are continuously exposed to a dynamic environment dictated by the metabolic and nutritional status of the organism. Certain instances, such as exposure of the organism to nutrient excess or deprivation, extremes in temperatures, xenobiotics, and radiation, cause damage to cellular components and disruption of cellular processes. It has long been recognized that cells are adept at compensating for changes in their environment by altering certain cellular processes. The mobilization of such coping mechanisms is designed to maintain or recover proper function, overcome stressful conditions, and increase the chance for survival (**Figure 1**).

In the past several years, there has been increasing evidence linking endoplasmic reticulum (ER) stress with development of diseases, including certain types of cancers (1–5). In the case of cancers, especially non-inherited cancers that arise from genome damage, the cells capitalize on the ER stress response, which may be adaptive and advantageous at the cellular level, but deleterious to the organism. In this review, we discuss ER stress and genome damage in relation to cancer development. We provide observations supporting a link between different corrective strategies that cells adopt, which may lead to malignancies.

The accumulation of unfolded and misfolded proteins disrupts ER homeostasis and leads to the activation of the classic coping mechanism termed the unfolded protein response (UPR) (**Figure 1**). The UPR is initiated by the molecular chaperone glucose-regulated protein 78 (GRP78). GRP78 not only binds to the misfolded and unfolded proteins, but also regulates the transmembrane ER stress sensors, namely protein kinase RNA like ER kinase (PERK), inositol-requiring protein 1α (IRE1α), and activating transcription factor 6 (ATF6) (6–8).

Each ER stress sensor activates a separate arm of the UPR to facilitate immediate changes to a set of cellular functions designed to temporarily arrest general protein synthesis, and to produce active transcription factors that ultimately facilitate correct protein folding, degradation of proteins that cannot be properly processed, and regain of ER function. Under extreme conditions, these strategies may not be sufficient to alleviate the ER stress and thus require the removal of the malfunctioning cells. In such cases, cells undergo controlled cell death by activation of the apoptotic pathway. In some situations, certain adaptive strategies provide these cells with a selective growth advantage over other cells (**Figure 1**). This selective advantage could permit cells to survive and propagate even under chronic ER stress.

ER STRESS AND CANCER

The high proliferative rates and inadequate vascularization of solid tumors culminate in a very unfavorable microenvironment. The low pH, low oxygen tension, and low nutrient supply result in an accumulation of misfolded proteins and ER stress, which could signal cell death (9, 10). Cancer cells, however, have developed a

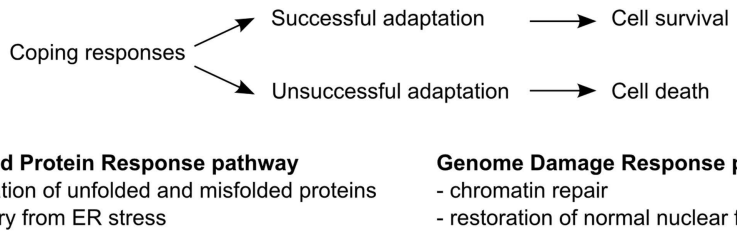

FIGURE 1 | Coping response mechanisms. The unfolded protein response (UPR) pathway operates to restore correct folding of proteins and recovery of the ER from stress. The genome damage response (GDR) pathway enables repair of damaged DNA, histones, and other DNA binding proteins and restores normal nuclear function.

capacity to survive these extreme conditions, despite the presence of ER stress, through modulation of the UPR response (11–14).

It has been observed that GRP78, a dominant regulator of the ER stress response, is increased in a variety of cancer types including breast, brain, lung, colon, prostate, skin, and some other malignancies (2, 12, 15–20). This chaperone is associated with prolonged cell survival, mainly by preventing ER stress-induced apoptosis and thereby promoting cell malignancy, metastatic development, and resistance to anti-cancer agents (12, 14, 21, 22). High levels of GRP78 are also associated with rapid proliferation and malignancy of tumors (12, 14). In breast cancer cells that express estrogen receptor α [NR3A1], the estrogen-mediated increase in GRP78 abundance confers improved resistance to ER stress and cell proliferation, both of which can be decreased through siRNA-mediated knockdown of estrogen receptor α (12). Similarly, up-regulation of GRP78 has been shown to increase growth of a glioma cell line whereas its down-regulation inhibits tumor development (14). The reduction of GRP78 in glioblastoma cell lines and solid tumors treated with a chemotherapeutic agent increased the expression of CHOP and caspase 7, leading to cell apoptosis and inhibition of tumor formation (11, 14). Moreover, the anti-tumor agent HKH40A decreases GRP78 not only at the transcriptional level but also at the protein level by directly binding GRP78 to facilitate its degradation (11). Based on these characteristics, GRP78 is considered as a biomarker of cancer progression (21).

The components of the UPR pathway have also been implicated in cancer (2, 13, 21, 23). Mutations in IRE1α have been found in some human malignancies (24, 25). Under hypoxia, the effector of the IRE1α pathway, spliced XBP1 (XBP1s), is one of the factors involved in tumor growth and survival. It promotes cancer cell survival under low oxygen conditions by forming a transcriptional complex around hypoxia-inducible factor-1, a major gene regulator under hypoxic conditions (26). This transcription factor is also involved in human breast tumorigenesis as well as in the progression of triple negative breast cancer (26, 27). Similarly, the PERK pathway can contribute to cell survival and growth through ATF4, a transcription factor that induces pro-survival genes (28, 29). ATF4 is overexpressed in solid tumors and is essential for tumor cell survival in various mouse and human cancers whereas elimination of ATF4 in cancer cells induces apoptosis (29). PERK can also facilitate tumor growth by upregulating vascular endothelial growth factor (VEGF) and thereby inducing angiogenesis in tumors (28). Tumors derived from PERK-deficient mouse embryonic fibroblasts are considerably smaller compared to those derived from wildtype embryonic fibroblasts as a result of their impaired ability to stimulate angiogenesis (28).

Despite ample examples suggesting that the activation of the UPR is essential to cancer cell survival and tumor development, there are also indications that ER stress may provide protection against cancer (3, 30, 31). In particular, it has been shown that XBP1 is protective against intestinal tumorigenesis (3). Prostatic cancer cells have been shown to produce high levels of UDP-*N*-acetylglucosamine pyrophosphorylase 1, which reduces ER stress in these cells and facilitates their growth (30). The flavonoid baicalein has also been shown to induce ER stress in hepatocellular cancer cells, resulting in increased apoptosis (31). Interestingly, in this same study, increased IREα and eIF2α activation provided a survival advantage to theses cancerous cells. This finding highlights the paradoxical role of the UPR in cancer and our incomplete understanding of how signaling pathways may favor cell death or survival under different conditions (32–34). Whichever the outcome produced by ER stress, it is clearly apparent that the UPR plays a critical role in cancer biology.

GENOME DAMAGE AND CANCER

Genome damage can be caused by a number of endogenous and exogenous genotoxic factors, including reactive oxygen species, altered cell metabolism, xenobiotics, and radiation (35, 36). These factors lead to DNA strand breaks, collapsed DNA replication forks, and damage to histones as well as other DNA-binding proteins (35). In response to chromatin damage, cells can establish a genome damage response (GDR) to repair damage to both DNA and nuclear proteins, adapt to genome damage, and reestablish nuclear function (**Figure 1**). Adaptation to genome damage can lead to cell survival but also chromatin alterations, which may have severe consequences for tissue function and physiology (36).

The GDR is orchestrated by several factors encompassing sensors, transducers, and effectors proteins (**Figure 2**), which require post-translational modification and accumulation of proteins to assemble multiprotein foci at the sites of DNA lesions (37–39). In general, activation of GDR involves temporary cell cycle arrest, local inhibition of transcription, and relaxation of chromatin to facilitate repairs. This process requires post-translational modification of proteins including the activation of the kinases ataxia

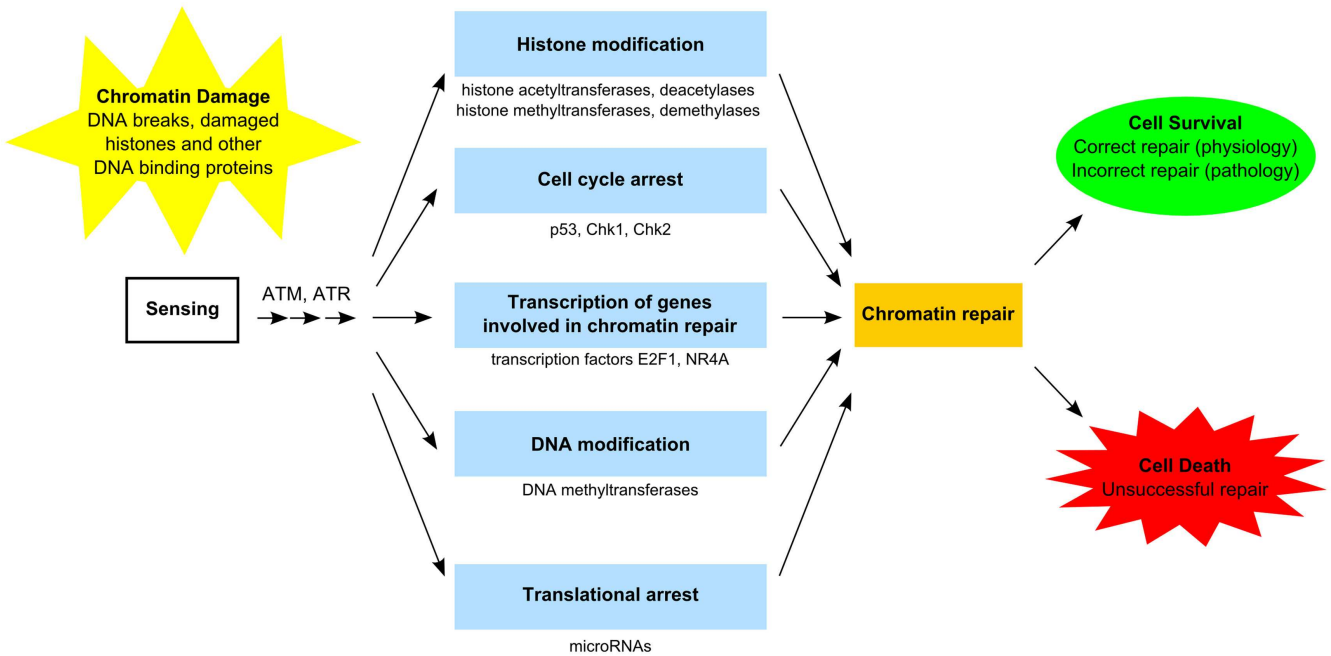

FIGURE 2 | Functional components of the GDR pathway. Sensing of damaged DNA, histones, and other DNA-binding proteins results in activation of specific kinases [e.g., ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) proteins] (37–40). Access to damaged chromatin is facilitated by histone modification involving histone acetyltransferases/deacetylases and histone methyltransferase/demethylases as well as by DNA modification involving DNA methyltransferase (37, 38, 48–50). Certain transcription factors (e.g., E2F1, NR4A) stimulate genes involved in chromatin repair (45). Translational arrest is facilitated by microRNAs (e.g., mIR-155, miR-18a) (51, 52). Cell cycle arrest (e.g., via p53, Chk1, Chk2) may be required in order to complete chromatin repair (41–44). Unsuccessful chromatin repair due to extensive damage commits the cell to die. Successful chromatin repair enables cells to survive and restore normal function. In certain cases, incorrect repair escapes quality control surveillance and leads to altered cell function, which may provide the cell with a survival advantage, but manifest as pathology at the organismal level.

telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR), which phosphorylate transducer proteins at the damages sites, including the histone H2A.x (H2AX139ph), which anchors some important effector proteins required for damage repair and cell cycle arrest (37–40). ATM and ATR also activate the serine–threonine checkpoint effector kinases, Chk1 and Chk2, which regulate a number of proteins involved in transcription, cell cycle progression and apoptosis, including the tumor suppressor protein p53 and BRCA1 (41, 42), and the cell cycle regulator proteins Cdc25 and Wee1 (43, 44). Phosphorylation of transcription factors, notably E2F1, NR4A, ATF2, and Sp1, also facilitate DNA repair in a transcription-independent fashion, by direct interaction with damaged DNA, and subsequently the co-localization of other DNA repair proteins (45). In addition to post-translational modifications, genotoxic lesions and DNA damaging agents can also trigger nucleosomal remodeling via eviction of resident histones and reincorporation of new histones into the reassembled nucleosomes after damage repair (37, 46–48).

Local transcriptional arrest associated with GDR may be transient or stable (49, 53). Linked to this arrest are numerous histone modifications, including hypoacetylation of histone H4, increased histone H3K9me3 and H3K27me3, and decreased histone H3K4me3 (49, 50). Histone chaperones, which transfer histones to the nucleosomes, are required for transcriptional reinitiation following DNA damage (49, 54). GDR also involves the participation of ATP-dependent chromatin remodeling complexes, including the switch/sucrose non-fermentable (SWI2/SNF2), imitation switch (ISWI), inositol requiring 80 (INO80), and chromodomain helicase DNA-binding protein, which mediate nucleosome sliding and histone displacement promoting access for DNA repair proteins (37, 38, 48).

Non-coding RNAs are also known to affect DNA repair and genome instability. Indeed, DNA damage responsive microRNAs have been shown to be misexpressed in cancer cells and to affect chemotherapy sensitivity (55–57). It has also been shown that down-regulation of Dicer and Ago2, two essential microRNA processing components, reduced cell survival and checkpoint response after UV-induced DNA damage (58). Moreover, the miR-18a was shown to downregulate ATM expression, reduce DNA damage repair, and sensitize breast cancer cells to γ-irradiation treatment (51). Also, up-regulation of the miR-24 decreases H2AX and renders cells more vulnerable to DNA damage induced by γ-irradiation and genotoxic drugs (59). Another microRNA, miR-155, was shown to reduce the levels of RAD51, a recombinase required to repair double strand breaks by DNA homologous recombination, and consequently decreased DNA repair and enhanced sensitivity to ionizing radiation in human breast cancer cells (52).

UPR AND GDR CROSSTALK

There is accumulating evidence suggesting that ER stress and GDRs are intertwined. Indeed, ER stress induced with tunicamycin treatment or glucose deprivation decreases genomic DNA damage repair by stimulating proteasomal degradation of Rad51 (60). On the other hand, down-regulation of PERK enhances DNA damage repair in irradiated cancer cells (61). Interestingly, induction of ER stress recruits the histone acetyltransferase p300 to the GRP78 promoter and this correlates with increased histone H4 acetylation and GRP78 gene expression (62). Increased GRP78 transcription is associated with the recruitment of arginine histone methyltransferase, PRMT1 (62). It was suggested that arginine methylation of MRE11 by PRMT1 regulates the activity of MRN complex, which is required for proper DNA damage checkpoint control (63). Therefore, it appears that increased GRP78 transcription from ER stress can also facilitate DNA damage repair. These contradictory effects further demonstrate our incomplete understanding of the stress signaling pathways and how they interact to determine cell fate. However, it also illustrates how ER stress can cause chromatin remodeling and affect the GDR pathway. If GDRs are impaired by alterations in the UPR, this can affect DNA integrity and subsequently increase risks of carcinogenesis.

Signaling from both ER stress and DNA damage also appear to result in similar chromatin remodeling changes to respond to cellular insults. Increased H3K14 acetylation as a consequence of ER stress has been observed, and this can activate the expression of other target ER stress response genes (64). Similarly, GDR results in increased H3K14ac, which promotes the binding of BRG1, an ATPase component of SWI2/SNF2 complex, to H2AXph139 at the sites of DNA damage enabling chromatin remodeling for DNA repair (65, 66). Phosphorylation of the histone H2Ax also enables recruitment of other chromatin remodeling complexes including INO80 and SWR1, and the histone acetyltransferase complex NuA4 to facilitate DNA repair (67–70). Therefore, H3K14ac and H2AXph139 seem to be important in connecting ER stress and GDR.

Chromatin remodeling has also been shown to occur as a result of hypoxia and heat stress, two common causes of ER stress that also have effects on GDR (71). Hypoxia-induced ER stress leads to global deacetylation and methylation of histones in the proximity of genes involved in the hypoxia-inducible factor-1-mediated response (72–74). This facilitates transcription of the genes needed for an adaptive response to hypoxia (75, 76). Yet, there is evidence confirming that hypoxia can lead to defective DNA repair, genomic instability, and consequently, to cellular transformation (76). In addition, it has been well documented that chromatin remodeling in response to heat stress results in increased transcription of heat shock proteins (77). These proteins have been shown to reduce accumulation of H2AXph139, decrease DNA damage repair, and increase radiation sensitivity and genome instability (78, 79).

While there is evidence demonstrating crosstalk between the UPR and GDR, it is not well understood at this time. Increased reactive oxygen species appears to be a common by-product of most cellular insults, ER stress, and DNA damage included (80). Oxidative stress can modulate multiple signaling pathways through activation of common transducers and transcription factors (81).

ROLE OF UPR AND GDR IN CARCINOGENESIS

Classically, the development of cancer is largely associated with inherited or acquired mutations of specific genes that regulate cell cycle, proliferation, and apoptosis (82, 83). However, similar effects can be seen with epigenetic changes, which, alone or associated with genetic mutations, can alter the expression of tumor suppressor genes (84–87). There are many examples of chromatin changes that lead to cancers. Hypermethylation of the DNA repair gene BRCA1 has been associated with both breast and ovarian cancer (88–90). Aberrant promoter methylation of the Kelch-like-ECH-associated protein 1 gene, which codes for an adaptor protein involved in degradation of cell survival and anti-apoptosis gene products, has been linked to a poorer prognosis and increased carcinogenesis in breast cancer patients (91). Hypermethylation of tumor suppressor genes has been observed in renal carcinomas and hematopoietic cancers (92–94). Hypermethylation of the cell cycle regulation gene RB1 and cyclin-dependent kinase inhibitor genes, CDKN2B and CDKN2A, which are, respectively, associated with the ocular tumor, retinoblastoma (95), and various leukemias and lymphomas (93). Histone deacetylation has been associated with a more aggressive form of acute myeloid leukemia (AML) through its repressive effect on the tumor suppressor gene death-associated protein kinase 1 (96). AML has also been associated with changes in histone methylation patterns (97). Finally, chromatin remodeling agents, including inhibitors of histone deacetylases, histone lysine demethylases, and DNA methyltransferases, have been tested for the treatment of various cancers (94, 98–102).

Since both ER stress and GDR coping mechanisms affect chromatin remodeling and DNA repair, adaptations based on these mechanisms could lead to emergence of malignant cells with self-renewal properties due to both genomic and epigenomic alterations. For example, hypermethylation of promoter regions around ER stress response genes have been implicated in the development of alcohol-induced liver cancer (103). GRP78-deficient mice fed large quantities of alcohol throughout their lives show high incidence of hepatic tumors, and correlate with hypermethylation of ATF6, which upregulates genes involved in ER-associated degradation to deal with the accumulation of misfolded proteins (103). Also, increased GRP78 stimulates the VEGF receptor 2 and subsequently VEGF-induced endothelial cell proliferation, which facilitates angiogenesis and tumor survival and growth (104–106). The apparent contradictory effect on neoplasticity as both inhibition and promotion of cancer progression, predicted by GRP78 abundance, suggests that the nature and context of coping response activation are important determinants of the outcome.

Acetylation of H3K14 has also been implicated in cell survival and carcinogenesis, both with respect to the UPR and GDR. Increase in H3K14 acetylation in response to ER stress results in stimulation of transcription, promoting cell survival (64). Increased H3K14ac during GDR enhances access of BRG1 to the sites of DNA damage to promote chromatin remodeling required

for DNA repair (65, 66). However, in addition to promoting DNA repair, BRG1 has been associated with cancer development. For example, BRG1 was shown to impair the recruitment of BRCA1 to DNA damage sites, which is important in DNA damage repair and in the maintenance of genomic stability (107); to activate the melanoma inhibitor of apoptosis gene (108); and to support oncogenic transcriptional program, including Myc (109), for the survival of leukemic cells (110). Finally, chromatin changes in response to genotoxic conditions have been shown to alter the regulation of the Hedgehog–Gli signaling pathway, which has been implicated in genome instability and in several types of cancers (111–113).

SUMMARY

Coping mechanisms are designed to correct, minimize, or overcome damage caused by harsh environments, and promote cell survival. The UPR pathway is mobilized in response to the accumulation of unfolded proteins and to ultimately regain ER homeostasis. Similarly, the GDR pathway operates in response to chromatin damage and to restore normal nuclear function. Some adaptive strategies allow cells to overcome defects in cellular function through metabolic adaptation and gain a survival advantage, such as in certain types of malignancies. A better understanding of the interplay between UPR and GDR pathways may provide new insights into the pathogenesis of cancers, which could give rise to more effective anti-cancer therapies.

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A role for naturally occurring alleles of endoplasmic reticulum aminopeptidases in tumor immunity and cancer pre-disposition

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Endoplasmic reticulum aminopeptidase 1 and 2 (ERAP1 and ERAP2) are key components on the pathway that generates antigenic epitopes for presentation to cytotoxic T-lymphocytes (CTLs). Coding single nucleotide polymorphisms (SNPs) in these enzymes have been associated with pre-disposition to several major human diseases including inflammatory diseases with autoimmune etiology, viral infections, and virally induced cancer. The function of these enzymes has been demonstrated to affect CTL and natural killer cell responses toward healthy and malignant cells as well as the production of inflammatory cytokines. Recent studies have demonstrated that SNPs in ERAP1 and ERAP2 can affect their ability to generate or destroy antigenic epitopes and define the immunopeptidome. In this review, we examine the potential role of these enzymes and their polymorphic states on the generation of cytotoxic responses toward malignantly transformed cells. Given the current state-of-the-art, it is possible that polymorphic variation in these enzymes may contribute to the individual's pre-disposition to cancer through altered generation or destruction of tumor antigens that can facilitate tumor immune evasion.

Keywords: aminopeptidase, antigen presentation, cancer, SNP, cytotoxic responses, adaptive immunity, innate immunity, polymorphism

PART I: ERAP1/2 AND CANCER

MECHANISMS USED BY THE IMMUNE SYSTEM TO FIGHT CANCER

The immune system plays a dual role in cancer, on one hand suppressing tumor growth by eliminating cancer cells or inhibiting their outgrowth, and on the other hand promoting tumor progression by selecting tumor cells able to survive inside an immunocompetent host (1–7). The dynamic process that integrates these opposing functions of the immune system – host protection and tumor progression – is termed "cancer immunoediting" and consists of three phases: (i) elimination, (ii) equilibrium, and (iii) escape.

In the elimination phase, cells of innate and adaptive immunity work together to identify and destroy nascent cancer cells, before they become clinically apparent tumors. If a cancerous cell variant is not eliminated in this phase, it may enter the equilibrium phase and remain in a state of functional dormancy under control of cells and mediators of the adaptive immunity. In this phase, the immune system controls tumor growth but does not eliminate it. Cancer cells that acquire mutations can become: (i) invisible to adaptive immunity, i.e., antigen-loss variants or tumor cells that develop defects in antigen-processing or presentation, (ii) insensitive to immune effector mechanisms, or (iii) induce an immunosuppressive state within the tumor microenvironment, enter the escape phase and grow progressively eventually becoming visible tumors. The achievement of the last phase is indicative of a failure in the adaptive immune cells to provide protection from tumor development due to selection of poorly immunogenic tumor cell variants during the equilibrium phase.

Many different cells belonging to the innate and adaptive immunity play an active role in cancer control, from the earliest stages of transformation to the terminal phase of widespread metastasis. The immune cells most involved in the host protection from development of cancer are natural killer (NK) cells and cytotoxic T-lymphocytes (CTLs).

NK cells provide the first immune defense against infections and tumor transformation through recognition and killing of aberrant cells. Their function is finely tuned by the interaction of activating and inhibitory receptors with their specific ligands expressed on target cells (8). Activating receptors recognize ligands expressed on stressed, infected, or transformed cells, whereas inhibitory receptors, prevent NK cell activation upon interaction with major histocompatibility complex (MHC) class I molecules expressed on target cells (9–14). The reduced surface expression of MHC class I molecules and acquisition of activating ligands in virally infected and transformed cells make these cells particularly vulnerable to NK cell-mediated killing.

CTLs constantly monitor peptide-MHC (pMHC) class I complexes on the cell surface and eliminate virally infected or transformed cells expressing novel peptides derived from abnormal gene products. The generation of these peptides is central in the regulation of CTL and NK cell responses against altered cells. Aberrant antigenic peptide generation can lead to either immune

evasion or to immune responses against normal cells, initiating or sustaining autoimmune reactions. Antigenic peptides are generated by the concerted action of multiple components of a biochemical pathway termed MHC class I antigen-processing and presentation.

PATHWAY OF ANTIGEN-PROCESSING AND PRESENTATION

Antigen generation generally starts with the ubiquitin–proteasome pathway where proteins are tagged for proteolytic destruction by the proteasome although other cytosolic peptidases often play important roles (15). The immune cell variant of the proteasome, called immunoproteasome, is more efficient in generating longer peptides, which are often N-terminally extended, compared to the final antigenic epitopes (16). A fraction of these precursors are transported by a specialized transporter [Transporter Associated with antigen-Processing (TAP)] into the endoplasmic reticulum (ER) for further processing. Inside the ER, the precursors are further trimmed by ER-resident aminopeptidases, ER aminopeptidase 1 (ERAP1) and ER aminopeptidase 2 (ERAP2), which excise the N-terminal extensions generating mature antigenic peptides (17, 18). These peptides then bind onto nascent MHC class I molecules with the help of a multi-protein complex termed the peptide loading complex. The pMHC class I complexes are then translocated to the cell surface where they can interact with specialized immune system (19).

ROLE OF ERAP1 AND ERAP2 IN THE GENERATION OF ANTIGENIC EPITOPES

The key role of ERAP1 and ERAP2 in the generation of antigenic epitopes has been repeatedly demonstrated in several cell lines and mouse models. Reduction of ERAP1 expression by RNA interference results in drastically defective presentation for some antigenic epitopes (17, 18, 20, 21) and enhanced presentation or no effect for others. Based on these studies, it has become apparent that ERAP1 has a complex, multifaceted role on the generation of the immunopeptidome (22–24).

In mice models, the complete loss of ERAAP expression (the mouse homolog of ERAP1) specifically inhibited surface expression of MHC class I, but did not affect the expression of MHC class II molecules (17). The expression of pMHC class I on the cell surface depends on the quantity as well as the quality of the peptide supply. In ERAAP-deficient cells, the reduction of MHC class I molecules was due to faster dissociation of pMHC class I from the cell surface rather than to a slower rate of pMHC class I assemble in ER. As a result, ERAAP is important for generating optimal peptides that yield stable pMHC class I complexes (25). Moreover, Hammer et al. observed that the absence of ERAAP disrupts the pMHC class I repertoire in professional and non-professional antigen-presenting cells. From *in vivo* studies emerged that ERAP1 plays an important role in immune response to viruses, either enhancing or reducing CTL responses to particular viral epitopes and, thereby, helping establish immunodominance hierarchies (25). Undauntedly, expression of endogenous pMHC class I is essential for the generation and maintenance of the normal $CD8^+$ T cell responses. Splenocytes from ERAAP-deficient mice display an alternative repertoire of peptides as well as differences in the stability of pMHC class I molecules characterized from a diminished ability to elicit HY-specific $CD8^+$ T cell responses. Interestingly, immunization of ERAAP-deficient mice with splenocytes from wild-type mice resulted in potent $CD8^+$ T cell responses, suggesting that ERAP1 plays an important role in modifying antigenic peptides and, paradoxically, its absence enhances immunogenicity (25, 26).

ERAP2, the second aminopeptidase demonstrated to be involved in antigen trimming in the ER, is highly homologous to ERAP1 but has distinct specificity (27–29). ERAP1 and ERAP2 have been suggested to perform antigenic peptide trimming in a coordinated manner by forming a functional heterodimer (18, 30). Saveanu et al. performed RNA interference to examine the roles of ERAP1 and ERAP2 in trimming of various precursors of the model HIV env epitope, using two different cell lines. The effect of ERAP2 knockdown on cell-surface MHC class I expression and epitope presentation was similar to that of ERAP1 knockdown, suggesting equivalent functions of the two enzymes in the cells studied. Also the greater effect of the double knockdown in some cases suggests that each enzyme can function independently, so that their effects are additive (18).

Overall, the exact effect of the ERAP1 and ERAP2 activities on antigen presentation can be highly variable and difficult to predict. Any factor that can influence the generation of the immunopeptidome may contribute to this, including the cell line used, the MHC class I alleles, whether the cell contains immunoproteasomes or constitutive proteasomes, the activities of cytosolic aminopeptidases and the sequence of the epitope studied. Regardless, ERAP1 and ERAP2 are undoubtedly important factors that influence the generation of the immunopeptidome, with ERAP1 having a dominant role (18, 22, 25).

ERAP1 IN INNATE IMMUNITY

ERAP1 has been found to play important roles in innate immune responses. Namely, ERAP1 has been involved in the shedding of cytokine receptors including the type I TNF receptor (TNFR1), type I IL-6 receptor (IL-6Ra), and type II IL-II decoy receptor (31–33). Additionally, macrophages were found to produce a secreted form of ERAP1 in response to interferon-γ and liposaccharides through a TLR-mediated mechanism that leads to enhanced phagocytosis (34, 35). Similarly, human PBMCs exposed to ERAP1 externally are activated and show enhanced production of cytokines and chemokines, through mechanisms involving the NLRP3 inflammasome (Aldhamen et al. *J Innate Immunity*, in press). This secreted form of ERAP1 was found to be enzymatically identical to the ER-retained form and only differ in glycosylation patterns consistent with maturation through the secretory pathway. Since ERAP1 does not contain an ER retention signal it has been hypothesized that it is normally retained inside the ER through interactions with specific ER-resident proteins and can be secreted when these interactions are saturated or disrupted (34).

The function of ERAP1 in regulating antigen presentation can also lead to altered NK cell immune responses. ERAP1 knock-out mice exhibit exaggerated innate immune responses early during pathogen recognition and show increased activation of NK and NKT cells and production of inflammatory cytokines (36). ERAP1 silencing in T cell lymphoma RMA results in tumor rejection in syngeneic mice by triggering NK cells and subsequently T cell

($CD4^+$ and $CD8^+$) anti-tumor responses. This rejection does not depend on a simple quantitative reduction in surface MHC class I expression, but is rather the result of changes in the MHC class I-peptide repertoire, because replacement of endogenous peptides with high-affinity mature antigenic peptides was sufficient to rescue the inhibitory activity of NK cells (37). Furthermore, ERAP1 knock-out mice show high frequencies of terminally matured as well as licensed NK cells expressing Ly49C and Ly49I receptors consistent with enhanced NK activation by pro-inflammatory stimuli in those mice (36). Together, these findings suggest an important role for ERAP1 in modulating innate immune responses during the earliest stages of pathogen recognition, a role that may be of particular importance to immune responses toward malignantly transformed cells.

ALTERED LEVELS OF ERAP1 AND ERAP2 CAN FACILITATE TUMOR IMMUNE EVASION

The enzymatic activity and expression levels of the mouse homolog of ERAP1, ERAAP, have been demonstrated to be key for the immune evasion of tumor cells in two distinct murine models. In one study, the authors showed that ERAP1 down-regulation was sufficient to stimulate the cytotoxic activity of NK cells and to result in tumor growth arrest (37). In another study, down-regulation of ERAP1 elicited specific CTL responses against a cryptic tumor-associated antigen that was normally destroyed by ERAP1, resulting in tumor growth arrest and enhanced survival (38). These two studies clearly demonstrated that ERAP1 expression can be critical for immune evasion of solid tumors. Moreover, by using cell lines with two different levels of ERAAP expression levels James et al. established that the induction of anti-tumor immune responses can be titrated based on ERAAP activity, laying the groundwork for the hypothesis described here (38).

In humans, defective expression of components of the antigen-processing machinery has been associated with the progression and clinical outcome in several types of cancer. The availability of specific ERAP1 and ERAP2 antibodies has allowed researchers to investigate the expression and tissue distribution of these enzymes in a large number of tumor cells of various origins.

In one study, expression of ERAP1 and ERAP2 was detected in all tumor cell lines examined, including melanoma and various type of carcinomas, although at highly variable levels and independently of each other (39). The amount of ERAP1 appears to be more closely coordinated with cell-surface HLA class I molecules, suggesting a secondary involvement of ERAP2 in the generation of ligands for HLA class I molecules at least on quantitative levels. However, this study did not consider that ERAP2 is missing in 25% of the population (40). Notably, upregulation of ERAP1 and ERAP2 in ERAP-low tumor cells was found to enhance HLA class I surface expression, suggesting that abnormal HLA class I levels in tumor cells may result from defective expression of these enzymes (39).

In another study, a heterogeneous expression of ERAP1 and ERAP2 was detected in a panel of 28 melanoma cell lines (41). A concordant expression between mRNA and proteins for these genes was detected in many cell lines, except four in which an aberrant ERAP2 transcript resulted in total absence of ERAP2 protein expression.

Expression of ERAP1 and ERAP2 was subsequently investigated in 39 different cell types derived from 24 normal non-lymphoid tissues and their malignant counterparts (42). In normal tissues, expression of ERAP1 and ERAP2 was limited to epithelial components. The two enzymes were co-expressed, singly expressed or not expressed, depending on the cell type. HLA class I expression appeared to be independent of ERAP1 and ERAP2 expression and only in nine cell types it was coordinated with both enzymes. In tumor samples, the expression of either or both enzymes was retained, lost, or acquired as compared to the normal counterparts, depending on the tumor histotype. Loss of at least one enzyme was the most frequent phenotype accounting for 86% tumors. Remarkably, in four types of carcinomas (breast, ovary, liver, and lung carcinomas) arising from normal counterparts co-expressing ERAP1 and ERAP2, none of the 26 tested samples retained this phenotype. ERAP1 was lost in all tested breast, ovary, and lung carcinomas and in 6 out of 7 liver carcinoma samples tested, whereas ERAP2 was retained only in 9 out of 26 samples. The double-negative phenotype was significantly associated with lack of detectable HLA class I molecules. Thus, *in vivo* transformation affects the expression of ERAP1 and ERAP2, together and individually, leading to losses, gains, or imbalances.

In another study, expression of ERAP1 and ERAP2 was investigated in 300 normal kidney tissues and 334 renal cell carcinoma lesions (43). A heterogeneous and discordant expression of the two enzymes was detected in the different regions of the normal kidney epithelium. In renal cell carcinomas, ERAP1 and ERAP2 appear to have a different behavior, being the first more frequently up-regulated and the latter more frequently down-regulated, as compared to the normal counterpart. None of the clinical parameters investigated was found to be associated with ERAP1 and ERAP2 expression.

ERAP1 expression was also tested in 101 cervical carcinoma patients including adenocarcinomas and squamous cell carcinomas, and correlated with clinical outcome (44). ERAP1 expression was observed in most cases (85 out of 101) and overall never totally lost. Partial ERAP1 loss was significantly associated with reduced overall survival and disease free survival. In multivariate analysis, ERAP1 down-regulation was demonstrated to be an independent predictor for worse overall survival and disease free survival, and significantly associated with lymph node metastases.

In another study, ERAP1 expression was examined in 50 esophageal carcinoma lesions and compared with clinicopathological parameters (45). In these tissues, ERAP1 expression was lost or down-regulated in 20 and 28% of cases, respectively, and significantly associated with the depth of tumor invasion. The authors showed that ERAP1 expression was partially or totally lost in cervical intraepithelial neoplasia and cervical squamous cell carcinoma as compared to normal epithelium of uterine cervix (46), but association with clinical outcome was not investigated.

Taken together, these studies suggest that normal function of ERAP1 and ERAP2 is required for NK cell- and T cell-mediated anti-tumor immunity. As a result, the deregulated expression of these enzymes found in tumors may cause improper

antigen-processing and contribute to escape for host immune surveillance.

PART II: ERAP1/2 SNPs

ERAP1/2 SNPs AND ASSOCIATION TO HUMAN DISEASE

Both ERAP1 and ERAP2 are naturally polymorphic and more than a dozen coding single nucleotide polymorphisms (SNPs) in their genes have been associated with pre-disposition to a variety of human diseases primarily of autoimmune etiology (**Figure 1**; **Table 1**). Such links are more evident for diseases that are strongly associated with particular HLA class I alleles, implying that the role of ERAP1 and ERAP2 in disease pathogenesis is through the pathway of antigen-processing and presentation. The most prominent example is the association of ERAP1 with Ankylosing Spondylitis (AS), a chronic inflammatory rheumatopathy of the lower spine with an autoimmune etiology. GWAS studies have consistently shown that various ERAP1 coding SNPs are associated with pre-disposition to AS (47–49). The variants that appear to confer risk susceptibility are: rs17482078-C (R725Q), rs30187-T (K528R), rs2287987-T (M349V), rs26653-C (R127P), rs10050860-C (D575N), and rs27044-G (Q730E), with each individual one contributing odd ratios of about 1.3–1.4 (47, 48, 50). Due to the strong linkage disequilibrium in the ERAP1/2 locus, specific haplotypes have been associated with either pre-disposition or protection against AS. A pre-disposing haplotype has been proposed to comprise of 730E, 575D, and 528K and a protective one of 528R, 276I, and 127P (51). Another pre-disposing haplotype also carries an ERAP2 SNP (ERAP1 730Q, ERAP1 528K, and ERAP2 392N) (52). Evans et al. showed that ERAP1 contribution to AS could be primarily attributed to rs30187-T (528K), with secondary effects found for rs10050860-C (575D) and rs17482078-C (725R) (48).

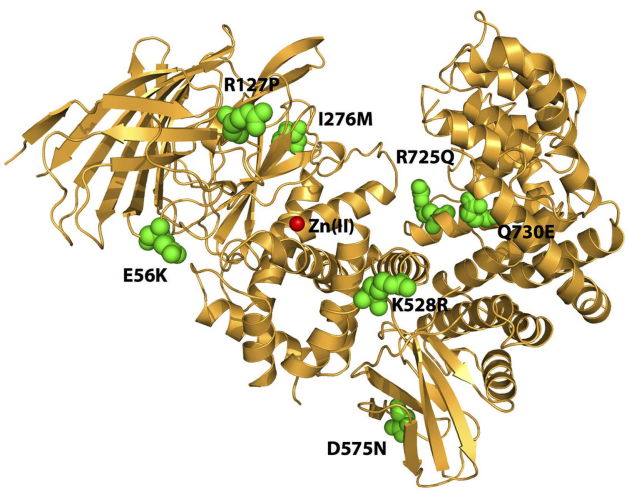

FIGURE 1 | Schematic representation of the crystal structure of ERAP1 (PDB code: 3MDJ). The amino acids at the sites of polymorphic variation that have been found to either affect enzyme function or to associate with pre-disposition to cancer are indicated by green spheres. The catalytic Zn(II) atom at active site of the enzyme is shown by a red sphere. Note that the polymorphic sites are distal to the active site and distributed throughout the enzyme.

Most notably ERAP1 is associated with AS only in the presence of at least one HLA-B27 allele, which is also the greatest known AS risk factor (48, 49) suggesting that the pathogenic effect is mediated through aberrant generation of HLA-B27 ligands either in the context of arthritogenic self-peptides or the generation of pro-inflammatory non-canonical HLA-B27 structures.

The association of ERAP1 with diseases in the context of specific HLA class I alleles is also observed in the cases of Behçet disease (BD) and Psoriasis. A GWAS study in a Turkish population, where BD is frequently encountered, identified rs10050860-T (575N) and rs17482078-T (725Q) as increased risk variants, with odd ratios significantly higher for HLA-B51 positive individuals (53). Similarly to HLA-B27 and its association to AS, HLA-B51 is the greatest known risk factor for BD. A recent study showed that six positions within the peptide-binding cavity of MHC-I molecules are primarily responsible for the association of particular HLA alleles (including HLA-B51) with BD, implying that aberrant peptide binding by HLA-B51 is central to the pathogenesis of BD (54). Another GWAS study using a population of European ancestry demonstrated that rs30187-T (528K) increases risk for psoriasis, but only in the presence of at least one copy of the allele HLA-Cw*0602 (55). In addition to these well-established cases, ERAP1 SNPs, with rs30187 as a prominent example, have also been associated with other diseases. Specifically, the polymorphism rs30187-C (528R) has been associated with essential hypertension (56) and the rs30187-T (528K) variant with Multiple Sclerosis (57).

ERAP2 has been associated with AS independently of ERAP1. Two functional SNPs have been described: coding rs2549782 (K392N) and rs2248374, a SNP that greatly influences ERAP2 expression (49). Furthermore, another ERAP2 SNP (rs2910686) was associated with AS in HLA-B27-negative cases (49). ERAP1 and ERAP2 SNPs can form haplotypes that may be more relevant to disease pathogenesis (52). The ERAP2 coding SNP rs2549782 (K392N) is also linked with preeclampsia and with resistance to HIV-1 infection (58–61). The minor allele G is significantly associated with preeclampsia in African-American and Australian populations, but not in a Chilean population (58, 60, 61). A case-control study in a cohort of Italian HIV-exposed seronegative individuals showed that the homozygote GG genotype (encoding Lys/Lys) is overrepresented in the HIV-exposed seronegative sample (59). Recently, Kuiper et al. identified variants near the ERAP2 locus that are associated with birdshot chorioretinopathy (BCR) (62). This GWAS study identified rs7705093-T (OR: 2.3), to be in perfect linkage disequilibrium with rs10044354, a variant that affects ERAP2 protein expression levels, with individuals homozygous for the C allele showing almost none ERAP2 expression. BCR is a useful prototype of disease-HLA association since it exhibits the strongest documented HLA class I association for a human disease (>95% of cases carry the HLA-A29 allele) implicating antigenic peptide processing and presentation in the pathogenesis of this disease.

Overall, a very strong genetic link between ERAP1/2 SNPs and HLA-associated autoimmunity has been established and has contributed to our understanding of the pathogenesis of these diseases. The genetic variability of ERAP1 and ERAP2 appears to constitute part of the natural variability of immune responses

Table 1 | Most common ERAP1 and ERAP2 SNPs, relation to disease, HLA, and functional consequences.

	ERAP1/2 SNP	Disease association	HLA class I link	Affects
ERAP1	rs3734016 (E56K)	HPV-induced cervical carcinoma		Expression levels
	rs26653 (R127P)	Ankylosing spondylitis, HPV-induced cervical carcinoma		Expression levels
	rs2287987 (M349V)	Ankylosing spondylitis		
	rs30187 (K528R)	Ankylosing spondylitis, psoriasis, essential hypertension, multiple sclerosis	B*27 Cw*0602	Activity and specificity
	rs10050860 (D575N)	Ankylosing spondylitis, Behçet disease		Activity
	rs17482078 (R725Q)	Ankylosing Spondylitis, Behçet disease	B*51	Activity
	rs27044 (Q730E)	Ankylosing spondylitis, HPV-induced cervical carcinoma		Activity and specificity
ERAP2	rs2549782 (K392N)	Ankylosing spondylitis, preeclampsia, resistance to HIV infection		Activity and specificity
	rs2248374 (non-coding)	Ankylosing spondylitis		Expression levels

and may therefore contribute to pre-disposition to any number of diseases actively fought by the adaptive immune response.

POLYMORPHIC STATE OF ERAP1/2 AFFECTS ENZYME FUNCTION AND ANTIGEN GENERATION

The multitude of genetic and population studies linking ERAP1 and ERAP2 SNPs to pre-disposition to autoimmunity and viral infections prompted several research groups to examine the effects of the identified polymorphic variations on the enzyme's biological function and molecular mechanism. To date, most studies have focused on the most well disease-associated SNPs, namely K528R, D575N, R725Q, and Q730E.

The SNP at position 528 has been repeatedly demonstrated to influence ERAP1 enzymatic activity. Various *in vitro* studies have shown that the 528R variant exhibits less enzymatic activity, compared to the 528K (48, 63–66). Cell-based experiments have also demonstrated the importance of that position: HeLa cells transfected with ERAP1 528R and a HLA-B27 peptide precursor displayed a reduced amount of HLA-B27 molecules on the cell surface compared to ERAP1 528K transfectants (64). Furthermore, the presentation of specific MHC class I epitopes was negatively influenced in cells transfected with the 528R variant and their N-extended precursors, as shown by CTL activation assays (67, 68).

Studies regarding polymorphic positions Q730E, D575N, and R725Q have been more complicated to interpret. *In vitro* assays have suggested that position 730 may or may not have an influence in the enzymatic activity, depending on the substrate used (63, 64). Regardless, a cell-surface HLA class I expression assay showed that this SNP could influence the generation of a specific HLA-B27-restricted epitope (64). Some studies showed that position 575 does not influence ERAP1 activity *in vitro* (48, 63), while another study showed that the 575N variant exhibits greater activity compared to 575D (66). Regarding position 725, Evans et al., using recombinant enzymes, showed that 725Q negatively influences enzymatic activity toward a model fluorigenic peptide substrate (48), but an *in vitro* CTL activation assay did not find any influence of that position to the presentation of an HLA-B27 epitope (68). The complex landscape of functional effects found for these SNPs may be attributed to differences in substrates or assays used, or to differences in background SNPs that are not always consistent between studies. Indeed, in several studies the effects of ERAP1 SNPs have been found to be strongly epitope dependent and to include effects on other mechanistic aspects of ERAP1 peptide trimming, such as substrate inhibition and product activation phenomena (64, 66, 69).

SNPs in ERAP1 and ERAP2 are often co-inherited as complex haplotypes and may have strong synergism with each other. Unfortunately, most functional studies until now have focused on analyzing the effects of single SNPs and as a result the functional effects of particular disease-associated ERAP1/ERAP2 haplotypes are not always clear especially since the synergisms between individual SNPs are not known. Recently, Seregin et al. analyzed two ERAP1 haplotypes of five SNPs for their effects on HLA-B27 restricted presentation in relation to the pathogenesis of AS and concluded that the high-risk haplotype resulted in reduction in presentation of multiple antigens (70). In another study, Reeves et al. identified nine separate naturally occurring haplotypes in a small population sample based on the five most disease-related SNPs (positions: M349V, K528R, D575N, R725Q, and Q730E) (67). They evaluated the trimming activity of these alleles using a CTL activation assay and showed that each allele exhibits a different activity that is not only dependent on the allele itself but also on the N-terminal extension of the peptide. This study was recently extended to AS patients, showing that ERAP1 haplotypes were clearly stratified in individuals with AS compared to healthy controls and that these functional alleles were poor in generating optimal peptide ligands for HLA-B*2705 (71). A study focusing on the combined effects of positions 528 and 575 showed that the latter position was dominant in determining enzyme activity (66). Recently, Chen et al. demonstrated that the polymorphic variation in position 730 is critical for rescuing the reduced CTL activation found in the presence of the 528R SNP (68). Overall, ample evidence for strong synergism amongst ERAP1 SNPs are available, although we have very little insight on how these SNPs that are scattered all throughout

the ERAP1 structure can cooperate to affect the activity of the enzyme.

The influence of ERAP1 polymorphic context has also been studied in the context of the HLA-B27 restricted immunopeptidome and the pathogenesis of AS. Garcia-Medel et al. showed that the presence of ERAP1 with all the AS-pre-disposing polymorphisms (349M, 528K, 575D, 725R, and 730Q) ensured efficient peptide trimming and a higher HLA-B27 stability, compared to the ERAP1 with the AS-protective SNPs (349V, 528R, 575N, 725Q, and 730E) (72). Extending this work, Martin-Esteban et al. showed that synergism between SNPs at positions 528 and 575 can have important effects on cell-surface HLA-B27 presented ligands and more recently Garcia-Medel et al. showed that the particular B27 subtype is critical in determining these effects (66, 73). It appears that the interplay between ERAP1 SNPs and HLA class I subtypes is the key to determining the cell-surface immunopeptidome and concomitant cytotoxic responses.

Two ERAP2 SNPs have been shown to have specific effects on the enzyme's biological function: the coding polymorphism rs2549782 (N392K) and the non-coding rs2248374. ERAP2 polymorphism N392K alters the activity and specificity of ERAP2 in a manner much more pronounced compared to the effects described for all coding SNPs of ERAP1 (74). Specifically, *in vitro* cell-based analysis showed that the 392N variant is much more effective in trimming hydrophobic N-terminal residues from antigenic peptide precursors compared to 392K. In contrast, the rs2248374-G allele can determine ERAP2 expression levels by inducing mRNA instability and non-sense mediated decay (40). The biological consequence of that frequently encountered allele (about 0.5 frequency in six different populations studied) has also been demonstrated; rs2248374-G homozygotes produce no detectable ERAP2 and have reduced levels of MHC class I expression on B-cell surfaces.

Similarly to ERAP1, these ERAP2 SNPs often organize to distinct haplotypes. SNP rs2248374 is in linkage disequilibrium with rs2549782 in various populations studied, thus effectively allowing only the expression of 392K variant (40). A noteworthy exception is the Chilean population where the two SNPs are not in linkage disequilibrium, allowing the expression of 392N variant (61). Nevertheless, no Chilean genotype was found homozygous for the 392N allele, implying a possible negative selection for individuals that are homozygous for that allele. Interestingly, the recent association of ERAP2 expression with pre-disposition to the inflammatory autoimmune disease BCR solidified the idea that ERAP2 polymorphic variation and its effects on antigen-processing may have important repercussions on adaptive immune responses (62).

ERAP1 SNPs AND PRE-DISPOSITION TO VIRALLY INDUCED CANCER

Genetic variations in genes encoding components of the antigen-processing pathway have been associated with risk of occurrence malignancy and in particular with cervical carcinoma. Mehta and colleagues analyzed the effect of different ERAP1 SNPs and haplotype combinations on the risk of developing human papillomavirus (HPV)-induced cervical carcinoma.

In the first study, the authors identified two common ERAP1 polymorphisms, R127P and Q730E, significantly associated with increased risk of cervical cancer (75). A haplotype combination consisting of four SNPs, including the minor alleles at R127P and Q730E loci and a major allele at the TAP1 R651C and LMP7 Q145K loci, was significantly associated with a threefold increased cervical carcinoma risk (75). The authors estimated that 12% of all cervical carcinoma cases were attributable to the occurrence of this haplotype combination (75).

In a subsequent study, the same group investigated which genetic ERAP1 variation affected tumor progression and overall survival in cervical carcinoma patients, and provided the first indication of association of ERAP1 SNPs with ERAP1 protein expression (76). Genotype distributions at the R127P, I276M and K528R were significantly associated with the presence of lymph node metastases. Heterozygosity of E56K and minor allele homozygosity at the R127P loci were significantly associated with decreased overall survival. Multivariate analysis performed on E56K and R127P genotypes combined with prognostic factors, revealed that the two SNPs loci were not independent predictors of survival. The R127P variant and the E56K–R127P haplotype were significantly associated with ERAP1 protein expression, with heterozygosity of both individual SNPs and the haplotype consisting of a major allele at the E56K locus and a minor allele at the R127P, being significantly associated with normal ERAP1 expression and better overall survival. Heterozygosity for E56K–R127P haplotype was found to be an independent predictor for overall survival and lymph node metastasis.

The association between ERAP1 SNPs and cervical carcinoma risk or patient survival may be explained by different mechanisms, all related to altered ERAP1 function. Genetic variations at these individual SNPs have been found to affect the expression and stability of transcripts and proteins, to reduce trimming activity or modify substrate specificity (48, 64, 76). All these effects may affect the ability of HPV to establish persistent infections, but also the ability of transformed cells to evade immune surveillance. The information gathered by these studies has helped to establish a new paradigm on how the polymorphic variation of the adaptive immune system can play a role on both cancer development and prognosis. Although the importance of viral control in the pre-disposition to virally induced cancer is easy to understand, this work has opened the possibility that variable immune responses in the population may also play important roles to cancer pre-disposition.

ERAP1 AND ERAP2 SNPs AND PRE-DISPOSITION TO CANCER – A HYPOTHESIS

Current knowledge on the roles of ERAP1 and ERAP2 in the human immune response have gradually led to the maturation of the hypothesis that the naturally occurring polymorphic variation in ERAP1 and ERAP2 may play significant roles in the pre-disposition to certain cancers, as well as their progression and prognosis. More specifically, the rational behind this hypothesis is based on the following points:

1. CTL and NK cell responses against malignantly transformed cells are important for eradicating tumor cells often at the early stages of carcinogenesis.
2. Establishment of solid tumors often requires adaptive measures from the transformed cells that lead to the evasion of

immune responses. Such adaptive measures include changes in antigen-processing and presentation in order to suppress the presentation of tumor-specific antigenic epitopes while also evading NK-cell recognition.

3. Polymorphic variation in ERAP1 and ERAP2, alters their function and their ability to generate antigenic peptides and controls cytotoxic reactions against antigen-presenting cells. This is now well established in autoimmunity and viral infections.
4. ERAP1 and ERAP2 expression levels, which undoubtedly affect effective enzyme activity, are consistently altered in tumors, presumably as an adaptive measure for immune evasion.
5. ERAP1 function can regulate innate immune responses and the production of inflammatory cytokines, a function that can either help eradicate cancer cells or contribute to localize inflammation that can promote tumor growth.

As a result, the polymorphic variability in ERAP1 and ERAP2 may affect the severity of early cytotoxic responses toward transformed cells and influence their chances to accrue genetic adaptations that will allow them to evade the immune system (**Figure 2**). Individuals carrying particular ERAP haplotypes in combination with specific HLA class I alleles may therefore be more prone to carcinogenesis, not because of facilitated malignant transformation but because they have an immune system that is less effective in mounting strong cytotoxic responses against cancer cells. Additionally, the function of ERAP1 in regulating inflammatory cytokine production may contribute to these effects by influencing the inflammatory state of the tumor microenvironment.

CONTROLLING ERAP1 AND ERAP2 ACTIVITY FOR TREATING CANCER

In addition to the possible role of the activity of ERAP1 and ERAP2 on the normal immune control of cancer, a series of recent studies have highlighted that these two enzymes may be important pharmacological targets for boosting immune responses to established cancers. In one study, the genetic down-regulation of ERAP1 in cancer cells that establish solid tumors in mice resulted potent NK cell-mediated cytotoxic responses (37). In another study, the genetic down-regulation of ERAP1 in cancer cells resulted in strong CTL responses against a cryptic tumor antigen normally destroyed by over-active ERAP1 (38). In both studies, tumor

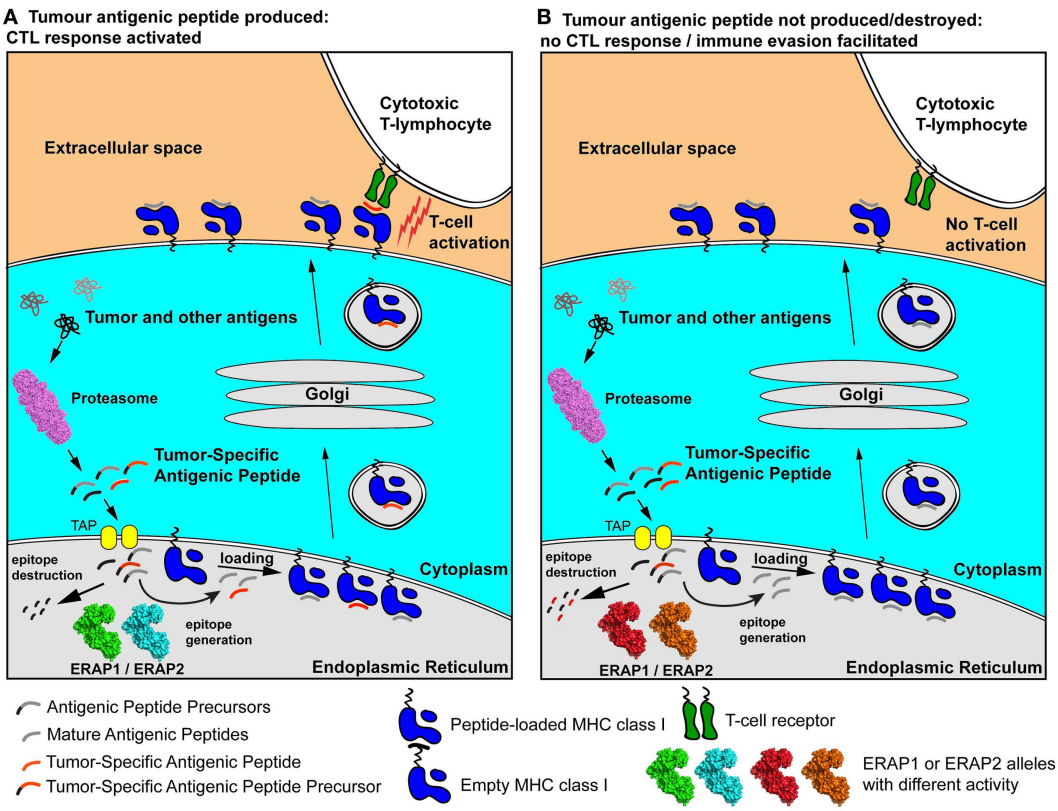

FIGURE 2 | Schematic representation of two extreme scenarios leading to either effective cytotoxic responses against a cancer cell (A) or immune evasion (B). A tumor-specific antigenic epitope or its N-terminal extended precursor is generated by the proteasome and transported into the ER. There, it is further processed by ERAP1/ERAP2 alleles. In case A, the "green" and "cyan" allele effectively generate the mature epitope (red line), which is then loaded onto nascent empty MHC class I and presented to the cell-surface, activating antigen-specific T cells. In case B, hyper-active or hypo-active ERAP1/ERAP2 alleles (in red and orange) either fail to produce the mature epitope or destroy it by generating peptides too small to bind onto MHC class I molecules. As a result, the epitope is not presented on the cell surface and no T cell activation occurs, facilitating immune evasion. The allelic state of ERAP1 and ERAP2 can therefore influence anti-tumor adaptive immune responses pre-disposing individuals to certain cancers by facilitating immune evasion at early stages of malignant development.

growth was halted resulting in clear therapeutic outcome in mice. In the latter study, the effect was partially reproduced using the small molecular weight metallopeptidase inhibitor Leucinethiol (38). A recently developed potent ERAP1/ERAP2 inhibitor was successfully used to induce CTL responses against the cancer cells used in that study (77). Apart from the obvious extension of these studies conclusions to cancer immunotherapy, one additional conclusion may be drawn: inhibition of ERAP1 activity was not necessarily complete in either study, indicating that even moderate modulation of ERAP1 activity, such as seen in the normal polymorphic variation in this enzyme, may be sufficient to radically affect the potential of cancer immune surveillance.

CONCLUDING REMARKS – FUTURE RESEARCH DIRECTIONS

In summary, we review the state-of-the-art on the role of ERAP1 and ERAP2 in adaptive and innate immune responses and their role on disease pathogenesis. Combination of knowledge on the role of polymorphic variation on those enzymes and disease pre-disposition with their role in cancer development has led to the formulation of a hypothesis on their direct role on cancer pre-disposition and prognosis. If this hypothesis holds to be true, haplotype analysis of components of the antigen-processing machinery may contribute to our understanding of cancer predisposition and complement other genetic findings on biochemical pathways that control malignant transformation. Because of the genetic heterogeneity of tumors however, it may be difficult to discern such effects at the tumor level and population studies may have to focus on the overall influence of this genetic locus on predisposition to specific cancers. Ongoing and future GWAS studies on cancer patients may yet reveal the importance of immune system variation to developing and fighting cancer, contributing to personalized treatments.

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Endoplasmic reticulum chaperones and oxidoreductases: critical regulators of tumor cell survival and immunorecognition

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Endoplasmic reticulum (ER) chaperones and oxidoreductases are abundant enzymes that mediate the production of fully folded secretory and transmembrane proteins. Resisting the Golgi and plasma membrane-directed "bulk flow," ER chaperones and oxidoreductases enter retrograde trafficking whenever they are pulled outside of the ER by their substrates. Solid tumors are characterized by the increased production of reactive oxygen species (ROS), combined with reduced blood flow that leads to low oxygen supply and ER stress. Under these conditions, hypoxia and the unfolded protein response upregulate their target genes. When this occurs, ER oxidoreductases and chaperones become important regulators of tumor growth. However, under these conditions, these proteins not only promote the folding of proteins, but also alter the properties of the plasma membrane and hence modulate tumor immune recognition. For instance, high levels of calreticulin serve as an "eat-me" signal on the surface of tumor cells. Conversely, both intracellular and surface BiP/GRP78 promotes tumor growth. Other ER folding assistants able to modulate the properties of tumor tissue include protein disulfide isomerase (PDI), Ero1α and GRP94. Understanding the roles and mechanisms of ER chaperones in regulating tumor cell functions and immunorecognition will lead to important insight for the development of novel cancer therapies.

Keywords: endoplasmic reticulum, localization, redox, Ca^{2+} signaling, cancer, immunity

INTRODUCTION

The endoplasmic reticulum (ER) is the location of oxidative protein folding, a mechanism that enzymatically manufactures fully folded secretory and transmembrane proteins. These two groups of proteins make up about 10 and 20% of a typical mammalian proteome, respectively (1). Ribosomally produced polypeptides for these two groups of proteins are first targeted to the ER membrane, where they interact with the Sec61 protein translocation channel (translocon) using their signal peptide (2). At this location, polypeptides undergo cytosolic folding that continues during translocation to the ER lumen (3, 4). Subsequently, the interaction with immunoglobulin binding protein (BiP/GRP78), a major ER-lumenal chaperone, initiates the production of secretory and transmembrane proteins (5, 6). If polypeptides are glycosylated they subsequently interact with the lectin chaperones calnexin and calreticulin (7), as well as oxidoreductases including protein disulfide isomerase (PDI) and related family members such as ERp57 (8). The oxidizing activity of these proteins is kept intact by oxygen- or hydrogen-peroxide consuming oxidoreductases such as Ero1α (9). Thus, ER chaperones and oxidoreductases cyclically interact with the ongoing flow of polypeptides emerging from the translocon. The flow of these proteins is massive. Using a vesicular stomatitis virus G protein (VSVG) fusion with green fluorescent protein (GFP), it has been estimated to amount to 7,000 molecules per second for this model protein alone (10, 11).

This observation raises the question as to how cells handle this quantity of export and how they ensure that exported proteins are segregated from resident ER chaperones and oxidoreductases. Early research using glycosylated short peptides had indicated that ER–Golgi trafficking occurs via non-specific "bulk flow" (12). However, this intuitive model may not be correct, since positive signals are not only needed for export from the ER (13, 14), but also for transit toward the Golgi complex (15). Moreover, secretory proteins are actually actively excluded from retrograde trafficking, which describes the trafficking route from the Golgi complex back to the ER (15). Conversely, most ER oxidoreductases and chaperones are equipped with a C-terminal KDEL motif that serves to interact with the KDEL receptor, a retrieval receptor that re-establishes ER localization for proteins with such a motif (16, 17). Therefore, given these efficient retention mechanisms, it makes little sense that tumor immunorecognition should be influenced by ER-restricted chaperones and oxidoreductases, when this mechanism is dictated by the properties of the cell surface. Nevertheless, in a cancer setting, proteins of this group can become localized to the plasma membrane or even secreted (18). When this occurs, ER oxidoreductases and chaperones become important regulators of tumor growth, but also of tumor immune recognition. For instance, the escape of calreticulin from the ER leads to the generation of an "eat-me" signal on the surface of tumor cells (19). Surface BiP/GRP78 is a target for antibody-based experimental

therapies as well (20, 21). Understanding how these proteins target to the plasma membrane could therefore lead to important insight for the development of immune-based cancer therapies.

ER RETRIEVAL OF CHAPERONES AND OXIDOREDUCTASES

To ensure their residence to the ER and their availability for further work on newly synthesized polypeptides, chaperones and oxidoreductases are continually recycled back to the ER (22). Lumenal ER chaperones and oxidoreductases use the lysine-based C-terminal KDEL sequence for this purpose to interact with the KDEL receptor, a sorting receptor that cycles between the Golgi complex and the ER, first discovered by the Pelham lab in 1990 (16, 17). The KDEL receptor is part of a group of transmembrane proteins that all retrieve luminal ER proteins (23). These transmembrane receptor proteins typically use cytosolic, C-terminal lysine-based motifs (KKXX) to travel from the Golgi complex to the ER on a retrograde trafficking route (24). One example is the KKFF motif in the case of the lectin ERGIC53 (25).

KKXX motifs retrieve ER transmembrane proteins by mediating interaction with the coatomer protein complex, also called COPI (26). This is a multi-subunit protein complex composed of seven distinct proteins termed coatomer whose subunits are termed α, β, β′, γ, δ, ε, and ζ COPs (27). The binding of dilysine-bearing cargo molecules on α and β′ subunits nucleates the formation of COPI coats (28, 29). This step also requires the activation of ADP-ribosylation factor 1 (Arf1) (30). Upon pinching off from within the Golgi complex or the ER–Golgi intermediate compartment (ERGIC), retrograde vesicles are uncoated, following GTP hydrolysis on Arf1 mediated by Arf GTPase activating proteins (ArfGAPs) 1, 2, and 3 (31). These vesicles then migrate into the proximity of the ER. Here, a Soluble NSF Attachment Protein Receptor (SNARE) complex becomes important for retrograde trafficking. This trans-SNARE complex forms via Dsl1p (yeast) or Zw10 (mammals) with incoming COPI-derived vesicles (32, 33). These vesicles then fuse with the ER membrane at a site termed ER import sites, whose existence so far has only been demonstrated in plants (34). Trafficking from the Golgi complex to the ER is also under the control of Ras-related GTPases, members of a large regulatory protein family that serve as address tags for intracellular trafficking (35). Rab6 and Rab2 likely work in sequence to facilitate retrograde transport mediated by coatomer and directed to the ER (36–38), whereas Rab18 might regulate coatomer-independent trafficking from the Golgi to the ER (39). Together, the retention of ER chaperones and oxidoreductases clearly requires a large set of proteins, whose identity and mechanisms are now fairly well understood, despite some important outstanding questions (40).

In addition to COPI-mediated retrieval, some ER chaperones and oxidoreductases are retained in this organelle by other retention mechanisms (41). One type of mechanism requires the interaction of ER-resident proteins with COPI adaptors or helper proteins, exemplified by the interaction of a calnexin cytosolic acidic cluster motif with the sorting adaptor PACS-2 that dictates the extent of calnexin ER retention (42). This is particularly important, as calnexin does not have a canonical KKXX motif, but rather a di-arginine-based C-terminal motif involved in its retention (43).

Another way how ER transmembrane proteins are excluded from ER export is by the length of their transmembrane domains. This is demonstrated with artificial 17 transmembrane residue constructs that are unable to enter ER exit sites (ERES), whereas 22 residue long transmembrane domains allow for inclusion into Golgi-destined vesicles (44). The length of these transmembrane domains might facilitate inclusion into specific ER membrane domains (44). Some ER proteins use their transmembrane domains to enter a retrieval cycle similar to KDEL-tagged ER lumenal proteins. This is the case with sarcoendoplasmic reticulum calcium transport ATPase (SERCA) (45). Some of these proteins use the retrieval receptor Rer1 for their localization to the ER, as is the case for rhodopsin or components of the γ-secretase complex (46–48).

Endoplasmic reticulum lumenal chaperones and oxidoreductases further depend on the nature of the ER environment to achieve their typical distributions (49). This phenomenon is best understood for the ER oxidoreductase Ero1α (50). This lumenal ER protein lacks a KDEL motif, but uses interactions with other ER oxidoreductases (PDI and ERp44) to stay within the ER, but only under oxidizing conditions (51, 52). A similar mechanism is used by peroxiredoxin 4 (53). Less is known about the ability of Ca^{2+} binding domains to assist to ER retention, as is known to occur in the case of the ER chaperone calreticulin (54). While the depletion of ER lumenal Ca^{2+} is a known inducer of ER stress, the disruption of calreticulin ER localization is uniquely dependent on Ca^{2+}. Calreticulin is not secreted upon induction of an ER stress with, for instance, tunicamycin (55). Potentially, this finding could indicate a requirement of Ca^{2+} binding to achieve a retrievable conformation of ER chaperones and oxidoreductases and specifically calreticulin. Such a hypothesis would be consistent with known alterations of protein conformation upon the loss of bound Ca^{2+} (56) and a general loss of chaperone–protein interactions within the ER upon the loss of free Ca^{2+} (57, 58). Either consequence could then lead to a loss of KDEL retrieval, either via masking of the KDEL sequence or via saturation of KDEL receptors (59). A similar Ca^{2+}-dependent mechanism appears to determine the retention of BiP/GRP78 in the ER (60, 61). Together, ER localization of chaperones and oxidoreductases is lost or reduced upon the interference with retrieval receptors, upon modulation of the oxidative conditions of the ER and upon loss of ER Ca^{2+}.

ER CHAPERONES AND OXIDOREDUCTASES ON THE PLASMA MEMBRANE OF TUMOR CELLS

At first glance, the ER retention of chaperones and oxidoreductases appears like an abstract problem of interest only to very specialized cell biologists. Although cell types such as thyrocytes and immature thymocytes retain ER chaperones and oxidoreductases less efficiently, it is not known what the exact biological significance of this finding is (62, 63). However, over the past few years, information has emerged that ER chaperone and oxidoreductase retention in the ER is a critical sentinel mechanism that signals ER stress to the immune system (64). This is not unexpected, since ER chaperones such as calreticulin are functionally linked to the immune system and mediate the folding of major histocompatibility complex (MHC) class I (64). Through this function, ER chaperones and oxidoreductases already exhibit

a tight link to the immune system via the regulation of intracellular peptide presentation by MHC class I on the plasma membrane (65). Accordingly, lost retention of ER chaperones and oxidoreductases upon ER stress impairs MHC class I expression on the surface (66). Surprisingly, however, this is not the only consequence. Calreticulin is normally enriched on the rough ER (rER) (67, 68). However, in cells undergoing ER stress, in particular following the depletion of ER Ca^{2+}, calreticulin, PDI, BiP/GRP78, and GRP94 escape ER retention and retrieval (69). These cell surface-exposed chaperones and oxidoreductases can present antigens to the immune system (calreticulin, GRP94), serve as anchors for leukocytes (PDI), but can also activate pro-survival signaling pathways (BiP/GRP78) (18). In addition, ER stress generated from lost ER localization of chaperones and oxidoreductases leads to mitochondrial dysfunction and also triggers the activation of the NLRP3 inflammasome (70). In an organism, these metabolic and chemical changes lead to increased blood flow and leukocyte delivery to cells, where ER stress occurs (71). Therefore, the mechanisms that retain ER chaperones and oxidoreductases communicate ER stress to the immune system via multiple readouts: they determine MHC class I surface exposure, they influence the activation of inflammation, but they also signal the intracellular stress status to the immune system when found on the plasma membrane.

CALRETICULIN: A DAMP ON THE PLASMA MEMBRANE OF TUMOR CELLS

The appearance of ER chaperones and oxidoreductases on the plasma membrane corresponds to a danger-associated molecular pattern (DAMP) (72). DAMPs are molecules that are normally intracellular, but become exposed on the plasma membrane in stressed, damaged, or dying cells, as well as in tumor cells (73). Their presence on the cell surface leads to the recruitment of innate inflammatory cells, following the interaction of surface DAMPs with pattern-recognition receptors (PRRs) (74). An example for this is CD91, found on the surface of dendritic cells (DC) and other antigen-presenting cells (APC), which interacts with the ER-derived DAMPs calreticulin and GRP94 on stressed or dying cells (75). Upon formation of a complex between these proteins, a potent "eat-me" signal is generated and phagocytosis of calreticulin or GRP94-bearing stressed cells is initiated (19, 76). In contrast, CD47 acts as an inhibitor of this activity of calreticulin by interfering with the calreticulin–CD91 complex formation (64, 76).

This mechanism is particularly important in the cancer scenario (**Figure 1**), where calreticulin is today one of the most extensively studied DAMPs that dictates the immunogenicity of cancer cells (19, 77). Importantly, calreticulin exposure on the plasma membrane is triggered upon treatment with different

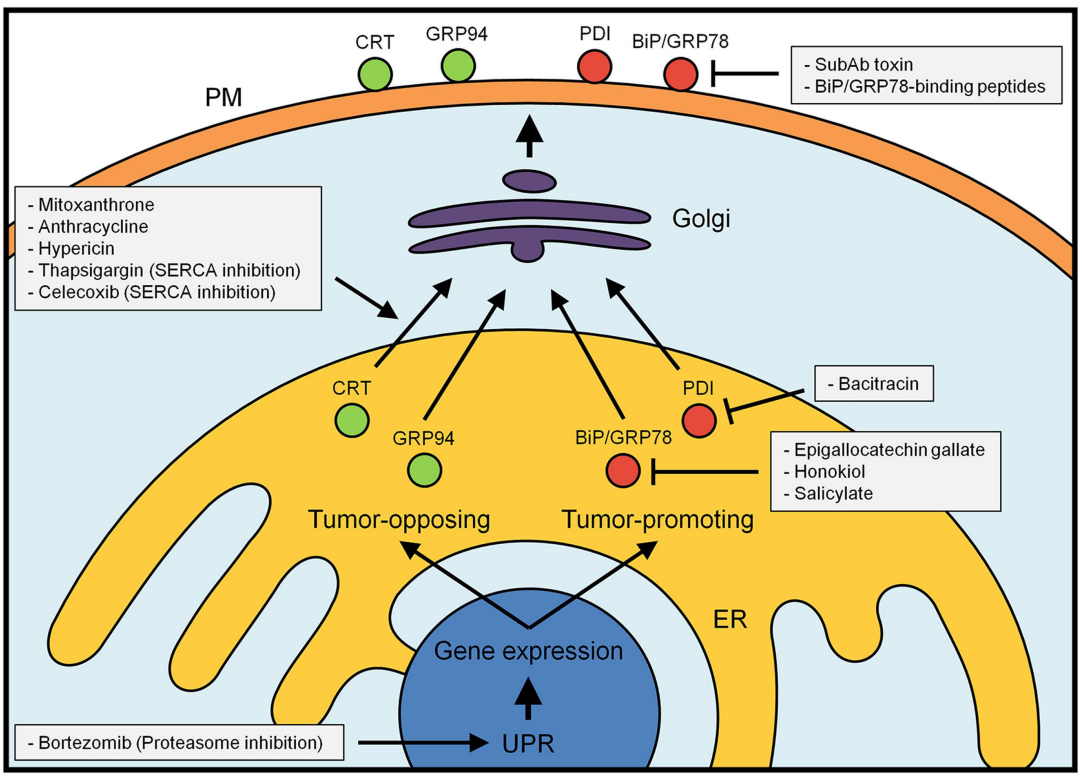

FIGURE 1 | Major tumor-promoting (red) and tumor-inhibitory (green) ER chaperones and oxidoreductases. Their localization and function within the ER can be favorably influenced by a number of currently known drugs (for details see text). At the plasma membrane, inhibitory binding peptides can stop the tumor-promoting activity of this class of proteins. In contrast, simple modulation of the unfolded protein response (UPR) is expected to induce both tumor-promoting and tumor-blocking responses.

chemotherapeutic stimuli, including cisplatin and the anthracyclines doxorubicin, idarubicin, and mitoxantrone (19, 78). However, it is not clear whether calreticulin remains in the membrane of the stressed cell or is transferred over to immune cells (79). Regardless of the exact location of this extracellular calreticulin, stressed and apoptotic cells are subsequently engulfed and eliminated (80). This activation of the immune system can be exploited via the injection of calreticulin-coated cancer cells. Once in the blood stream, these abnormal cells can trigger a tumor-specific immune-response that eventually may activate an anti-tumor immune-response *in vivo* (81).

An additional important prerequisite for the immunoelimination of tumor cells using the calreticulin "eat-me" signal is autophagy: the inhibition of autophagy significantly increases the amounts of calreticulin on the surface of stressed tumor cells, suggesting that autophagy-competent cancer tissue may be less susceptible to calreticulin-mediated immunorecognition of tumor cells (82, 83). In contrast, autophagy promotes the secretion of ATP upon ER stress, another tumor DAMP (84, 85). Interestingly, not only calreticulin on the plasma membrane, but also its overall expression is frequently enhanced in tumor tissue, potentially indicating that this chaperone could indeed provide an avenue for future cancer immunotherapy (86). Specifically, calreticulin overexpression is associated with the development and progression of pancreatic cancer (87). However, studies on infiltrating ductal breast carcinomas (IDCAs) were not able to detect an involvement of calreticulin in the development of a humoral immune-response (88). In defense of the calreticulin role as a protective mechanism against cancer, none of these studies have investigated the intracellular distribution of calreticulin in the respective tumor scenario. Consistent with this caveat, hepatocellular carcinoma has been found associated with high levels of circulating anti-calreticulin antibodies (89). In addition, serum IgG levels of anti-calreticulin autoantibodies have been found to be significantly higher in bladder cancer patients than in normal controls, leading to the proposal of anti-calreticulin antibodies as a novel biomarker for bladder cancer progression (90). It is currently unclear whether the injection of a fragment of recombinant calreticulin blocks tumor growth using these or other mechanisms (91, 92).

ERp57, GRP94, Ero1α, AND PDI: FUNCTIONS BEYOND IMMUNORECOGNITION FOR TUMOR CELL MIGRATION

Other ER chaperones and oxidoreductases also show aberrant targeting to the plasma membrane. One example is ERp57, which is critical for the peptide loading complex for MHC class I together with calreticulin (93). Similar to what occurs with calreticulin, ERp57 also appears on the cell surface following anthracycline treatment. Importantly, ERp57 might not act as a DAMP itself, but rather as a prerequisite for calreticulin surface targeting (94, 95). The expression level of ERp57 in cancer does not provide much insight about its role in cancer, since bladder and gastric cancers appear to be characterized by low levels of calreticulin and ERp57 (96, 97).

GRP94 (also called gp96) is another prominent chaperone of the ER that has a much smaller set of client proteins when compared to calreticulin (98). Its substrates include toll-like receptors (TLRs), important sensors of DAMPs (99). This Hsp90 family protein can escape ER retention like calreticulin, and is found secreted from pancreatic cells and hepatocytes (100, 101). In contrast, tumor cells are decorated with surface-bound GRP94 (102, 103). On this localization, GRP94 acts as a DAMP similar to calreticulin (104) and in parallel to surface-exposed Hsp90 (105). In addition, GRP94 also binds HER2 on the surface of breast cancer cells, and regulates its cancer-promoting activity (106). Interestingly, cell surface GRP94 may interact with the CD91 receptor, like calreticulin, albeit with unclear functional significance (107, 108). Breast cancer tissue is characterized by the over-expression of GRP94 that may modulate the ability of tumor cells to migrate (109).

The oxidoreductase PDI is a central enzyme in the formation of disulfide bonds in secreted proteins (110). This protein also localizes in significant amounts to the cell surface of platelets, CHO, and pancreatic cells, as well as thyrocytes (62, 111–113). Here, it modulates surface-exposed thiols (113, 114) and cellular adhesion of immune cells via the association with integrins (115, 116). This mechanism also determines the ability of T helper cells to migrate through the extracellular matrix (117). PDI expression is tied to tumor vascularization that is often low and results in the activation of the hypoxia-dependent transcription factor HIF-1α (118). This transcription factor then promotes the upregulation of the oxidoreductases PDI and Ero1α (119–121). Subsequently, increased PDI and Ero1α expression also induces the production of vascular endothelial growth factor (VEGF), which, in turn, enables hypoxic tumors to improve angiogenesis (120, 121). Similar to GRP94, the levels of PDI and Ero1α have also been found to correlate with the invasiveness of glioma and the metastatic ability of soft tissue sarcoma, due to the role of PDI in mediating the interaction of cells with integrins (122, 123). Although Ero1α is secreted from hypoxic tumor cells, we currently do not known whether this occurs *in vivo* and what the function of surface or extracellular Ero1α is (67).

BiP/GRP78, AN INHIBITOR OF TUMOR CELL APOPTOSIS AND IMMUNORECOGNITION

Compared to PDI, more is known about the role of BiP/GRP78 for cancer cells, and specifically when found on the plasma membrane. BiP/GRP78 is over-expressed in many cancers, a hallmark that is associated with aggressive growth, invasive properties, and therapeutic resistance (124). This chaperone is a major regulator of ER protein folding and ER stress (125). By binding hydrophobic surfaces on newly synthesized polypeptides, BiP/GRP78 is first in line for ER protein folding, a role that becomes accentuated when misfolded polypeptides accumulate within the ER. Under that condition, also termed ER stress, BiP/GRP78 binds to unfolded proteins in its ATP-bound form, mediates their folding at the expense of ATP and is released when GDP is exchanged with GTP (126, 127). Folding is typically achieved through multiple rounds of binding and release of BiP/GRP78. Interestingly, when BiP/GRP78 acts as a chaperone, it dissociates from the ER transmembrane stress sensor proteins inositol requiring enzyme 1 (Ire1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6) that are then able to trigger the unfolded protein response (UPR) (128). This intracellular signaling pathway activates the transcription of numerous ER chaperones and oxidoreductases to protect the cell from accumulated unfolded proteins, but also acts

as an activator of apoptosis (129). Notably, BiP/GRP78 itself is a transcriptional target of the UPR via ER stress-responsive elements that can bind to ATF6 (130).

In cancer tissue, the UPR is frequently constitutively active, because solid tumors are poorly vascularized, leading to low oxygen delivery for mitochondria and low glucose delivery for glycolysis, both a cause of low ATP availability for ER protein folding (131). Consistent with this, BiP/GRP78 has been found over-expressed in prostate, head and neck, melanoma, breast, lung, brain, gastric, colon, and hepatocellular carcinomas (132). High levels of BiP/GRP78 act first of all as suppressors of apoptosis (133, 134), based on its role as a suppressor of the UPR (18), but also from its ability to sequester ER-associated pro-apoptotic Bcl2 family proteins such as Bik (135). Over-expression of BiP/GRP78 also inhibits pro-apoptotic Ca^{2+} transfer from the ER to mitochondria in astrocytes. This likely occurs due to the inhibitory action of BiP/GRP78 on the inositol-1,4,5 trisphosphate receptors (IP3Rs), major Ca^{2+} release channels of the ER (136, 137). As expected from these tumor-promoting roles of BiP/GRP78, high levels of this ER chaperone lead to poor prognosis in breast cancer (138).

As a side effect, the UPR not only leads to elevated expression of BiP/GRP78 in tumor tissue, but also leads to aberrant localization of this ER chaperone to the cytosol, mitochondria, and the plasma membrane (124). Cell surface BiP/GRP78 is apparently directly tied to its expression level that is under the control of the UPR, suggesting that high expression of this chaperone leads to saturation of the KDEL receptor retrieval mechanism (61). This phenomenon has been found in prostate, ovarian, and gastric cancer, as well as melanoma (21, 139–141). In some tumors, so much BiP/GRP78 escapes from the ER that secretion results, accompanied by the production of autoantibodies (142). These autoantibodies can promote or inhibit proliferation and apoptosis, but also interfere with phosphoinositide 3-kinase (PI3K), Akt, and MAP kinase pathways with the consequence of increased survival in several types of tumors (143, 144). This latter activity depends on the activating, physical interaction of cell surface BiP/GRP78 with PI3K that subsequently results in the activation of its downstream target Akt (145, 146). This activity of surface BiP/GRP78 may depend on α2-macroglobulin (α2M*), since the association between the two proteins triggers Akt phosphorylation in a PI-3 kinase-dependent manner (147, 148). In contrast, low levels of BiP/GRP78 tend to have opposite effects in mice and result in decreased activity of PI3K signaling in prostate and leukemia cancer models (149, 150).

Like calreticulin, BiP/GRP78 also influences the way cancer cells interact with the immune system. However, whereas calreticulin provides an "eat-me" signal, cell surface BiP/GRP78 protects insulinoma and fibrosarcoma cells from cytotoxic T lymphocytes (151, 152). In addition, BiP/GRP78 also interacts with MHC class I on the cell surface, although the functional significance of this observation is currently unclear (153).

AVENUES OF INTERFERENCE WITH ER CHAPERONES IN CANCER

Increased expression and cell surface appearance of ER chaperones and oxidoreductases have emerged as critical hallmarks of cancer cells and as consequences of low tumor vascularization that results in hypoxia. The observations outlined in our review suggest this insight may be used to develop new strategies to treat cancer (**Figure 1**) (154). In cancer, an approach under consideration consists in triggering the UPR (155). A number of compounds are currently in preclinical studies or Phase II/III trials and typically attempt to prevent the pro-survival readout of the UPR. This approach led to marked decrease of cancer growth in a multiple myeloma xenograft model (156). A promising strategy appears to be the combination of such drugs with bortezomib, a blocker of the proteasome and inducer of ER stress (157). With this combination of drugs, stress-inducing bortezomib primes cancer cells for death that becomes inevitable, once an inhibitor of protective UPR responses is added to the mix. A similar approach aims to target the redox-modulatory role of ER chaperones and oxidoreductases using the PDI inhibitor bacitracin, which acts as a potent booster of the chemotherapeutics fenretinide and bortezomib (158). Due to the toxicity of bacitracin, novel PDI inhibitors are currently under development (159). Another way how ER redox modulation can be used as an adjuvant for cancer chemotherapy is by interfering with the redox-sensitive activity of SERCA to allow for increased cell stress in tumor tissue due to reduced ER Ca^{2+} content (160, 161). Given the tumor-promoting (e.g., BiP/GRP78, PDI) and tumor-opposing (e.g., calreticulin) activities of ER chaperones and oxidoreductases, current knowledge suggests more pinpointed approaches are needed to increase efficacy of ER-targeted cancer chemotherapeutic strategies.

Interestingly, the SERCA inhibitor thapsigargin and the non-steroidal anti-inflammatory drug celecoxib both make use of the interference with ER Ca^{2+} content as a weapon against tumor cells (**Figure 1**) (162–164). Under this condition, calreticulin, PDI, BiP/GRP78, and GRP94 escape ER retention and retrieval (69, 165). This effect is similar to an indiscriminate activation of the UPR, which turns on ER chaperone and oxidoreductase production, and leads to the saturation of KDEL as well as di-lysine-based retrieval to the ER (166, 167). Similar to blanket interference with the UPR, this approach is bound to have tumor-promoting and tumor-opposing effects: while calreticulin and GRP94 will appear as immune system targets on the cell surface of tumor cells, PDI and BiP/GRP78 will have tumor-promoting effects as promoters of cell mobility and blockers of apoptosis.

Ideally, an efficient therapy would aim to generate cell surface calreticulin to serve as an efficient "eat-me" signal on tumor cells, while down-regulating or inactivating BiP/GRP78 on the plasma membrane, which acts as tumor-promoting. To achieve such a goal, it would be helpful to understand the molecular machinery of calreticulin plasma membrane exposure, currently known to require the triggering of PERK, the cleavage of caspase-8, and the functioning of SNARE proteins (168). Mitoxantrone, an anthracycline that robustly influences these mechanisms and leads to calreticulin surface exposure, is currently in clinical trials against lymphoma (**Figure 1**) (169).

In contrast to calreticulin, the requirements for BiP/GRP78 cell surface exposure are less understood. As a sole factor, the transmembrane protein MTJ-1 has been identified as critical for BiP/GRP78 surface translocation (170), possibly via the catalysis of ATP exchange through its J domain (171). Interestingly, photo-dynamic therapy (PDT) using the ER-localized

photosensitizer hypericin may be such a magic bullet: not only does it result in the reduction of SERCA activity (172), but this treatment specifically results in the surface targeting of only calreticulin, and not BiP/GRP78 (84). Promisingly, this treatment causes tumor regression in BALB/c mice inoculated with colon carcinoma (173).

Conversely, inhibitory agents against BiP/GRP78 could create tumor cell specificity of UPR-targeted anti-cancer strategies (**Figure 1**). Approaches include the selective destruction of BiP/GRP78 on the surface with the bacterial toxin SubAb that cleaves and inactivates this chaperone (174). This strategy delayed the growth of multiple cancer xenografts in mice (175, 176). Cancer cells also respond well to the inhibition of the BiP/GRP78 ATPase activity with epigallocatechin gallate, honokiol, and salicylate (177–179). It is currently unknown whether these effects stem from inhibiting the activity of MTJ-1 that is needed to transport BiP/GRP78 to the plasma membrane (170). Xenograft growth of tumors is also inhibited in the presence of BiP/GRP78-binding peptides that obstruct the chaperone's folding pocket (180). Importantly, these peptides bind specifically to tumor cells and abrogate their growth *in vivo* (181, 182). Such a strategy might be particularly important following the surgical removal of tumor tissue or in combination with chemotherapeutic approaches (183, 184).

CONCLUSION

Endoplasmic reticulum chaperones and oxidoreductases have emerged as unlikely regulators of tumor growth. While neither being directly connected to the regulation of cell division and growth, nor the triggering of apoptosis, they instead frequently acquire new functions unrelated to their classic ER roles in a cancer setting. These new roles coincide with their relocation from the ER to the plasma membrane. In most cases, this occurs because the UPR triggers the production of more chaperones and oxidoreductases that eventually saturate the KDEL retrieval machinery. Once at the plasma membrane, ER chaperones and oxidoreductases serve as DAMPs for the immune system (calreticulin, GRP94) or modulators of tumor hallmarks (BiP/GRP78, PDI). The exploitation of this group of proteins as cancer therapeutic targets will require a detailed understanding of their intracellular and extracellular roles. Our current knowledge has identified chaperones that serve as DAMPs, whereas modulators of tumor hallmarks including cell death and metabolism are typically tumor-promoting. Specific triggers and inhibitors of the functions of ER chaperones and oxidoreductases will help direct cancer therapeutic approaches in the right direction. This insight warrants further investigation on this class of proteins.

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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 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	Propynoic 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 β1 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 $CD8^+$ and $CD4^+$ 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 potently 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 SIRPα 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

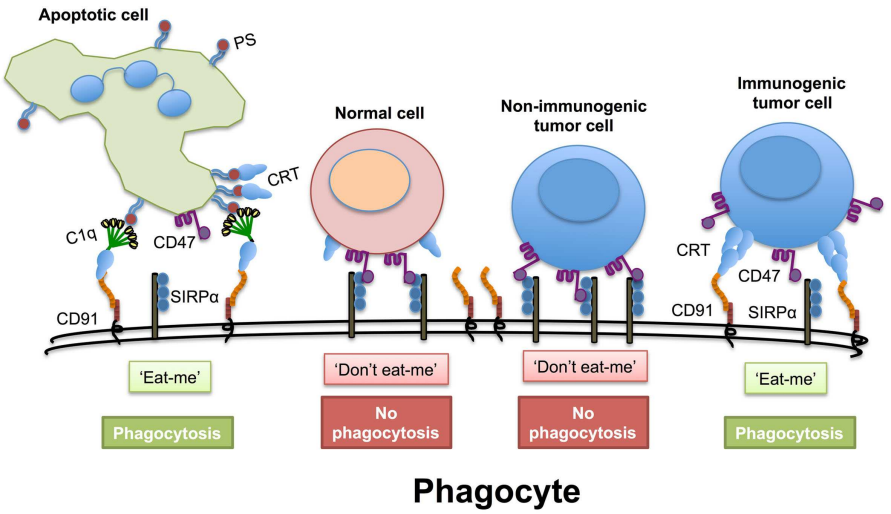


FIGURE 1 | Disruption of the "don't eat me" signal. Cells express the "don't eat me" signaling molecule CD47 on their cell surface that interacts with SIRPα on phagocytes. This must be overridden when cells are preparing to die. During apoptosis, normal cells express greater amounts of phosphatidylserine (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– SIRPα "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
AUTOIMMUNE DISEASES		
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)
CANCERS		
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)

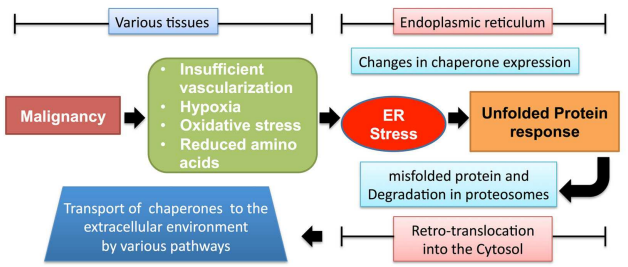
chaperones are susceptible to attack by reactive oxygen and nitrogen species, leading, e.g., to nitrosylation. Such post-translational modifications may make ER chaperones sufficiently "foreign" as to elicit an immune response. Whether the initiation of an immune response to ER chaperones is simply a 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,



Calreticulin expression	Tissue	Cancer	Reference
Increased	Urine	Bladder	(82)
Increased	Serum	Lung	(83)
Increased	Breast	Breast	(84)
Increased	Ovarian	Ovarian	(85)
Decreased	Endometrium	Endometrial	(86)
Increased	Liver	Hepatocellular carcinoma	(87)
Increased and decreased	Hyperplasia & intraepithelial neoplasia	Prostate	(88, 89)

FIGURE 2 | Tumor factors that lead to changes in chaperone expression during ER stress. Once tumors begin to proliferate in various tissues, the local microenvironment begins to become "stressed" leading to a change in metabolic and vascular demands. The ER is required to increase the rate of protein production, involving the synthesis, folding, and secretion of proteins involved in the production of tumors. This furthers stresses the ER organelle, leading to protein production errors, triggering the unfolded protein response pathway to remove incorrectly folded proteins from the ER for degradation in the cytosol via retrotranslocation to the proteasome. Some unfolded proteins are accompanied by chaperones, and these now enter the cytosol, where via a number of proposed mechanisms can leave the cell (82–89).

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 secretary 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^+/H^+ transporter inhibitors or alkalizing 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).

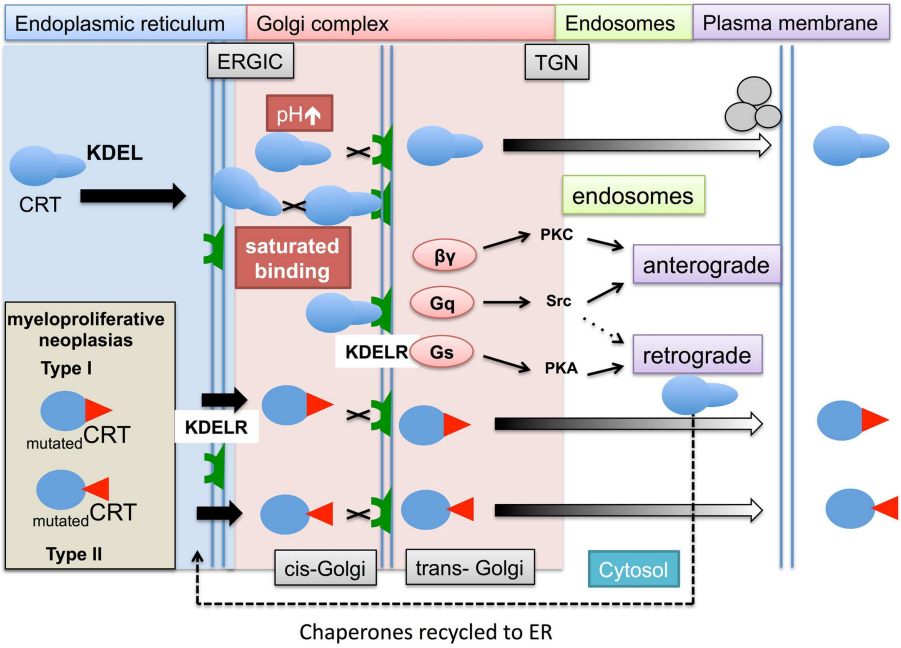


FIGURE 3 | The role of KDEL ligand and receptor in chaperone retrieval and retention within the ER and escape into the cytosol. Within the ER, membrane bound and soluble chaperones assist in the folding (not shown) and transport of glycoproteins to the cell surface. During this process the chaperones, e.g., calreticulin (CRT) escort their cargos between the ER and Golgi complex. Upon chaperone docking to the KDEL receptors (KDELR) via their KDEL ligand, the KDELR activates a number of G proteins (βγ, Gq, and Gs) and kinases (PKC, PKA, and Src), which allows released proteins to be transported via the secretory anterograde pathway toward the plasma membrane, while chaperones are returned to the ER via a retrograde pathway. There are a number of situations in which the process of chaperones interacting with their KDEL receptors might be impaired. During ER stress induced by tumorgenesis, the ER chaperone production increases and may lead to increased saturation of the KDEL receptors with chaperones. In addition, the optimum acid pH can increase during cell stress reducing KDEL ligand/receptor interaction. In hematopoietic cells carrying Type I (52 bp deletion) and Type II (5 bp insertion) mutations in the carboxyl terminus of calreticulin, may result in lack of binding of chaperones to the KDEL receptors. This leaves the chaperones vulnerable to being trafficked by a number of secretory and alternative mechanisms into the cytosol and ultimately out of the cell.

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**).

RETROTRANSLOCATION 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

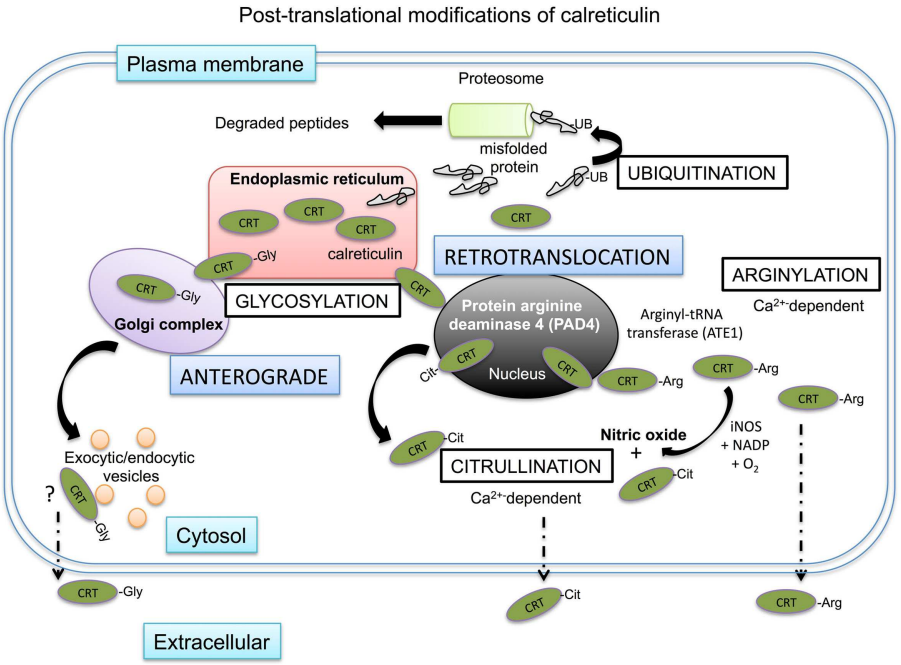


FIGURE 4 | Intracellular post-translational modifications of calreticulin. Mis-folded proteins directly leave the ER and are ubiquitinated in the cytosol before degradation in the proteasome. Calreticulin has been shown to be transported to the cytosol possibly via the nucleus. Within the nucleus, calreticulin is exposed to protein arginase deaminase 4 (PAD4) where it may be citrullinated before being shuttled to the cytosol in association with nuclear export proteins. There is no evidence of calreticulin being ubiquitinated in the cytosol, but it does encounter arginyl-tRNA transferases, which can arginylate the protein. The addition of arginine on the protein can be further citrullinated in the cytosol in the presence of iNOS as a byproduct of nitric oxide production from the conversion of arginine to citrulline. Both citrullinated and arginylated isoforms of calreticulin have been found outside of the cell, where they exert specific biological functions. Artificial glycosylation of calreticulin leads to the secretion of calreticulin out of the cell via the secretory pathway and glycosylated isoforms of calreticulin have been observed in human myeloid cells.

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 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 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 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., anthracycins, 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 anthracyclin therapy (21).

The ER stress response via PERK and eIF2α was found to be involved in the translocation of calreticulin to the cell surface during ICD. When PERK phosphorylates eIF2α, 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 eIF2α (21). Similarly, hypericin mediated photodynamic therapy induced eIF2α phosphorylation and PERK activation (50). When CT26 cells were depleted for PERK or when a non-phosphorylatable form of eIF2α was expressed, this completely abolished calreticulin/ERp57 exposure, whereas it did not affect the sensitivity toward anthracyclin induced cell death. In contrast, eIF2α 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 eIF2α hyperphosphorylation (131). Furthermore, the disruption of the PP1/GADD34 complex, a complex that is involved in the dephosphorylation of eIF2α 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^{2+} 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 anthracylines 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 $^1O_2$ 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 eIF2α was only minimally phosphorylated upon cisplatin treatment. Of note, thapsigargin treatment alone was also inefficient for induction of calreticulin exposure, although it did phosphorylate eIF2α (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^{2+} 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^{2+} 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 ore 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^{2+} 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/γ-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/γ-ray/UV-ray 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 anthracylines 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 IFNγ upon interaction with autologues 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 anthracylines 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 *ex 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, γ-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 release 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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Is it all that bad when living with an intracellular protozoan? The role of *Trypanosoma cruzi* calreticulin in angiogenesis and tumor growth

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The immune system protects against disease, but may aberrantly silence immunity against "altered self," with consequent development of malignancies. Among the components of the endoplasmic reticulum (ER), important in immunity, is calreticulin (CRT) that, in spite of its residence in the ER, can be translocated to the exterior. *Trypanosoma cruzi* is the agent of Chagas disease, one of the most important global neglected infections, affecting several hundred thousand people. The syndrome, mainly digestive and circulatory, affects only one-third of those infected. The anti-tumor effects of the infection are known for several decades, but advances in the identification of responsible *T. cruzi* molecules are scarce. We have shown that *T. cruzi* CRT (TcCRT) better executes the antiangiogenic and anti-tumor effects of mammal CRT and its N-terminus vasostatin. In this regard, recombinant TcCRT (rTcCRT) and/or its N-terminus inhibit angiogenesis *in vitro*, *ex vivo*, and *in vivo*. TcCRT also inhibits the growth of murine adenocarcinomas and melanomas. Finally, rTcCRT fully reproduces the anti-tumor effect of *T. cruzi* infection in mice. Thus, we hypothesize that, the long reported anti-tumor effect of *T. cruzi* infection is mediated at least in part by TcCRT.

Keywords: *Trypanosoma cruzi* calreticulin, angiogenesis, cancer, infectivity, c1q

INTRODUCTION

The immune system protects against disease. However, abnormally silenced protective immunity against "altered self" may lead to the development of malignancies. As such, cancer represents a prominent example of defective immunological surveillance.

Components of the ER play key roles in the development of protective immunity. Among these components, is calreticulin (CRT) that, in spite of its residence in the endoplasmic reticulum (ER), can be translocated to the extracellular milieu, where it displays immune modulating capacities. Work from several laboratories indicates that CRT is an interesting ER candidate to manipulate anti-cancer immunity.

According to the World Health Organization (WHO), Chagas' disease is endemic in 21 countries, with about 8 million infected people (1). The disease is considered one of the most important neglected tropical infections worldwide, because it causes 15,000 deaths per year and 0.7 million disability adjusted life-years (2). The impact of this parasite on domestic and wild animals (reservoirs) (3) is unknown.

The disease is endemic in Latin America. However, it has now gone global, affecting several hundred thousand people, mainly South American immigrants, in the USA, Canada, Europe, Oceania, and Asia (4), where transmission is independent of the protozoan. In countries without arthropod vectors, transmission is through blood products (5), organ transplants (1, 5), or congenital (6). Infection can also occur *per os* through parasite-contaminated food (7, 8).

The most frequent treatments for Chagas' disease have been the administration of Benznidazole or Nifurtimox, with reported efficacy in up to 80% of acute cases after a 60-day course, but with frequent severe side effects and drug resistance (9). Although these drugs reportedly may cure the disease in the acute phase, particularly in children, their efficacy in adults, in the indeterminate or chronic phases, has not been determined.

About 80 years ago, Roskin, Exemplarskaja, and Kliueva, investigators from the former Soviet Union postulated an anti-cancer activity of *Trypanosoma cruzi*, based on a toxic effect of this parasitic infection, or parasite extracts, over different tumors, both in experimental animals and humans (10, 11). More recently, it was described the parasite capacity to infect preferentially tumor cells as compared to normal host cells (12). In general, these data suggest an antagonism between *T. cruzi* infection and tumor growth (12). Herein, we will review the available information with regard to possible molecular mechanisms underlying the anti-tumor effects of *T. cruzi* infection, with emphasis on the experimental rational basis leading to the proposal that the parasite utilizes its calreticulin (TcCRT) to protect its host against neoplastic aggressions. We have provided experimental evidences indicating that TcCRT is an antiangiogenic molecule that inhibits proliferation, migration, and capillary morphogenesis in several *in vitro*, *ex vivo*, and *in vivo* (*in ovum*) assays (13–15). On the other hand, TcCRT inhibits the growth of a mammary adenocarcinoma and a melanoma in different experimental animal models (13–16).

IS IT ALL THAT BAD WHEN LIVING WITH AN INTRACELLULAR PROTOZOAN?

The work from the investigators from the former Soviet Union, proposed that *T. cruzi* infection potential as a biotherapy for cancer treatment (17–19), opened possibilities for several research lines. They produced a "cancerolytic toxin" [Kliueva and Roskin (KR) preparation], from *T. cruzi* lysed cells. In humans, affected by a variety of tumors, these "toxins," reduced tumor growth, pain, local inflammation, and bleeding (18). Controversial results followed and the situation, complicated by World War II and "The Cold War," interrupted or greatly delayed this work (10). Thus, the mechanism and the molecular component responsible for the biotherapy effect have remained largely unknown.

Of note is the proposal in Science journal in 1948, by the immunologist Theodore S. Hauschka and Margaret Blair Goodwin that tumor-bearing mice, concomitantly infected with the lethal *T. cruzi* strain died within 8–13 days post infection. They observed that weight loss in tumor-bearing infected animals was important, *and that tumor growth was almost completely suppressed.* When, in tumor-bearing animals, the infection was treated, the tumors resumed their usual growth rate, and the hosts died of cancer (20). Thus, presence of the parasites was necessary for tumor inhibition. However, the authors' view (20) that tumor and parasites compete for nutrients with consequent inhibition of the former does not seem now completely satisfactory given the information emerging during the last few years that we review and discuss below.

Earlier this century, experimental data obtained from rats infected with *T. cruzi* parasites and carcinoma induced by 1,2-dimethylhidrazyne, demonstrated that chronic infection may enhance resistance against tumor growth (21). More recent reports, evaluated the tumor-tropism-parasite capacity to infect host cancer cells rather than normal cells. Normally, the invasiveness (tissue-distribution of parasites of different strains of *T. cruzi*) in mice, primarily demonstrated a parasite tropism toward heart tissues, since 46% (40–65%) of parasites are found in this organ. The liver and kidney contained 3–4 times less parasites and even less was found in the spinal cord. Finally, only 3–4% were found in the brain, spleen, and lymph nodes. However, the presence of a tumor in the host leads to *T. cruzi* redistribution between the tissues: the parasites found in the tumor accounted for 18% in the decrease of heart invasion (now down to 28%) and increased invasiveness of spleen and lymph nodes (12). Nevertheless, a relationship between these findings and tumor development was not addressed in these studies.

More recently, a role for *T. cruzi* infection in controlling tumor growth has been revisited at least in two laboratories, including ours (22, 23). Junqueira et al. (23) reported that the use of a recombinant non-pathogenic *T. cruzi* clone as vector of a testis tumor antigen (NY-ESO-1) is efficient in generating T cell-immune responses and protection against cancer cells, thus delaying tumor development in mice.

Most recently, we have corroborated that *T. cruzi* infection greatly reduces the growth of a mammary adenocarcinoma.

CALRETICULIN

Calreticulin, a 46 kDa pleiotropic protein, participating as a chaperone and in calcium homeostasis (24), has been described in different organisms such as humans (25), insects (26, 27), nematodes (28–31), protozoa (32–35), and plants (36).

Calreticulin, mainly residing in the ER of all nucleated cells (37), contributes in different processes such as the control of glycoprotein folding quality and binding to monoglucosylated glycans with high mannose content. CRT is also present in the cytosol, nucleus, secretory granules, on the plasma membrane and also free in the extracellular environment (37). There, CRT modulates the immune response against apoptotic cancer cells (38–42). The mechanisms involved in CRT translocation and release to the extracellular milieu are still unknown (43). CRT also promotes cutaneous wound healing (44–46), cell adhesion (37), nuclear export of some steroid hormone receptors (47–49), and the stability or translation of a variety of RNAs (50–54). CRT reaches the cytosol and nucleus by a C-terminal domain-dependent retrotranslocation, after ER calcium depletion (55).

Calreticulin has a globular N-terminus (N), a proline-rich (P) domain, and an acidic C-terminus (37). An S-domain (aa 160–289), within N and P, binds complement component C1, a "danger signal detection module" that initiates the classical complement activation pathway (56, 57). The primary CRT sequence starts with a signal peptide and ends with a KDEL-ER retention sequence (58). Human CRT (HuCRT) and its N-terminal fragment binds laminin (59), with antiangiogenic properties *in vitro* and *in vivo* (60, 61) and inhibit the growth in several tumor models (62–64). Vasostatin, is a CRT 180 amino acid N-terminal fragment, a potent endogenous inhibitor of angiogenesis and suppressor of tumor growth. Vasostatin inhibits vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation and interactions in matrigel and induces cell apoptosis under limiting oxygen availability (65).

TcCRT AND INFECTIVITY

Trypanosoma cruzi, may use its CRT, a putative universal apoptosis cell marker (39, 41, 42), in an "apoptotic mimicry" strategy to generate "eat me" signals (i.e., by capturing C1 in the area of flagellum emergence), thus facilitating the invasion of host cells. C1q bridges the parasite molecule with host cell surface receptors (66), most likely CRT known as cC1qR (67). Thus, host C1, upon binding to the trypomastigote surface, also promotes parasite infectivity (68). The parasite molecule responsible for recruiting this complement component has been identified as TcCRT (69, 70). Increased parasite infectivity is paralleled by significant increases in TcCRT mRNA levels during early (cell contact and penetration) infection stages of a VERO cell line. In spite of its lysine–aspartic acid–glutamic acid–leucine (KDEL)-ER retrieval sequence, TcCRT does translocate from the ER to the parasite area of flagellum emergence. An augmented capacity to recruit C1, an important "eat me" signal for phagocytic cells follows, thus leading to increased infectivity (41, 68–72).

The TcCRT–C1q interaction can be decreased by anti-TcCRT $F(ab')_2$ antibody fragments (lacking the C1-binding Fc domains) (73). Indeed, passive immunization of mice with these fragments resulted in important decreases in infectivity and improved clinical parameters (69).

Of particular interest and conceivable consequences in pathology is the possibility that, in *T. cruzi* infected individuals, the parasite molecule may promote autoimmune mechanisms (74).

Trypanosoma cruzi CRT also binds complement mannan binding lectin (MBL) and Ficolins (75). Together with C1(q,r,s) they are three complement "danger signal" recognition macromolecular modules. Genetically, structurally, and functionally related, they differ in the nature of the recognized danger signals (22). After binding C1(q,r,s), TcCRT or its S and R central domains inhibit the classical pathway of human complement, in a calcium-independent manner (69, 72, 76). More recently, we have also proposed that L-Ficolin binds TcCRT, thus inhibiting the lectin pathway, a likely alternative or concomitant *T. cruzi* strategy to inhibit the host immune response (75). The roles of MBL and Ficolins in the infectivity process are still under study.

TRYPANOSOMA CRUZI CALRETICULIN, A MOLECULE WITH ANTIANGIOGENIC AND ANTI-TUMOR PROPERTIES

Inhibition of tumor angiogenesis, proposed as a cancer therapy almost 40 years ago (77), is a complex process to form new blood vessels, thus providing the necessary supply of nutrients, oxygen, and ways for waste disposal (78). Antiangiogenesis is currently applicable to a wide variety of tumors, frequently as a supplement to other therapies (79).

Our description of TcCRT provides alternative or concomitant explanations for at least an important part of the anti-tumor effect of this parasite infection. Most likely, TcCRT anti-tumor properties derive from its antiangiogenic properties (13, 73). By direct interaction with endothelial cells, probably through a Scavenger-like receptor, TcCRT acts as a potent angiogenesis inhibitor (13, 14, 71). Antiangiogenic agents may generate a primary stressing challenge to a variety of tumor cells. On the other hand, many tumors have a notorious capacity to produce an array of proangiogenic molecules. Of note are VEGF, the platelet-derived endothelial cell growth factor (PD-ECGF), and the acidic and basic fibroblast growth factors (aFGF and bFGF) (80). Thus, tumor growth and metastasis are indirectly, but importantly promoted by these factors.

Angiogenesis modulators behave differently across species. TcCRT and its N-terminal vasostatin-like domain (N-TcCRT) were studied in mammals, *Homo sapiens sapiens* included (13). Thus, recombinant TcCRT (rTcCRT) and its N-terminal domain inhibit capillary growth *ex vivo* in *Rattus rattus* aortic rings, morphogenesis, proliferation, and chemotaxis in human umbilical cord endothelial cells (HUVECs) (13) and *in ovum* angiogenesis in the *Gallus gallus* chorioallantoid membrane (CAM) assay (14). These are valid correlates of important features of angiogenesis *in vivo*. In most of these assays TcCRT was more effective, in molar terms, than HuCRT (13). Of particular interest is the fact that, in the CAM assay, the antiangiogenic TcCRT effect was fully reverted by polyclonal antibodies against rTcCRT (15). We are currently investigating whether the anti-tumor effect of *T. cruzi* infection is reverted by $F(ab')_2$ anti-TcCRT antibody fragments, derived from these immunoglobulins. In such a case, a formal causal link between externalized TcCRT and the anti-tumor effect of *T. cruzi* infection would be established.

In agreement with the previously described facts, inoculation of rTcCRT inhibits by 60–70% the time-course development of a murine mammary metrotexate multiresistant adenocarcinoma (TA3-MTX-R), with a higher efficiency than the human counterpart (13) (**Figure 1**).

CONCLUDING REMARKS

Recombinant TcCRT, and most likely translocated native TcCRT, mediate mechanisms relevant in the host/parasite interplay: (i) through a central S domain (aa 159–281), it interferes with the earliest stages of the complement activation; (ii) C1, bound to the parasite, promotes infectivity (69); and (iii) through an N-terminal domain (20–193), it interacts directly with endothelial cells and inhibits angiogenesis (13). Thus, prolonged host–parasite interactions may be promoted. Several of these features are variably conserved in the HuCRT, but with lower equimolar efficiency. Thus, when the parasite and human chaperones are compared in equimolar terms, the former displays stronger antiangiogenic effects in *in vitro*, *ex vivo*, and *in vivo* (22, 70) and this is reflected in the compared anti-tumor effects.

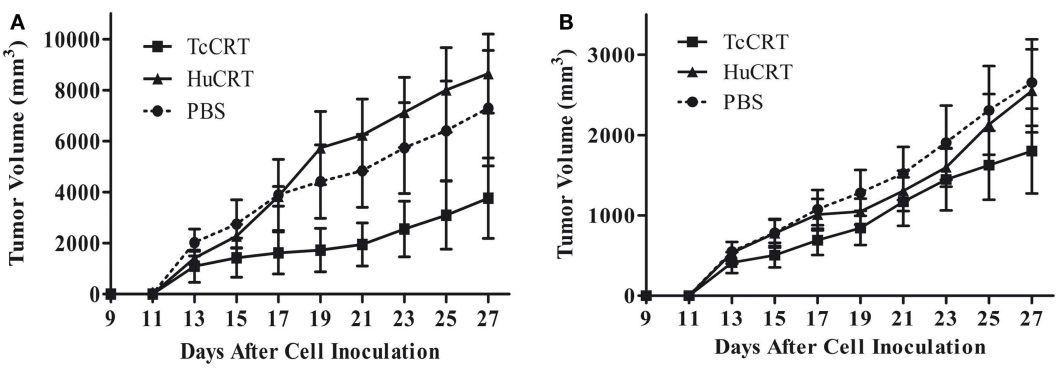

FIGURE 1 | *Trypanosoma cruzi* CRT-mediated tumor growth inhibition. In both experiments, 5×10^5 murine A/J mammary tumor (TA3 MTXR) cells were inoculated s.c. in A/J female mice, five animals per group. **(A,B)** Together with tumor cells, and every other day, the animals were inoculated s.c. with 50 μg TcCRT or HuCRT or solvent. While TcCRT had a similar anti-tumor effect in both experiments ($p = 0.0078$), HuCRT did not show that effect under these conditions (13). In both experiments, the tumor size was determined with a digital caliper (Mitutoyo Corp., Japan), in a double blind procedure. The formula ($\pi/6 \times \text{length} \times \text{width}^2$) was used. Data were statistically validated by Wilcoxon Signed Rank test, GraphPad Prism 4. Reproduced with permission from PLoS Neglected Tropical Diseases (13).

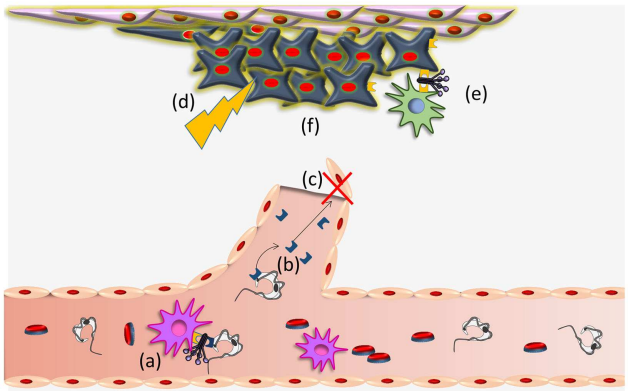

FIGURE 2 | *Trypanosoma cruzi* CRT participates in infectivity and anti-tumor process. (a) TcCRT, exposed on the parasite surface, binds C1q thus inhibiting the classical pathway of the complement system. TcCRT/C1q interaction participates in the infectivity process binding CRT present on mammalian cells. (b) TcCRT is translocated to the parasite surface and secreted. This TcCRT in the extracellular milieu binds to endothelial cells, (c) inhibiting angiogenesis. (d) This inhibition provokes a stressful environment in the tumor (decreased nutrients and oxygen supply, accumulation of metabolic waste products, etc.) (e) that induces CRT exteriorization on tumor cells. External tumor CRT captures C1, a signal that increases phagocytosis of tumor cells and consequent immunogenicity and (f) reduction of the tumor growth. Whether TcCRT also binds tumor cells *in vivo*, thus promoting tumor immunogenicity, has not been demonstrated.

Perhaps the TcCRT antiangiogenic effects reflect a parasite evolutionary adaptation to protect its host integrity and, as a necessary consequence, its own (71). Concomitantly, by decreasing angiogenesis, access of immunocompetent cells to the sites of parasite locations may be impaired, as well as subsequent inflammatory consequences, both with possible benefits to the aggressor, although the second strategy could also benefit the host from exaggerated immune reactivity.

The ability of TcCRT to delay solid tumor growth may represent an evolutionary adaptation with consequences in host survival and increased possibilities for the parasite to expand its genome. Based on fundamental Darwinian principles, cancer (i.e., mammary, cervix-uterine, prostate, lung, stomach, among others), taken altogether, have prevalence equivalent to an epidemic. These cancers may have exerted a selective pressure on the parasites, to develop molecular mechanisms to protect their hosts. Our experimental evidences indicate that externalized TcCRT, through its antiangiogenic properties may explain, at least in important part, such mechanisms (70).

Trypanosoma cruzi CRT-mediated antiangiogenesis, may provoke a stressful environment in the tumor (decreased nutrients and oxygen supply, accumulation of metabolic waste products, etc.) that induce CRT exteriorization on tumor cells. External CRT captures C1, a signal that increases phagocytosis of tumor cells and consequent immunogenicity (16). These possibilities are summarized in **Figure 2**. Other stressful agents (i.e., UV, anthracyclins) (39–42, 81) also mediate CRT translocation with similar immune stimulating consequences. The possibility that a concomitant immune boost, mediated by other means, is promoted by the infection (23), is also conceivable.

Finally, given the current evidences, the old proposal that tumor and parasites compete for nutrients with consequent inhibition of the former (20), now seems less likely.

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The endoplasmic reticulum chaperone GRP170: from immunobiology to cancer therapeutics

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Glucose-regulated protein 170 (GRP170) is the largest member of glucose-regulated protein family that resides in the endoplasmic reticulum (ER). As a component of the ER chaperone network, GRP170 assists in protein folding, assembly, and transportation of secretory or transmembrane proteins. The well documented cytoprotective activity of intracellular GRP170 due to its intrinsic chaperoning property has been shown to provide a survival benefit in cancer cells during tumor progression or metastasis. Accumulating evidence shows that extracellular GRP170 displays a superior capacity in delivering tumor antigens to specialized antigen-presenting cells for cross-presentation, resulting in generation of an anti-tumor immune response dependent on cytotoxic $CD8^+$ T cells. This unique feature of GRP170 provides a molecular basis for using GRP170 as an immunostimulatory adjuvant to develop a recombinant vaccine for therapeutic immunization against cancers. This review summarizes the latest findings in understanding the biological effects of GRP170 on cell functions and tumor progression. The immunomodulating activities of GRP170 during interactions with the innate and adaptive arms of the immune system as well as its therapeutic applications in cancer immunotherapy will be discussed.

Keywords: endoplasmic reticulum, glucose-regulated protein 170, molecular chaperone, anti-tumor immunity, cancer vaccine

INTRODUCTION

The endoplasmic reticulum (ER) is the key organelle that plays a critical role in many cellular processes, including protein synthesis, post-translational modification, and proper folding (1). Molecular chaperones in the ER lumen, through non-covalent interactions with their client proteins, catalyze or regulate protein folding, complex formation, protein translocation, or degradation (2–6). The physiological and pathological stress conditions that disturb the highly oxidizing and calcium-rich ER environment can trigger unfolded protein response (UPR) (7, 8). The UPR is a protein quality control mechanism that aims to limit ER stress and restore ER homeostasis, in part by inducing the elevation of ER chaperones, which enhance the protein folding/refolding capacity of the ER and target misfolded proteins to the ER-associated degradation (ERAD) pathway for degradation (9, 10).

Glucose-regulated proteins (GRPs) are among the most abundant and well-characterized ER chaperones (10). As stress-inducible chaperones, GRPs were originally discovered in mammalian cells undergoing glucose deprivation (11–13). GRPs are functionally and structurally related to the heat shock proteins (HSPs) and belong to the HSP family (14–16). Unlike most of the HSPs that reside mainly in the cytosol and the nucleus, the GRPs are predominantly in the ER (15, 16). Major members of the GRP family include GRP78/Bip, GRP94/Gp96, and GRP170 [also known as oxygen-regulated protein (ORP) 150 and HYOU1] (15, 16). These GRPs are often induced by stressors that perturb the ER functions, e.g., hypoxia, nutrient deprivation, reducing agents, calcium depletion, low PH, hyper-proliferation, or viral infection (16, 17). Due to their cytoprotective and pro-survival activities (18–21), GRPs have been extensively studied in the context of cancer development and progression, including cellular signaling, proliferation, apoptosis, angiogenesis, metastasis, and resistance to therapeutics (10, 15, 22, 23). To target the autonomous tumor-promoting effect of intracellular GRPs, many approaches or agents are being developed and tested for their anticancer efficacy in the experimental models and in the clinic (15). Over the last two decades, a wealth of studies has demonstrated novel aspects of GRP functions, intracellularly or extracelluarly, in regulating innate and adaptive immune responses during interactions with the host immune system. This has provided new opportunities to develop

immune-based strategies for cancer treatment (16, 24–26). As the largest GRP and molecular chaperone in the ER, GRP170 has been less studied compared to other members in the same class. In this review, we highlight recent progress in chaperoning-based diverse activity of GRP170, and discuss the potential applications of exploiting the immunological features of this molecule to design novel anticancer therapeutics.

Grp170 AND ITS CHAPERONING PROPERTY

Grp170 was initially found in the early 1980s during a study of GRP induction by glucose starvation (13). More than 10 years later, we cloned the cDNA of mammalian GRP170 from Chinese Hamster Ovary cells (27). GRP170 has also been referred to as ORP 150 (28), which indeed is the unglycosylated form of GRP170 (28). Sequence analysis indicated that GRP170 represents a new stress protein family that is distantly related to, but different from, both HSP70 and HSP110 families (27). The HSP70, HSP110, and GRP170 families have been classified into the "HSP70 Super-Family" (14, 29). GRP170 consists of 999 amino acids, encoded by hypoxia upregulated 1 gene (*Hyou1*) that is located on the q arm of chromosome 11. Beside glucose starvation as a classical inducer of GRPs, including GRP170, other stressors, such as hypoxia, ischemia, perturbation of calcium homeostasis, proteasome inhibitors, and non-steroidal anti-inflammatory drugs (e.g., celecoxib) are also known to upregulate GRP170 expression (13, 30–36).

Predicted secondary structural modeling indicated that the overall organization of GRP170 is similar to that of HSP70 and HSP110, but with very little similarity in C-terminal regions (14, 27, 29, 37). GRP170 also has a high degree of homology to GRP78, the ER homolog of HSP70, as they all possess an N-terminal nucleotide binding domain (NBD) followed by a β-sheet domain, which acts as the substrate binding domain (SBD) and an α-helical domain at the C terminus. The increased size of GRP170 is due to the insertion of an acidic loop in their β-sheet domain and an extended C terminus following the α-helical domain (14, 38). It has long been known that GRP170 associates with the other major GRPs (e.g., GRP78) in the ER and interacts with immunoglobulin chains (39, 40), implicating its role in protein folding or assembly in concert with other GRP or ER chaperones. GRP170 was shown to be the most efficient ATP-binding protein in microsomal extract, and was suggested to assist the translocation of polypeptides into the ER via the transporter associated with antigen processing (TAP) (41, 42). The yeast counterpart of GRP170 (Lhs1; lumenal HSP70) displayed similar activity in transporting proteins into the ER (29, 43, 44). Biochemical studies demonstrate that GRP170 is significantly more effective in blocking heat-induced protein aggregation than other stress proteins or chaperones (37, 45–48), underscoring a superior chaperoning capacity of this only ER member of the large HSP70 family.

Protein folding mediated by the GRP78-centered chaperoning machinery in the ER is regulated by its bound nucleotide, i.e., cycling of ATP and ADP (49). ADP-bound GRP78 has a high affinity for unfolded or incompletely folded proteins, while exchange of ADP for ATP decreases the affinity of GRP78 for substrates lead to release of folded protein substrate (49, 50). Surprisingly, Grp170 was recently reported to function as a nucleotide exchange factor (NEF) for GRP78 in the ER (50–52), which also raised the question of GRP170 being an independent chaperone molecule. A recent study confirmed that Grp170 can directly bind to a variety of incompletely folded protein substrates *in vivo*, although the regulation of its substrate binding function is different than for conventional HSP70 (53). GRP170 and GRP78 can associate with similar molecular forms of two substrate proteins. However, while GRP78 is released from unfolded substrates in the presence of ATP, GRP170 remains bound (53), suggesting that binding of same substrate to different GRPs may result in distinct fates for their client proteins. These data further established the GRP170 as a bona fide chaperone. More studies are necessary to address the question as to why GRP170 in the ER possess the dual ability to bind to substrates or client proteins and to have NEF activity (54).

ER STRESS AND GRP170-CONFERRED CYTOPROTECTION

In addition to playing important roles in protein modification and folding, the ER as a major organelle also integrates and coordinates cellular responses to a variety of stressors (55, 56). Disturbance of the ER functions by inhibiting protein glycosylation or disrupting calcium homeostasis, oxidative stress, pathogen infection, can result in the accumulation of unfolded or misfolded proteins in the ER that will trigger the UPR. The induction of GRPs and ER-resident chaperone molecules, including GRP170, often used as an indicator of the UPR, can help overcome the excessive protein loading and maintain or recover ER functions (7, 57).

There are three major signaling pathways involved in the canonical UPR upon ER stress, which includes pancreatic ER kinase (PERK), inositol-requiring transmembrane kinase/endonuclease-1 (IRE-1), and activating transcription factor-6 (ATF6) (7, 58). These ER stress sensors upon activation will initiate a cascade of molecular events that help diminish the protein load by inhibiting protein translation or by limiting the pool of mRNAs available to enter the ER. They can concurrently engage a transcriptional program to upregulate a number of genes, e.g., ER chaperones including GRPs, X-box binding protein 1 (XBP-1), and ERAD components, which are crucial for protein folding, amino acid metabolism, and protein degradation (7, 59–61). Activated ATF6 transported to the nucleus induces the transcription of the GRPs mRNA by binding to the ER stress response element (ERSE) in their genes (58). Activated PERK phosphorylates eukaryotic initiation factor 2α (eIF2α), resulting in translation of the ATF4, which also binds to the ERSE sequence to increase the expression GRPs (58). Both ATF6 and ATF4 have been reported to mediate the UPR-dependent induction of GRP170 due to the existence of ERSE in its gene (30, 31, 62, 63). The IRE1α induces the unconventional splicing of XBP-1 mRNA and production of the longer isoform of spliced XBP-1 (XBP-1s), which stimulates the transcription of ER chaperone genes, including GRP170 (58, 64, 65).

Several lines of evidences support a cytoprotective role of intracellular GRP170 in response to ER stress. GRP170 can limit oxidized low density lipoprotein (ox-LDL)-induced ER stress and prevent subsequent cell apoptosis (66, 67). GRP170 executes this protective activity by maintaining calcium homeostasis and blocking calcium signaling through IP3 channels (67). Cytoprotection conferred by induction of GRP170 has also been shown in cellular responses to other ER stressors, e.g., proteasome inhibitors that cause excessive protein accumulation (30, 68), hypoxia,

ischemia-reperfusion (69–72), and glutamate-induced cytotoxicity (73). However, the conventional UPR signaling is not the only molecular mechanism involved in the induction of GRP170. In a high fat diet-fed mouse model, AMP-activated protein kinase (AMPK) was reported to mediate the elevation of GRP170, which ameliorated hepatic ER stress and lipotoxic death. Forkhead box O1 (FOXO1), which can directly bind to the promoter region of GRP170 gene, was identified as the critical transcription factor mediating the AMPK-enhanced GRP170 expression at both the mRNA and protein levels in hepatocytes (74). However, an animal study of COX-2 inhibitor (i.e., parecoxib)-mediated neuroprotection from cerebral ischemic reperfusion injury showed that the elevation of GRP170 was observed in the presence of suppression of FOXO1 activation (75). GRP170 was found to directly bind to ER stress sensors, such as PERK, ATF6, and IRE1α in vascular cells (66) or PERK and eIF2α in hepatocytes (74), which suggests that the ER-resident GRP170 may be able to retain those ER stressors inside of ER and maintain them in an inactive state, thereby preventing the activation of ER stress.

GRP170 IN CANCER DEVELOPMENT AND PROGRESSION

Tumor development is associated with cell hyper-proliferation, protein overexpression, and emergence and accumulation of mutated or misfolded oncogenic proteins, which often induce overexpression of GRPs and other ER chaperone molecules in cancer cells (10, 15, 76). Elevation of chaperone molecules may be required for the maintenance of the functions of those proteins essential for tumorigenesis or invasion. The deprivation of glucose and hypoxic condition in the tumor microenvironment, caused by poor vascularization in most of the neoplastic tumors, can act as ER stressors that activate the UPR in cancer cells to promote their survival (77–79). It has been well documented that many ER chaperones, including GRP78, GRP94, and calreticulin (CRT), are capable of protecting cancer cells against ER stress-induced cell death (76, 80–84). Upregulation of GRP78 (85–88) and GRP94 (89–91), likely due to the adaptive UPR in cancer cells, have also been associated with the poor survival or recurrence in cancer patients as well as tumor resistance to radiotherapy.

The levels of GRP170 were also shown to correlate with cancer invasiveness and GRP170 was suggested to be a potential prognostic factor in human breast cancer (92). In addition to altered expression of GRP170 in the different stage of breast cancer, the upregulation of GRP170 correlated with tumor lymph node invasion and decreased expression of estrogen receptor (93), implicating its potential involvement in cancer metastasis. Beside cytoprotection or resistance to cell death conferred by GRP170, other activities of GRP170 during tumor progression have been elucidated. Angiogenesis, formation of new capillaries from pre-existing vessels, is an important process in tumor growth and metastasis. GRP170 was shown to be required for the angiogenesis of C6 glioma tumors by facilitating the processing and secretion of vascular endothelial growth factor (VEGF), a major pro-angiogenic factor (94). Similarly, suppression of GRP170 using an antisense approach reduced the tumorigenicity of human prostate cancer cells through blocking of secretion of matured VEGF (95). GRP170 in bladder cancer cells was also found to chaperone matrix metalloproteinase-2 (MMP-2) for secretion, thereby promoting tumor invasion (96). While additional studies are needed to better understand the precise contribution of GRP170 in tumorigenesis, the chaperoning property appears to be a main underlying mechanism involved in its pro-tumor activity (**Figure 1**). Interestingly, it was recently found that ER-stressed tumors could propagate the stress signals to the neighboring cells (e.g., macrophages) via secretion of soluble mediators, which lead to an amplified inflammatory response that facilitates tumor progression (10, 97). It is not clear as to whether GRPs or ER chaperones in cancer cells contribute to this pro-inflammatory and pro-tumoral effect.

CANCER IMMUNOGENICITY ALTERED BY COMPARTMENTALIZATION OF GRP170

Glucose-regulated proteins or ER chaperones are initially considered to be exclusively intracellular proteins that only released into extracellular environment upon cell injury (98). GRP170, like other GRPs, resides normally in the lumen of ER due to an ER-retention signal, KNDEL, at its carboxyl terminus. However, it is now apparent that ER chaperones can be present on the plasma membrane or actively secreted into the extracellular environment (10, 99–101). The differential localizations of GRPs could potentially have distinct impact on cellular activities and the host response. The cell surface GRP78 acts as a multifunctional receptor that promotes cancer cell survival and proliferation by activating ERK and AKT (102, 103), PI3k (104), or NF-κB and AKT (105). In contrast, tumor cells forced to secrete GRP78 resulted in a tumor-reactive immune response and tumor rejection (106). Another ER chaperone CRT was recently reported to translocation to the tumor cell surface upon exposure to chemotherapeutic agents or ionizing irradiation (107, 108). The surface CRT serves as an "eat me" signal that triggers increased phagocytosis of dying tumor cells, cross-presentation of tumor antigens, and consequent anti-tumor immune response (109).

We have performed studies to determine the impact of extracellular secretion of GRP170 on tumorigenicity. In this regard, murine B16 melanoma cells (47), TRAMP-C2 prostate cancer cells (110), or CT26 colorectal cancer cells (111) were forced to express a secretable form of GRP170, in which its ER-retention sequence "KNDEL" has been depleted. We found that these cancer cells secreting GRP170 did not differ from their mock-treated controls in cell proliferation *in vitro*. However, the tumor growth was markedly suppressed *in vivo*, which was dependent on the presence of cytotoxic $CD8^+$ T lymphocytes (CTLs) and/or natural killer (NK) cells. This secretory GRP170 not only acted as a "danger" signal stimulating specialized antigen-presenting cells (APCs), such as dendritic cells (DCs), but also delivered tumor-derived antigens via its intrinsic chaperoning activity for priming antigen-specific CTLs (47). Using mass spectrometry analysis, we demonstrated that this secreted GRP170 was associated with tumor protein antigens (111), which is consistent with the intracellular chaperoning function of GRPs or ER chaperones that are essential for the activity of oncoproteins in cancer cells.

The studies of cell surface GRP94 (112) or GRP94 secreted from tumor cells (113–115) support our findings by demonstrating that the exposure of GRP94 to the immune system represents a highly

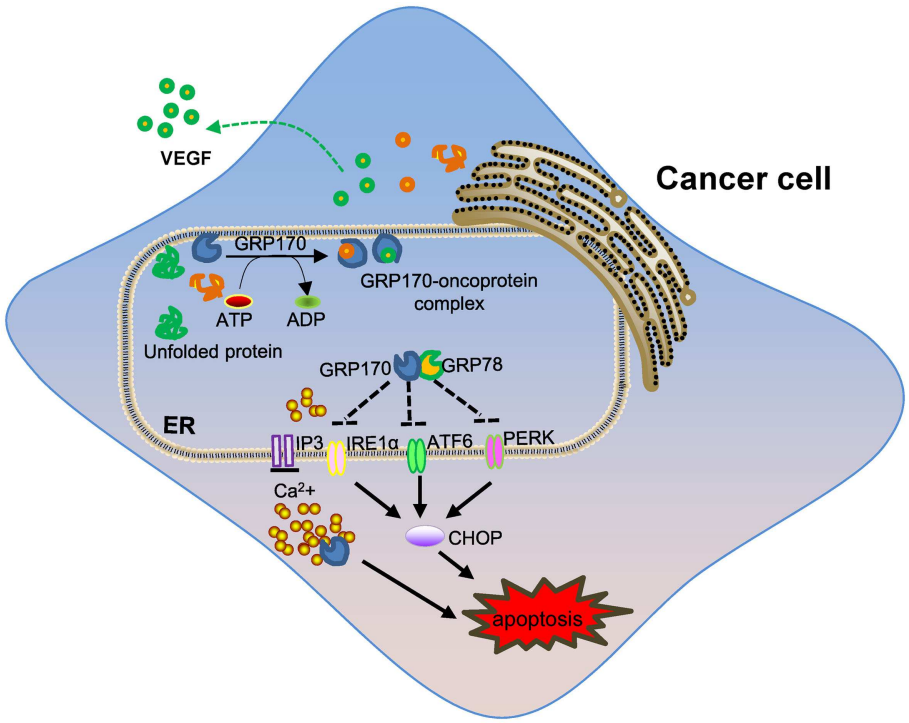

FIGURE 1 | The role of intracellular GRP170 in tumor development. ER-resident GRP170 together with other GRPs (e.g., GRP78) or chaperone molecules participate in post-translational modification and protein folding or transportation. Under stress conditions (e.g., ER stress), GRP170 exhibits cytoprotective activity by preventing protein aggregation or help protein repair and maintaining calcium homeostasis in the ER. GRP170 can directly bind to and possibly keep the ER stress sensors (e.g., PERK, ATF6, and IRE1α) in quiescent inactivate state to limit ER stress. The pro-survival effect of intracellular GRP170 and chaperoning of oncogenic or tumor-promoting factors (e.g., VEGF) may contribute to tumor progression and invasion.

immunogenic signal capable of inducing a potent anti-tumor immune response. Further support came from a study showing that enforced cell surface expression of GRP94 in a transgenic mouse conferred hyper-responsiveness to LPS and induced lupus-like autoimmune syndrome (116, 117). Thus, GRP170 or other ER chaperones may display dual biological or immunological activities during tumor progression or in response to therapeutic treatments, which depends on individual GRPs as well as their cellular compartmentalization. Intracellular or ER-resident GRPs play a general protective role that promotes the survival of cancer cells in stress or lethal conditions. However, the membrane-bound or extracellular GRPs could alter the immunogenicity of cancer cells and facilitate the immune recognition as well as immune-mediated destruction of cancers (10, 15, 16, 118–120). Manipulating cellular compartmentalization of GRP170 may be used to induce or restore protective anti-tumor immunity for cancer eradication. Genetically modified cancer cells with a capacity to produce secretory GRP170 have been successfully tested as a cell-based vaccine to generate a therapeutic anti-tumor response to the established tumors in mice (47, 110). We also demonstrated that intratumoral delivery of secretory GRP170 via an adenovirus promoted the anti-tumor efficacy of melanoma differentiation-associated gene-7 (mda-7), a cancer-specific therapeutic cytokine, by mounting systemic anti-tumor immunity that controls treated as well as distant untreated tumor lesions (121).

GRP170 AS AN IMMUNE ADJUVANT OF CANCER VACCINE

Glucose-regulated protein preparations or ER chaperones-derived from tumors are believed to carry individually distinct array of tumor antigens, which can be utilized to provoke a tumor-specific immune response in cancer vaccination or immunotherapy (25, 122–128). Our early studies showed that animals immunized with GRP170 purified from various murine tumors (e.g., colon tumor, melanoma, and fibrosarcoma) developed a robust anti-tumor immune response (126, 129). Tumor-derived GRP170, when compared to other chaperones, displayed a higher anti-tumor potency (129), which, we believe, is attributed to its superior protein or antigen-holding capacity (48). The substantially increased size of GRP170 due to the extension of its C-terminal domain could be a factor, which enables it to bind to and chaperone protein clients or antigens more efficiently (14, 16). Indeed, two independent regions in GRP170, i.e., the classical peptide-binding β-sheet domain and the C-terminal α-helix domain, have been identified that can execute chaperoning activities (37).

To overcome the hurdle of preparing autologous GRP vaccines for clinical use, which can be limited by a requirement of patient specimen and laborious procedures of vaccine production (130, 131), we have developed a recombinant chaperoning technology that exploits the exceptional antigen-holding capacity of GRP170 (132). The reconstituted chaperone complex of GRP170 and melanoma-associated antigen gp100 has been used

as a targeted vaccine to generate a strong anti-tumor immune response to aggressive, poorly immunogenic B16 melanoma in mice (132). Similar observations have been made in other vaccination studies that employed GRP170 to target different tumor antigens (133, 134). This recombinant vaccine approach has several advantages over autologous vaccines. The GRP170-antigen complex vaccine can be prepared in large quantities for use off-the-shelf. A large reservoir of antigenic epitopes in a protein antigen can stimulate polyepitope-directed T cells and potentially enhance the strength of the anti-tumor effect. Our recent comparison study of GRP170-protein antigen complex vs. GRP170-peptide antigen in the setting of therapeutic immunization against cancer strongly supports the idea of including protein antigen in this vaccine regimen (48). Use of defined antigenic target should also facilitate the monitoring of antigen-specific immune responses in the clinic. Additionally, the highly efficient antigen-holding property will permit the development of a multivalent vaccine against different antigenic targets (48).

EXTRACELLULAR GRP170 AND ANTIGEN CROSS-PRESENTATION

Dendritic cells are one of most efficient APCs for processing and presenting antigens to T lymphocytes. Cross-priming of the $CD8^+$ T cells by DCs plays a crucial role in the induction of antiviral and anti-tumor immune responses. The cross-presentation efficacy of DCs is determined by many factors, including their ability to capture antigens, the route of antigen uptake and trafficking, antigen stability, and the pathways by which processed antigen is loaded on the MHC class I molecules. Generally, two models have been proposed for antigen cross-presentation, i.e., the vacuolar pathway and the cytosolic pathway (135, 136). In the vacuolar pathway, internalized antigens remain in endolysosomal/phagosomal compartments, where they are degraded and loaded onto the recycling MHC class I molecules (137–139). In the cytosolic pathway, the endocytosed antigens are transported from endosomes or phagosomes into the cytosol for proteasome-dependent degradation, followed by peptide import and loading onto MHC class I molecules in the ER (140–143).

The choice of adjuvant is critical in the success of antigen-targeted, protein-based cancer vaccines because soluble protein antigens are typically poorly cross-presented by DCs. One of major tenets in the vaccine activity of GRPs and other chaperone molecules, including GRP170, is their high efficiency to introduce antigens into the endogenous antigen-processing pathway of APCs for cross-presentation and activation of CTLs (48, 132, 144–147). The interaction of intracellular GRP170 with TAP in the early studies suggested that endogenous GRP170 may assist with ER translocation of peptides (41, 42). Two scavenger receptors, SR-A and SREC-I, have been identified to contribute to the binding of GRP170 on APCs (148). However, the mechanism of cross-presentation enhanced by exogenously delivered GRP170 in the context of tumor vaccination is poorly defined.

Using a clinically relevant melanoma antigen gp100 carried by GRP170, we recently investigated the trafficking pathways of GRP170–gp100 complex in DCs. Surprisingly, we found that the GRP170 directed and enhanced gp100 efficiently to access the ER after their internalization. GRP170-facilitated gp100 processing and presentation was dependent on the ERAD machinery involving Sec61, which was shown to target gp100 for ubiquitination and degradation in the cytosol by the proteasome system and subsequent integration into the conventional MHC class I restricted antigen-processing pathway (149). Our data indicated that GRP170 can help the associated protein antigen escape from lysosomal degradation and shuttle the antigen into the ER compartment from the early endosomal compartment. Internalized GRP170 might be directly involved in the ERAD following vaccine uptake, because GRP170 in the complex enhanced the interaction of gp100 with several ERAD molecules (e.g., Sec61α, VCP/97, CHIP, and GRP78). We speculate that gp100 protein that is partially unfolded during the vaccine preparation and chaperoned by GRP170 serves as an ERAD target once accessing the ER. Since endogenous GRP170 also binds to Sec61α, it is likely that internalized GRP170 could become a part of the ER chaperone network and collaborate with other GRPs to guide retrotranslocation of gp100. The GRP170/Lhs in yeast was recently reported to facilitate the ERAD of the epithelial sodium channel by preferentially targeting the unglycosylated form of the protein, which relied on its holding function not NEF activity (150). In our studies, the transient co-localization of ER markers with early endosome marker suggested an interesting possibility of formation of ER/endosome fusion structure after vaccine captured by DCs, which may explain the route of the ER access of GRP170–gp100 complex from the extracellular environment. Intriguingly, the GRP170-peptide antigen complex was recently found to be transported into early and recycling endosome compartments, where antigen was processed (151). It appears that the distinct trafficking patterns are caused by the size or nature of the antigens (protein vs. peptide) chaperoned by GRP170 in vaccines.

ER-associated degradation is an essential protein quality control mechanism in the ER that retrotranslocates unfolded or misfolded proteins to cytosol for degradation in response to ER stress (152, 153). ERAD involving Sec61 and chaperone molecules have been implicated in the cytosolic pathway of antigen cross-presentation (137, 154). Several lines of evidence suggest that the ERAD components are present on or can be recruited to the endosome/phagosome in APCs to facilitate cytosolic translocation of antigen (155–158). However, ER access and ERAD-mediated processing of GRP170–antigen complex in the setting of therapeutic vaccination warrant more studies, which will result in a better understanding of the action of this molecular adjuvant and the optimization of GRP170-based targeted cancer vaccination strategies.

EXTRACELLULAR GRP170 AS AN ALARMIN AND INNATE IMMUNITY

Upon release from injured or stressed cells, certain chaperone molecules, including GRPs, are suggested to serve as alarmins or damage-associated molecular patterns to alert the host immune systems of cell or tissue stress and trauma (159, 160). It has been well established that GRP170-dependent tumor immunization offers effective treatment of malignancies in the experimental models. The acquired immunity enhanced by GRP170 through shuttling and presenting tumor antigens for T cell cross-priming is an essential component of this process. The previous studies

showed that GRP170 bound to DCs in a receptor-mediated fashion (i.e., scavenger receptors SR-A and SREC-I) (148) and GRP170 by itself could modestly induce DCs to upregulate MHC class II and co-stimulatory molecules (e.g., CD86) (120). Binding of GRP170 to DCs also stimulated them to produce pro-inflammatory cytokines (120). Although this stimulatory effect appears to be modest, it distinguishes GRP170 or other GRPs from other conventional adjuvants in vaccine design and formulation.

Vaccine adjuvants can be functionally divided into two major groups, toll-like receptor (TLR)-dependent and TLR-independent adjuvants (161, 162). TLR-dependent adjuvants, such as the Bacillus Calmette–Guerin (BCG) that is recognized by TLR2 and TLR4, act directly on DCs and promote their maturation and migration to the T cell area of the lymph node (161, 162). TLR-independent adjuvants, e.g., alum, increase antigen availability at injection site by adsorption and entrapment of antigens (163, 164). GRP170 along with other chaperone molecules, as self-proteins of mammalian origin, may be considered as the third functional group of adjuvants, because they increase the immunogenicity of antigens via preferentially delivering antigen cargos to DCs and enhancing antigen cross-presentation by DCs (120, 132, 148, 149, 165).

In addition to chaperoning intracellular antigenic polypeptides, GRP170 can efficiently bind to foreign pathogen-associated molecular patterns (PAMPs) in the extracellular environment and enhance the host response to pathogens. We recently showed that GRP170 interacts with microbial DNA, e.g., CpG oligodeoxynucleotides (CpG-ODN), a ligand for TLR9 (166). Chaperoning of CpG-ODN by extracellular GRP170 resulted in markedly increased internalization of CpG-ODN by macrophages. The internalized GRP170 was seen to directly associate with endosomal TLR9, suggesting that GRP170 chaperoning may enhance the sensing of CpG-ODN by its receptor (i.e., TLR9). As a result, complexing of CpG-ODN with GRP170 leads to enhanced activation of the MyD88-dependent signaling cascade and production of pro-inflammatory cytokines for pathogen clearance (166). Indeed, GRP170-amplified innate immune response protected mice from challenge with *Listeria monocytogenes* (166). These results revealed a previously unrecognized attribute of GRP170 as a superior DNA-binding chaperone. More importantly, the interaction of an evolutionarily conserved chaperone molecule with PAMPs in the extracellular milieu may play a critical role in the host response to pathogen. Interestingly, other than internalized GRP170, TLR9 was associated with major endogenous GRPs, including GRP170, GRP94, and GRP78 (166), suggesting that the outside-in GRP170 may function in concert with intracellular chaperone networks in modifying TLR9 signaling. This result, together with a recent work showing a critical requirement of the chaperoning of TLR9 by intracellular GRP94 for TLR9 functions (167), offers new insight into the dynamics of ancient chaperoning functions inside and outside the cell. Given that CpG-ODN can be used as an immunostimulatory adjuvant in cancer vaccination (168), the unique characteristics of GRP170 in amplifying CpG-ODN-induced immune activation provide a scientific rationale for including the CpG-ODN as a component in the recombinant GRP170 vaccine regimen for cancer immunotherapy.

Among all the biological and immunological activities of extracellular GRP170, e.g., enhanced endocytosis of protein antigen or CpG-ODN, increased ER access of protein antigen, increased association with TLR9, all these processes seem to intimately involve the intrinsic chaperoning property of GRP170. During investigation of vaccine potential of various deletion mutant of GRP170 (37), we found that only chaperoning competent mutants exhibited APC binding activities and could deliver tumor antigen (e.g., gp100) for inducing an antigen-specific anti-tumor immunity (132). Interestingly, two of chaperoning competent GRP170 mutants, although both contained no overlapping sequences, could still bind to APCs in a receptor-mediated fashion and stimulate tumor-inhibiting CTL response. Together, these findings support the notion that the ancient chaperoning property is the key denominator underlying the diverse biological and immunological effects of GRP170 and possibly those other immunostimulatory GRPs (**Figure 2**).

ARMING GRP170 WITH A PATHOGEN-DERIVED "DANGER" SIGNAL FOR IMPROVED ANTI-TUMOR POTENCY

Coupling antigen and an immunostimulating "danger" signal into the same vaccine delivery cargo is crucial for optimal antigen cross-presentation by DCs and priming of antigen-reactive T cells (169, 170). While certain chaperone molecules in the extracellular environment, including GRP170, possess direct immunostimulatory activity during interaction with APCs, they do not activate an innate immune response as efficiently or robustly as PAMPs, which strongly promote a vaccine response (171, 172). The modest innate-stimulating effect of GRP170 may not be sufficient to fully activate antigen-exposed APCs *in vivo*. We hypothesized that incorporating a pathogen-derived "danger" signal into the GRP170 backbone would enhance its immunostimulatory potency in therapeutic immunization against cancer. To test this concept, we engineered a chimeric chaperone, termed Flagrp170, by fusing GRP170 with the defined NF-κB-activating domain of Flagellin (173).

Flagellin is the principal substituent of bacterial flagella and the ligand for TLR5 (174–176). Since the NF-κB pathways in DCs are essential for its optimal functions (169, 177–179), this chimeric chaperone possesses two distinct features that are required for efficient cancer vaccine therapy: enhancing the cross-presentation of tumor antigens in the chaperone complex cargo and concurrently provoking the functional activation of DCs via engaging the NF-κB signaling (173). As expected, Flagrp170 strongly activated DCs, indicated by elevation of co-stimulatory molecules, such as CD40 and CD86, as well as production of pro-inflammatory and Th1-polarizing cytokine IL-12. Since only a small portion of Flagellin was present in the construct, it was surprising that Flagrp170 exhibited a similar effect as Flagellin in stimulating NF-κB and MAPK signaling, as well as phenotypic activation of DCs. This might be due to the ability of GRP170 to amplify the innate immune response, as we observed in the study of GRP170 interaction with CpG-ODN (166). Moreover, Flagrp170 was much more efficacious than Flagellin in promoting antigen cross-presentation, which can be explained by the superior intrinsic property of GRP170 in antigen shuttling and T cell cross-priming (149).

Intratumoral delivery of Flagrp170 using an adenovirus induced a superior anti-tumor response against treated B16 melanoma and distant lung metastases compared with unmodified GRP170 or Flagellin treatment (173), which indicates systemic

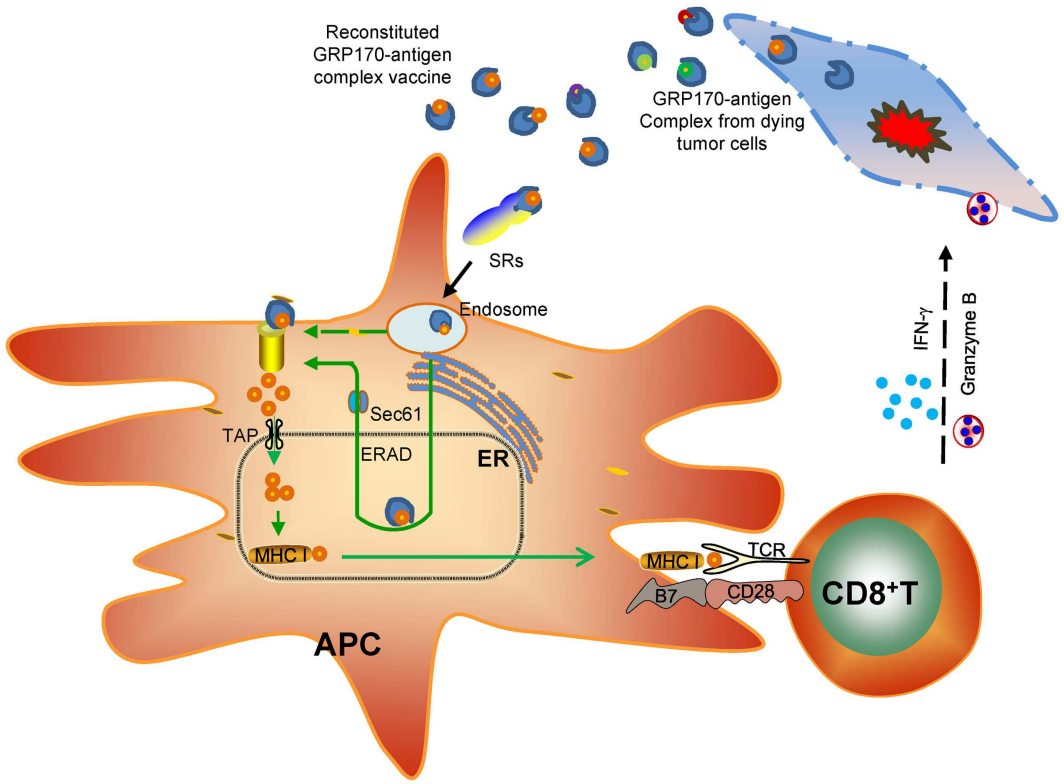

FIGURE 2 | Chaperoning-based immunological effects of extracellular GRP170 in cancer therapy. GRP170 isolated or released from cancer cells due to stress or injury is believed to chaperone tumor antigens. These tumor-derived GRP170-antigen complexes in the extracellular environment or reconstituted recombinant GRP170-antigen complex vaccine can be captured preferentially by specialized antigen-presenting cells (APCs) through the surface receptors (e.g., scavenger receptors, SRs). The GRP170 can direct the chaperone complexes to the endoplasmic reticulum (ER) and facilitate their interaction with the components of ER-associated degradation (ERAD) machinery (e.g., Sec61α). The antigen target will then be retrotranslocated to the cytosol for ubiquitination and proteasome-mediated processing. The generated antigenic peptides are transported by TAP and loaded on the MHC class I molecules. The MHC I-peptide cargo will traffick to the cell surface and prime $CD8^+$ T cells. Activation and expansion of antigen-specific $CD8^+$ T cells leads to eradication of antigen-positive tumor cells by releasing cytotoxic molecules (e.g., IFN-γ, granzyme B).

mobilization of tumor-reactive immune effector cells. Flagrp170 treatment was shown to drive Th1 polarization of the tumor microenvironment, characterized by high levels of IL-12 and IFN-γ, as well as tumor-infiltrating $CD8^+$ and NK cells (173). Mechanistic studies showed that depletion of $CD11c^+$ cells or lack of $CD8\alpha^+$ DCs attenuated the anti-tumor response generated by Flagrp170 therapy, suggesting that Flagrp170-enhanced activation of tumor-specific CTLs depends on these DCs for efficient antigen cross-presentation (173). Interestingly, Flagrp170 selectively activated the NF-κB signaling pathway in DCs, not in tumor cells, which suggests that Flagrp170 represents an ideal agent that may be exploited to condition the immunosuppressive tumor environment and to break immune tolerance established during tumor development or progression.

CONCLUSION

Glucose-regulated protein 170, as one of the largest GRPs and chaperone molecules in the ER, can protect cells during ER stress-triggered UPR and in other stressful and lethal conditions. The cytoprotective activity of GRP170 is also reflected in its elevation in certain cancer cells and resultant resistance of tumor cells to the induction of cell death, which supports a potential tumor-promoting role of GRP170 during cancer progression. While the intracellular chaperoning and NEF functions of GRP170 remain to be further defined, accumulating evidence has highlighted an immunoregulatory effect of GRP170 in the extracellular environment, indicated by its superior capacity in holding protein tumor antigens, facilitating antigen cross-presentation, enhancing T cell priming, and amplifying an innate immune response. These unique features have been exploited to develop GRP170 chaperone complex vaccine directed against defined antigenic target in cancers. The successful results derived from preclinical models have led to an ongoing phase I clinical trial of testing recombinant chaperone vaccine in melanoma patients. While this vaccine approach holds promise, more studies are needed to better understand the adjuvant action of GRP170 in therapeutic immunization. Studies are also needed with regard to the contributions of other DC subsets in GRP170-enhanced antigen cross-presentation, such as lymphoid organ resident $CD8\alpha^+$ DCs and dermal migratory $CD103^+$ DCs (180–183). Recently, the role of the IRE1α–XBP-1 pathway has been extended beyond UPR and was shown to be required for the differentiation of effector

$CD8^+$ T cells and development or function of DCs (184–186). It is of interest to explore the potential involvement of GRP170 and other GRPs in this context because both intracellular and extracellular GRPs are likely to actively participate in these processes given their documented roles in antigen transportation, processing, and presentation. Integrating a NF-κB-stimulating "danger" signal into the GRP170-based delivery cargo strongly enhances its immunostimulatory and anti-tumor efficacy, which warrants the future studies of Flagrp170 as a novel immunomodulating agent either alone or combined with conventional treatment modalities (chemotherapy and radiotherapy) to restore anti-tumor immunity in the tumor site to achieve *in situ* vaccination. It is also conceivable that this engineered GRP170 molecule can be used to design the new generation of targeted chaperone vaccine to deliver tumor protein antigens for the treatment of metastatic malignancies.

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Calreticulin as cancer treatment adjuvant: combination with photodynamic therapy and photodynamic therapy-generated vaccines

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Calreticulin is recognized as one of the pivotal damage-associated molecular pattern molecules alerting the host of the presence of distressed cells. In this role, calreticulin becomes exposed on the surface of tumor cells treated by several types of cancer therapy including photodynamic therapy (PDT). The goal of the present study was to examine the potential of externally added calreticulin for augmenting antitumor effect mediated by PDT. Recombinant calreticulin was found to bind to mouse SCCVII tumor cells treated by PDT. Compared to the outcome with PDT alone, cure rates of SCCVII tumors grown in immunocompetent C3H/HeN mice were elevated when calreticulin (0.4 mg/mouse) was injected peritumorally immediately after PDT. Such therapeutic gain with PDT plus calreticulin combination was not obtained with SCCVII tumors growing in immunodeficient NOD-scid mice. In PDT-vaccine protocol, where PDT-treated SCCVII cells are used for vaccination of SCCVII tumor-bearing mice, adding recombinant calreticulin to cells before their injection produced improved therapeutic effect. The expression of calreticulin gene was reduced in PDT-treated cells, while no changes were observed with the expression of this gene in tumor, liver, and spleen tissues in PDT-vaccine-treated mice. These findings reveal that externally added recombinant calreticulin can boost antitumor response elicited by PDT or PDT-generated vaccines, and can thus serve as an effective adjuvant for cancer treatment with PDT and probably other cancer cell stress-inducing modalities.

Keywords: calreticulin, photodynamic therapy, cancer vaccine, DAMPs, antitumor-immune response

INTRODUCTION

Calreticulin is primarily ER-residing chaperone protein with a broad array of cellular functions, including maintenance of adequate calcium levels, protein folding and trafficking, gene transcription regulation, cell adherence and migration, apoptosis and dead cell clearance, and immune responses (1, 2). Cancer therapy-mediated lethal insults that involve stress induction in the ER induce the translocation of calreticulin to the outer leaflet of the plasma membrane by an active process occurring before the appearance of morphological signs of apoptosis (3, 4). Such surface-exposed calreticulin serves as a powerful mobilizing signal to the immune system, which has inspired recognition of calreticulin as one of the most important damage-associated molecular patterns (DAMPs) (5). As danger signals, DAMPs alert the host that immune response is required to restore homeostasis (3, 6). Surface calreticulin expression on dying cancer cells is critical for their demise by immunological cell death (ICD) resulting in immune rejection of tumors with the same cells (7).

Photodynamic therapy (PDT) belongs to cancer modalities capable of inducing an abundance of DAMPs including surface-exposed calreticulin (5, 8). This therapy is established clinically for treatment of various malignant and non-oncological lesions (9). It works by localized generation of reactive oxygen species mediated by the transfer of energy absorbed by light-activated drugs (photosensitizers) to molecular oxygen (10, 11). Photooxidative lesions induced in PDT-treated cells provoke a strong oxidative stress that with many photosensitizers includes the ER. Such a type of stress was suggested to lead to the escape of calreticulin and other ER molecules to the cell surface (4). Ceramide and sphingosine-1-phosphate are ER-derived molecules that were likewise found exposed on the surface of PDT-treated cells and were also shown to act as DAMPs (12, 13). With PDT mediated by photosensitizer chlorin e6 (ce6), we detected cell surface-exposed calreticulin as early as 15 min post-treatment (Korbelik, unpublished).

Abundant engagement after PDT of various DAMPs, both cancer cell surface expressed and released from treated cells was suggested as one of the key elements responsible for the capacity of this modality to elicit a strong immune response against treated tumor (14). This PDT-induced antitumor-immune response can be enhanced by a variety of immunostimulating treatments for achieving superior tumor cures (15). The present report describes how such approach can include recombinant calreticulin protein. The research questions to be addressed were: first, can the recombinant calreticulin be used as an effective adjuvant to PDT and/or PDT-generated vaccine therapy? Second, what investigative directives should be followed when designing research for the comprehensive elucidation of the underlying mechanism of action?

MATERIALS AND METHODS

TUMOR MODEL

The squamous cell carcinoma model SCCVII syngeneic to C3H/HeN mice, a recognized model of head and neck cancer of spontaneous origin with absence of strong immunogenicity (16), was implanted subcutaneously into lower dorsal site of 7–9-week-old C3H/HeN or NOD-scid mice. The tumors were treated with PDT vaccine when they reached 5 mm and with PDT *in situ* at 7–8 mm size in largest diameter. The procedures used with mice were in compliance with the protocols approved by the Animal Care Committee of the University of British Columbia. Cultures of SCCVII cells were maintained in alpha minimal essential medium supplemented with 10% fetal bovine serum (Life Technologies, Burlington, ON, Canada).

PREPARATION OF RECOMBINANT HUMAN CALRETICULIN

Native recombinant human calreticulin was generated using yeast as expression host based on the methodology developed in previous work (17, 18). Whole coding sequence of human calreticulin gene (GenBank accession no. M84739) was PCR-cloned from human adult liver cDNA library (Clontech Laboratories, Saint-Germain-en-Laye, France) using primers that generate *Xba*I restriction sites. The PCR product was digested and cloned into the *Xma*JI site of pPIC3.5K shuttle vector (Invitrogen, Life Technologies) under control of *Pichia pastoris* AOX1 promoter. The pPIC3.5K-CRT plasmid was transformed into *P. pastoris* GS115 (*his4*) (Invitrogen) by electroporation after linearization with *Bgl*II restriction endonuclease. Several multi-copy integrants grown on various geneticin concentrations were screened for secretion of human calreticulin. Recombinant *P. pastoris* strain (GS115:hCRT) with the optimal secretion of human calreticulin was chosen for further studies. It was initially grown in the Ygly medium (2% peptone from soybean, 1% yeast extract, 1% glycerin, 2×10^{-5}% biotin). After 24 h incubation, the cells were transferred into Ymet medium (2% peptone from soybean, 1% yeast extract, 1% methanol, 2×10^{-5}% biotin) and incubated for 120 h, with 1% methanol supplanted every 8 h. Following this induction period, cells were separated from the medium by centrifugation at 10,000 *g* for 20 min. Yeast growth medium was subjected to prefiltration and microfiltration through filters with pore size of 1.6, 0.45, and 0.2 μM (cat. nos. FT-3-1101-047, 15406-47 and 15407-47-MIN, respectively, SartoriusStedim Biotech, Goettingen, Germany). After microfiltration, proteins were concentrated and transferred into the binding buffer (20 mM L-histidine, pH 5.5, 100 mM NaCl) through tangential ultrafiltration using cassettes with 100 kDa cut-off membranes (cat. no.VF20P3, SartoriusStedim Biotech). Protein sample was loaded onto the Q Sepharose FastFlow column (cat. no. 17-0510-10, GE Healthcare, Freiburg, Germany). Unbound proteins were washed from the column with 10 column volumes of binding buffer, while bound proteins were eluted with 15 column volumes NaCl gradient (100–500 mM). In the final step, the elution fractions were analyzed by SDS-PAGE, and the fractions showing ≥ 90% pure human calreticulin were pooled and dialyzed against storage buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 3 mM $CaCl_2$). The SDS-PAGE gel with the final calreticulin preparation used in the experiments with cells and *in vivo* is shown in **Figure 2**.

PDT AND PDT-VACCINE TREATMENT

Two different treatments involving photosensitizing drug exposure followed by light application were performed using photosensitizers Temoporfin for PDT and chlorin e6 (ce6) for PDT vaccines in standard doses established in previous extensive investigations. The protocol used for PDT-generated vaccine treatment was described in detail earlier (19, 20). Briefly, for each treatment group (6 mice), 1.2×10^8 SCCVII cells were exposed to ce6 (purchased from Frontier Scientific Inc., Logan, UT, USA) for 30 min at 0.5 μg/ml followed by 1 J/cm^2 of 665 ± 10 nm light. The light, delivered through a liquid light guide (model 77638, Oriel Instruments, Stratford, CT, USA), was produced by a 150 W QTH lamp-based high-throughput source with an integrated ellipsoid reflector and interchangeable interference filters (model FB-QTH-3, Sciencetech Inc., London, ON, Canada). This PDT treatment was lethal for around 70% of cells, most of them dying by apoptosis. After PDT treatment, the cells were left overnight (16 h) at 37°C in EX-CELL chemically defined protein- and serum-free medium (Sigma Chemical Co., St. Louis, MO, USA). The cells were then collected, concentrated by centrifugation for treatment with X-rays (60 Gy) followed in some treatment groups by the exposure to recombinant rabbit calreticulin (Abcam Inc., Cambridge, MA, USA) at the concentration of 15 μg per vaccine dose for 30 min on ice. The vaccine cells were then used immediately for peritumoral injection (2×10^7 per mouse). As a part of the routine PDT-vaccine protocol, mice received low-dose cyclophosphamide (50 mg/kg i.p.) 24 h before vaccination (for controlling immunoregulatory cell activity). The therapeutic effect of PDT vaccines was assessed by monitoring changes in tumor size.

For PDT treatment of SCCVII tumors, the host mice were administered Temoporfin (active pharmaceutical ingredient of Foscan, provided by Biolitec Research GmbH, Jena, Germany) at the dose of 0.1 mg/kg i.p., and 24 h later, the tumors were exposed superficially to 655 ± 10 nm light (using the source described above) with a dose rate of 80–90 mW/cm^2 while the mice were immobilized in specially designed holders. The mice were subsequently monitored for tumor growth and those showing no sign of palpable tumor at 90 days post-treatment qualified as cured. For testing the effect of PDT plus calreticulin combination, each mouse was injected peritumorally 400 μg of recombinant human calreticulin contained in 0.1 ml PBS immediately after photodynamic light treatment.

CALRETICULIN ASSOCIATION WITH PDT-TREATED CELLS

The protocol was identical as used with the PDT-generated vaccine (exposure of SCCVII cells to ce6-based PDT followed by 16 h post-incubation and then 30 min exposure to calreticulin on ice), except that the recombinant human calreticulin was used and its concentration was 20 μg per million cells. In one treatment group, mitoxantrone (M6545, Sigma) 0.5 μg/ml was present during the 16-h post-incubation period. Presence of calreticulin on the surface of SCCVII cells was assessed by staining the cells with chicken polyclonal antibody to calreticulin (reactive with both human and mouse protein) followed by Alexa Fluor 488-conjugated goat anti-chicken IgY both purchased from Abcam. For isotype control, ChromPure chicken IgY (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as the primary antibody.

Suspensions of stained cells were then analyzed by flow cytometry performed on Coulter Epics Elite ESP (Coulter Electronics, Hialeah, FL, USA) with 20,000 cells measured in each test. Fluorescence values per cell obtained with the isotype control were deducted from values recorded with the calreticulin antibody.

CALRETICULIN GENE EXPRESSION ANALYSIS

Total RNA was extracted from SCCVII cells or mice tissues using Trizol and cleaned up with Qiagen MinElute (Qiagen Canada Inc., Montreal QC, Canada). It served for performing gene expression analysis as described in detail in our previous reports (20, 21). Briefly, the extracted total RNA was used for creating complementary strand DNA transcript that was then amplified by quantitative RT-PCR in the presence of mouse calreticulin gene (NCBI Reference Sequence NM_007591.3)-specific primers CTGAATACAAGGGCGAGTGGA (forward) and GCATCGGGGGAGTATTCAGG (reverse) that were designed and tested in our laboratory. The expression of housekeeping gene glycerladehide-3-phosphate dehydrogenase (GAPDH) was also measured and used for normalizing the expression of calreticulin gene.

STATISTICAL ANALYSIS

Each experiment was repeated at least once. Statistical evaluation of tumor response to PDT or PDT-vaccine treatment was done using log-rank test, while the analysis of other data was based on Mann–Whitney test with the threshold for statistical significance set at 5%.

RESULTS

The PDT-generated vaccine protocol with SCCVII tumor model developed in our laboratory, centers on preparing single-cell cancer vaccines by using *in vitro* PDT-treated SCCVII cells post-incubated in culture for 16 h and then injected peritumorally into mice bearing SCCVII tumors (19). To determine whether the efficacy of PDT vaccine can be affected by modifying calreticulin involvement, before injecting them to mice the vaccine cells were altered either by 30 min exposure to calreticulin protein or by treatment with mitoxantrone (known to induce cell surface expression of calreticulin (22). The results are presented in **Figure 1**. Compared to untreated SCCVII tumors, PDT vaccine alone treatment produced a significant retardation in the growth rate of these relatively fast growing tumors. This therapeutic gain was further improved by both adjuvant calreticulin (15 μg per dose) and the exposure to mitoxantrone during the post-PDT incubation of vaccine cells (**Figure 1**). Thus, the time for all the tumors to reach the critical size of 200 mm^3 was extended from 10 to 27 days. The same calreticulin dose per mouse was used by Obeid et al. (22). Using the vaccine SCCVII cells treated with calreticulin or mitoxantrone alone without PDT had no significant impact on tumor growth rates compared to those with untreated tumors (not shown).

The finding that calreticulin can act as an effective adjuvant to PDT vaccine encouraged our further investigation for which we secured required quantities of recombinant calreticulin by developing its production from yeast carrying human calreticulin gene. The SDS-PAGE gel with the final calreticulin preparation used in the experiments with cells and *in vivo* is shown in **Figure 2**. This allowed us to examine the effects of calreticulin on the response of tumors treated *in situ* by PDT. For this study, SCCVII tumors were implanted subcutaneously either into immunocompetent syngeneic C3H/HeN mice or into immunodeficient NOD-scid mice. When tumors reached 7–8 mm in largest diameter, the mice were administered photosensitizer Temoporfin, and 24 h later, the tumors were exposed to PDT illumination. Since recombinant human calreticulin was administered directly into mice immediately after light treatment, its dose was escalated to 400 μg per injection. All PDT-treated tumors became impalpable and necrotic within 24 h but recurrence was detected with many mice within 2 weeks post-therapy. The results with immunocompetent mice (**Figure 3A**) showed that the adjuvant calreticulin treatment rendered a significant improvement in tumor response to PDT from marginally curative to the solid levels of about 40% cure rates. There was no evidence with PDT-treated tumors of any effect following peritumoral injection of the same volume of saline (not shown). No significant effect on tumor was observed following treatment with calreticulin alone. In immunodeficient mice, the adjuvant calreticulin was not effective in improving tumor response to PDT (**Figure 3B**) and again no effect was observed with calreticulin alone.

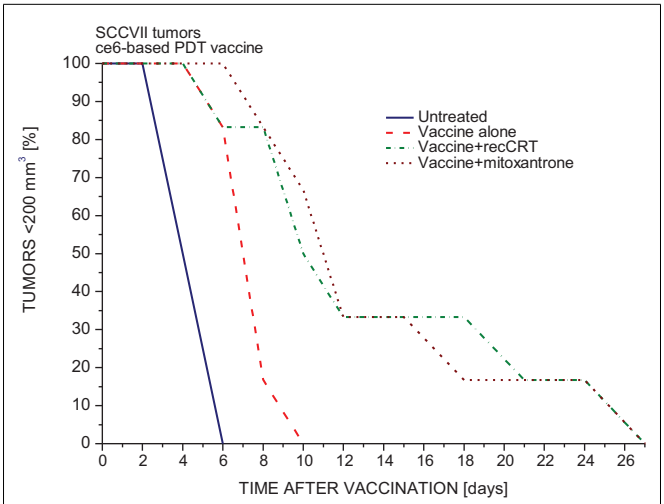

FIGURE 1 | The impact of calreticulin exposure or mitoxantrone treatment on the potency of PDT-generated vaccine. PDT vaccine was prepared by treating SCCVII cells with ce6-PDT (30 min exposure to 0.5 μg/ml ce6 followed by 1 J/cm^2 of 665 ± 10 nm light) and leaving them in culture for 16 h post-treatment incubation before they were injected (20 million/mouse) peritumorally into C3H/HeN mice bearing SCCVII tumors. With one treatment group, mitoxantrone (0.5 μg/ml) was present during the post-treatment incubation. Before injection, vaccine cells for one group of mice were exposed to recombinant rabbit calreticulin (15 μg per 20 million cells) for 30 min on ice. For other details of PDT vaccine protocol, Section "Materials and Methods." Each treatment group consisted of 6 mice. The response, based on two experiments, of SCCVII tumors to vaccine treatment is depicted as changes in the percentage of mice with tumors smaller than 200 mm^3 in time after therapy. Statistically significant difference ($p < 0.05$): untreated vs. vaccine alone groups, vaccine alone vs. vaccine with calreticulin groups, and vaccine vs. vaccine with mitoxantrone.

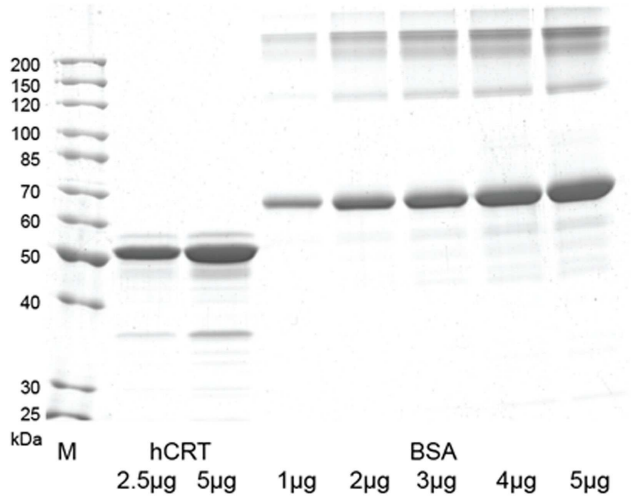

FIGURE 2 | SDS-PAGE gel with human calreticulin protein purified from yeast culture medium. The gel was loaded with 2.5 and 5 μg of human calreticulin and 1–5 μg of BSA (cat. no. B14, Thermo Fisher Scientific, Waltham, MA, USA) as amount control. M – Unstained protein ladder (cat. no. 26614, Thermo Fisher Scientific).

One of the proteins sharing with calreticulin the capacity to act as DAMP is heat shock protein 70 (Hsp70) (5, 23). We have shown that externally supplied Hsp70 protein binds to tumor cells that have sustained oxidative stress mediated by PDT (24). To test whether a similar property is exhibited by calreticulin, mouse tumor SCCVII cells were treated *in vitro* by PDT using photosensitizer ce6. After 16-h post-PDT culture at 37°C, the cells were transferred on ice and further incubated 30 min with recombinant human calreticulin added to the medium. The calreticulin dose range was 1–20 μg per10^6 cells, i.e., similar to the levels described for *in vitro* experiments by Obeid et al. (22). The cells were then stained with anti-calreticulin antibody for detecting the presence of this protein on the surface of cells using flow cytometry. The results show that the calreticulin protein present in the medium binds to PDT-treated cells but not to untreated cells (**Figure 4**). As shown before (8, 12), PDT treatment induced the presentation of endogenous calreticulin on the surface of cells. However, the calreticulin levels on these cells were significantly greater when in contact with the externally added calreticulin. Binding of this protein to PDT-treated cells could not be detected when less than 1 μg per million cells was added to the medium (not shown).

Another characteristic of Hsp70 is that its gene becomes upregulated not only in tumor cells directly treated by PDT but also in the livers and spleens of mice with PDT-treated or PDT vaccine-treated tumors (20, 24). In contrast, the expression of gene encoding calreticulin decreased in SCCVII cells treated by PDT (**Figure 5A**). The effect was PDT dose dependent, with close to 50% reduction found in cells 3 h after they received the medium tested PDT dose that attains around 90% cell kill. *In vivo*, no significant differences were found in the calreticulin gene expression in tumor, liver, and spleen tissues between untreated and PDT vaccine-treated mice (**Figure 5B**).

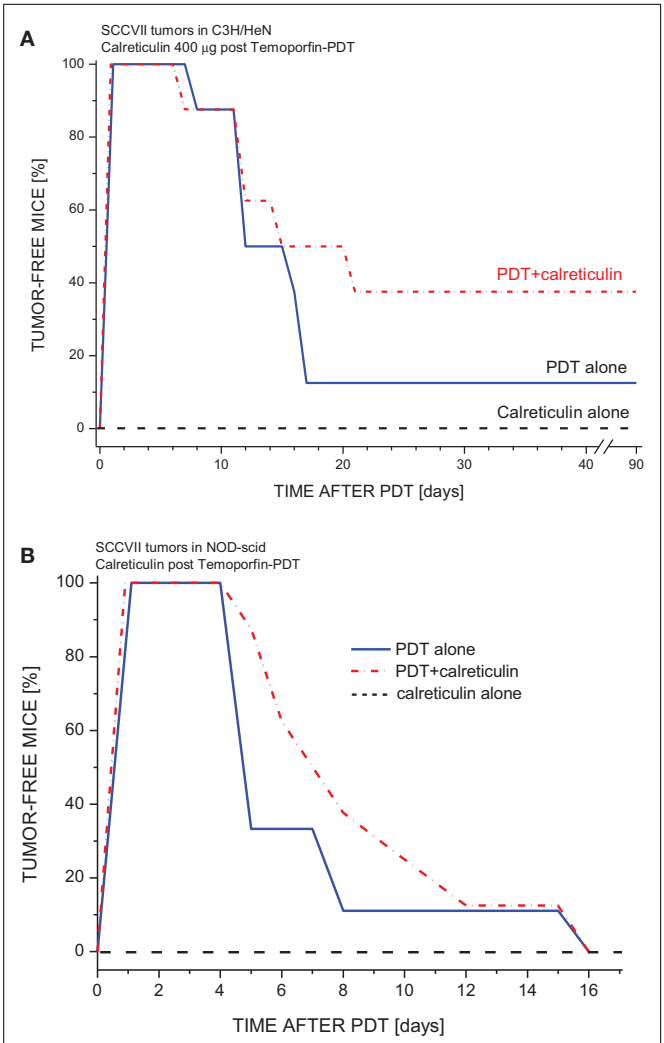

FIGURE 3 | The effect of tumor-localized calreticulin injection on PDT response of SCCVII tumors growing in immunocompetent or immunodeficient mice. The tumors growing either in **(A)** syngeneic immunocompetent mice (C3H/HeN) or **(B)** in immunodeficient mice (NOD-scid) were treated by Temoporfin-PDT (0.1 mg/kg i.p. Temoporfin followed 24 h later by 80 J/cm^2 light dose). Recombinant human calreticulin (400 μg/mouse) was injected peritumorally immediately after PDT light. Mice were then monitored for up to 90 days for signs of tumor regrowth. Each treatment group consisted of 8 mice. With C3H/HeN mice, the response of PDT alone and PDT plus calreticulin groups were statistically different ($p < 0.05$).

DISCUSSION

In one of the earliest reports on the therapy-induced translocation of calreticulin to the surface of tumor cells and its relevance, Obeid et al. (22) demonstrated that anthracyclines or their analog mitoxantrone are effective in achieving an such effect and that this is responsible for ICD produced by these drugs. In the same work, the authors showed that tumor cells treated by a chemotherapy agent mitoxantrone not only translocated intracellular calreticulin to their surface but were also able to bind externally added calreticulin protein. In accordance to these findings, our previous

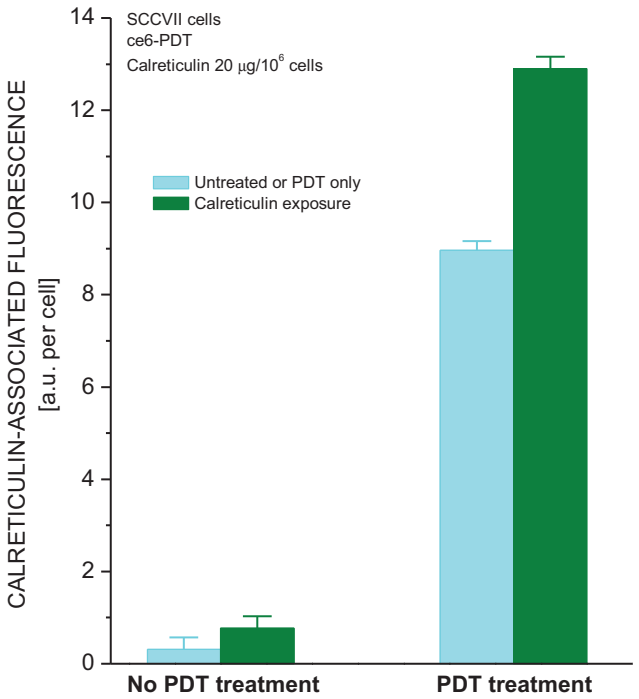

FIGURE 4 | Calreticulin binds to PDT-treated cells. The treatment of SCCVII cells with ce6-PDT and 16-h post-incubation was performed using the protocol for PDT vaccine preparation described in **Figure 1**. The cells were then concentrated by centrifugation and kept on ice in the presence of recombinant human calreticulin (20 μg per million cells). Cell suspensions were then stained with chicken antibody raised against calreticulin or with isotype control immunoglobulin, followed by Alexa Fluor 488-conjugated secondary antibody, and analyzed by flow cytometry. The data show the levels of cell surface-localized calreticulin on untreated and PDT-treated cells that were either unexposed or exposed to recombinant calreticulin. $N = 4$, bars are SD, *statistically significant difference ($p < 0.05$) compared to calreticulin unexposed PDT-treated cells.

investigation revealed the induction of surface calreticulin expression on SCCVII cells treated with this neoplastic agent as well as with PDT mediated by photosensitizer Photofrin (12), while the present study shows that calreticulin protein present in cell medium has no detectable affinity for binding to untreated cells but associates readily with PDT-treated tumor cells.

Our results demonstrate that this association with externally provided calreticulin renders PDT-vaccine cells therapeutically more potent (**Figure 1**). The similar benefit can be obtained by treating PDT-vaccine cells with mitoxantrone, which will further raise surface calreticulin levels originally prompted to emerge by PDT treatment. In the protocols for PDT of tumors *in situ*, the therapeutic gain seen with adjuvant calreticulin (**Figure 3**) can be expected to result from the association of this protein with cancer cells upon its administration by tumor-localized injection. Importantly, such therapeutic gain was not produced with the host mice deficient in mature lymphocytes (NOD-scid).

The capacity for binding to the surface of stressed tumor cells such as those treated by PDT calreticulin shares with another molecular chaperon and DAMP, hsp70 (24). Indeed, both of them could be binding to their common receptor CD91 on macrophages

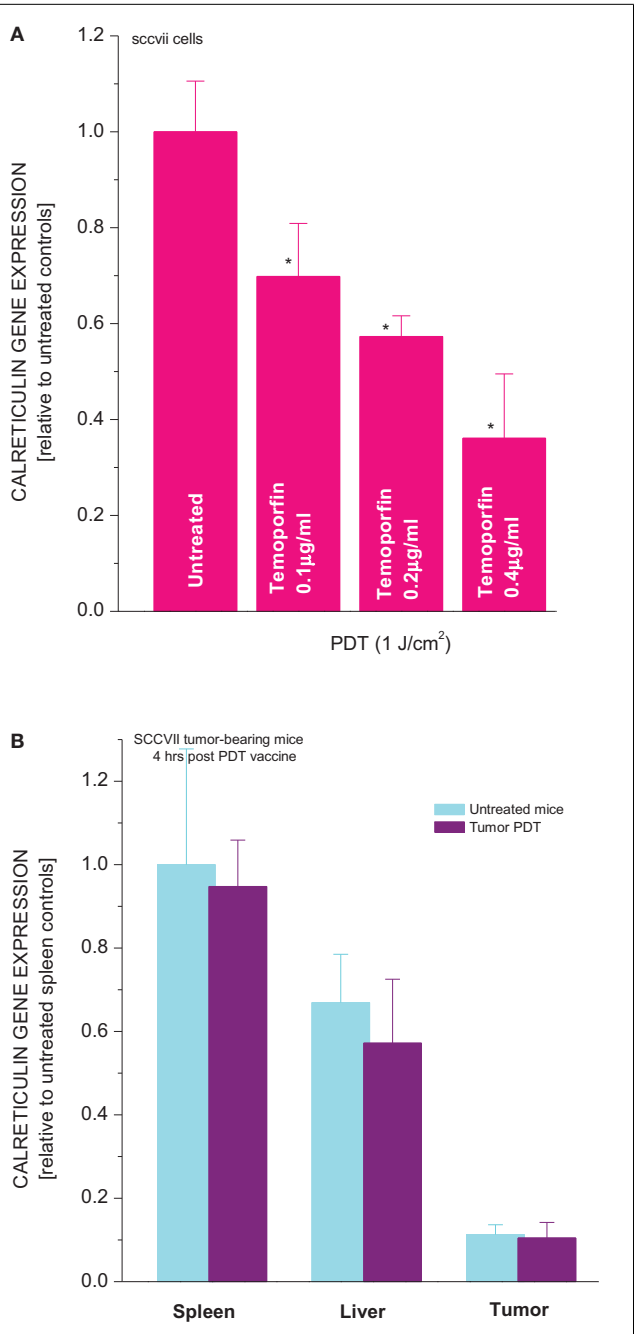

FIGURE 5 | Expression of calreticulin gene in PDT-treated cells and tumor, liver, and spleen tissues of mice bearing PDT-treated SCCVII tumors. In experiments *in vitro* **(A)** SCCVII cells were exposed to Temoporfin (0.1, 0.2, or 0.4 μg/ml for 24 h) and then treated with 655 ± 10 nm light (1 J/cm^2). The cells were collected 3 h after PDT and their total RNA isolated. In experiments *in vivo* **(B)** C3H/HeN mice with SCCVII tumors received tumor-localized PDT vaccine treatment as described for **Figure 1**. The mice were sacrificed 4 h later and their tumor, liver, and spleen tissues collected for total RNA isolation. The obtained total RNA samples were used for quantitative RT-PCR-based analysis of the expression of calreticulin gene. The results are presented as GAPDH-normalized mouse calreticulin gene expression relative to the same feature in untreated cells or in the same tissue of untreated tumor-bearing mice. $N = 4$, bars are SD, *statistically significant difference ($p < 0.05$) compared to calreticulin gene expression in PDT-untreated cells.

(25) but the identity of their binding partner on the surface of stressed tumor cells is not clear. Nonetheless, there are important differences between calreticulin and Hsp70. Unlike with calreticulin, tumor-localized recombinant Hsp70 protein injection after PDT has no impact on therapy outcome (Korbelik, unpublished results). On the other hand, as shown in **Figure 5**, calreticulin does not share with Hsp70 the capacity for gene expression and production to be upregulated in PDT-treated cells and be mobilized systemically as acute phase reactant, as evidenced by the upregulation of Hsp70 gene in the liver and spleen of mice bearing PDT-treated or a PDT vaccine-treated tumor (20, 24). Since the primary function of Hsp70 is expediting the repair or elimination of damaged intracellular proteins within stressed cells, it can be assumed that the cells will upgrade its gene while trying to survive. In contrast, the function of surface-exposed calreticulin is to facilitate the disposal of the cells doomed not to survive; in these cells, sufficient calreticulin can be supplied from the existing intracellular locations. This is in conformity with the finding that the surface exposure of calreticulin is entirely regulated in the cytoplasm by post-transcriptional and/or post-translational processes (22).

Calreticulin accumulated on the surface of tumor cells may modulate their death. Unlike Hsp70, which has inhibitory role in cell death pathways (26), the role of calreticulin is controversial due to reports suggesting its protection against apoptotic stimuli while other evidence infers its participation in the cell death pathway (4). The facts that tumor cells decorated with calreticulin are more efficiently phagocytosed by dendritic cells and have increased immunogenicity (22) are more consistent with the role of calreticulin as promoter of their death and disposal. Since surface-exposed calreticulin is one of the three DAMPs (together with ATP and HMGB1) attributed a key role in the immunogenic potential of ICD inducers (7), it seems rational to expect that its adjuvant properties described in the present study are also based on its role of promoting ICD. Calreticulin treatment adjuvant to PDT results in markedly increased levels of this protein exposed on the cell surface by adding from the external source to that of the internal origin already present. Evidently, the added calreticulin must become bound to certain (as yet unknown) molecules that appear on the surface of PDT-treated cells. This elevation of calreticulin levels on cancer cell surface appears to enhance its potential of promoting ICD, presumably by boosting their removal by antigen-presenting cells through phagocytosis pathways optimized for recognition of tumor antigens.

In summary, the present study demonstrates that externally added calreticulin protein, which has the ability to bind to tumor cells sustaining injury/stress from PDT treatment, can serve as an effective adjuvant augmenting tumor control with this therapy. Lack of such positive effect with tumors growing in immunodeficient mice indicates that the observed therapeutic gain with adjuvant calreticulin entails the engagement of adaptive immune system. The underlying mechanistic aspects remain to be uncovered in more detail with studies such as tumor immune infiltrate analysis. Based on the presented results, the hypothesis to be tested is that the adjuvant treatment with calreticulin provides a supplement DAMP material and promotes phagocytosis of PDT-treated tumor cells enhancing the development of antitumor-immune response (14, 22).

ACKNOWLEDGMENTS

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The ever-expanding immunomodulatory role of calreticulin in cancer immunity

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Calreticulin is a pleiotropic molecule that normally resides in the lumen of the endoplasmic reticulum (ER). Here, it has various functions, ranging from regulation of calcium homeostasis to ensuring proper protein folding. More recently, calreticulin gained special interest for its extracellular functions, where it has direct immunomodulatory activity. In this respect, calreticulin activates dendritic cells and macrophages. In addition, certain anti-cancer therapies induce the translocation of calreticulin from the ER to the cell surface of dying cancer cells, where calreticulin dictates the immunogenicity of these cells. Interestingly, treatment with tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) also induces membrane calreticulin exposure on cancer cells. As shown here, calreticulin directly interacts with TRAIL and its receptor-signaling complex, as well as with other TNF family members. Of note, TRAIL is a well known immunomodulatory molecule, and is expressed on the surface of natural killer T-cells. Therefore, calreticulin may have an as yet unrecognized wide(r) impact on immunity, with the TNF-ligand family modulating virtually all aspects of the immune response.

Keywords: calreticulin, TNF, tumor necrosis factor related apoptosis inducing ligand, immunomodulation, immunogenic cell death, complex formation

INTRODUCTION

The endoplasmic reticulum (ER)-resident protein calreticulin is a pleiotropic molecule with many functions in the ER, ranging from protein folding, calcium homeostasis, and regulation of loading of antigenic peptides into major histocompatibility class I (MHCI) (1, 2). Thus, ER-resident calreticulin has an important impact on development and correct execution of immunity. However, in recent years, calreticulin has taken center stage not for its ER-related functions, but for its newly uncovered immunomodulatory effects in the extracellular space. It has become evident that ER chaperones such as calreticulin function as danger associated molecular pattern molecules (DAMPs) once outside the cell (3). For instance, when present on the cell surface of dendritic cells (DCs) calreticulin functions as a receptor for autocrine-produced complement factor 1q (C1q), which is upregulated during CD40L/CD40 signaling, a key T helper signal required for effective DC maturation (4). In addition, soluble calreticulin promotes differentiation of CD1dhi $CD5^+$ B-cells into antibody-secreting cells (5). Further, soluble calreticulin induced TNF-α and IL-6 release from macrophages, which was regulated by scavenger receptor A (6).

A further prominent pro-immunogenic feature of extracellular calreticulin is its key role in the process of immunogenic cell death (ICD) (7). ICD is a type of apoptosis that is induced by certain chemotherapeutics or radiation, which promotes phagocytic uptake of apoptotic cells by professional antigen presenting cells, i.e., myeloid-derived DCs. Briefly, anthracycline or radiation therapy trigger the translocation of calreticulin to the pre-apoptotic tumor cell surface, which is dependent on the induction of an ER-stress response (8, 9). Cell surface exposed calreticulin then promotes clearance of the dying cancer cells by DCs, with subsequent release of ATP, HMGB1, and HSP70 from late apoptotic cells giving requisite cues for DC maturation and clonal T-cell expansion (10).

Immunogenic cell death has been described in a preclinical setting for an ever-expanding set of cytotoxic therapies, including many chemotherapeutics, radiation therapy, and photodynamic therapy. Interestingly, also treatment of cancer cells with a recombinant form of the tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) was reported to induce calreticulin exposure and ICD (11). TRAIL is a member of the TNF superfamily that can bind to a set of five receptors; TRAILR1 (DR4), TRAILR2 (DR5), TRAIL-R3 (DcR1, TRID), TRAIL-R4 (DcR2, TRUNDD), and soluble osteoprotegerin. Binding of TRAIL to its agonistic receptors TRAILR1 and TRAILR2 induces assembly of the so-called death inducing signaling complex (DISC) to the intracellular death domain (DD). The DISC contains the adaptor molecule Fas-associated protein with death domain (FADD) and the pro-form of initiator caspase-8, which is auto-proteolytically processed in the DISC. Activation of caspase-8 triggers a proteolytic caspase cascade that ultimately leads to the execution of apoptosis. Of note, the pro-apoptotic signaling of the DISC can be inhibited by recruitment of additional regulators, such as cFLIP (12).

TNF-related apoptosis inducing ligand is an important immune effector molecule that is expressed on various types of immune cells including natural killer (NK) cells, T-cells, and natural killer T-cells (NKT cells) (13). Expression of TRAIL on T-cells is further increased during T-cell receptor (TCR) stimulation in the presence of interferon-γ (IFNγ) (14). Similarly, IFNγ-mediated activation of NK-cells, monocytes, and DCs enhances the expression of TRAIL on these cells (15, 16). TRAIL and its receptors (TRAILRs) play an important role in anti-tumor immunity, e.g., being involved in the immune surveillance for (metastatic) cancer cells by liver NK-cells (17). Thus, the induction of calreticulin exposure upon treatment with recombinant TRAIL may be related to a possible immunoregulatory effect of TRAIL in the context of its normal role on immune effector cells. Interestingly, calreticulin can also interact directly with TRAIL in a catching-type ELISA, as well as with the immunoregulatory TNF family members such as CD40 ligand (CD40L) and FasL (18). In contrast, calreticulin did not interact with TNF-α, adiponectin, or CD30L. The reported activation of ICD upon treatment with recombinant TRAIL suggests that calreticulin may regulate the pro-immunogenic effect of TRAIL during anti-tumor immunity.

CALRETICULIN DIRECTLY INTERACTS WITH PRO-APOPTOTIC TNF-LIGANDS AND RECEPTOR COMPLEXES

Calreticulin was recently reported to bind to TNF family member FasL in the synovial fluid of rheumatoid arthritis (RA) patients. This FasL/calreticulin interaction inhibited FasL-induced apoptosis of Jurkat T-cells (19). Since patients with RA have elevated levels of calreticulin in their serum, this might inhibit apoptosis of inflammatory T-cells in this particular disease. Our own studies confirm a similar direct interaction of calreticulin with TRAIL, as evidenced by, e.g., fluorescent microscopy for calreticulin and TRAIL on the cell surface of TRAIL-treated A375M melanoma cells and not in control cells (**Figure 1A**). This is in line with the reported translocation of calreticulin to the cell surface of apoptotic HeLa cells by recombinant TRAIL (11). Interestingly, calreticulin also strongly co-localized with TRAILR2 on TRAIL-treated A375M cells (**Figures 1B,C**), with calreticulin, TRAIL, and TRAILR2 all being in one complex co-precipitated upon anti-HA-TRAIL immunoprecipitation experiments (**Figure 1D**). Similarly, calreticulin was immunoprecipitated in the FasL/Fas DISC (data not shown). The association of calreticulin with TRAILR2 occurs in a clear patched pattern on the cell surface (**Figures 1B,C**), a pattern corresponding to that of the patched foci to which calreticulin redistributed on the cell membrane of apoptotic neutrophils (20), on mitoxantrone treated CT26 colon cancer cells (21), and hypericin-treated bladder cancer cells (22).

The recruitment of calreticulin in distinct patches on the cell membrane has been shown to facilitate engulfment of apoptotic cells by phagocytes induced by UV radiation or anthracycline therapy (7, 20) (**Figure 2A**). These calreticulin-rich patches also contained high levels of phosphatidylserine (PS), an apoptotic marker that provides strong "eat me" signals for phagocytes (20). Correspondingly, calreticulin binds in a Ca^{2+}-dependent manner directly to the polar head of PS with high affinity ($K_D = 1.5 \times 10^{-5}$ M) (24). This recruitment of calreticulin into lipid rafts was dependent on ERp57 (21, 25). Of note, on the surface of viable cells calreticulin is associated with CD47, a prominent so-called "don't eat me" signal that blocks phagocytosis by binding to the inhibitory receptor SIRPα on phagocytes (26). During apoptosis, calreticulin dissociates from CD47 and associates with PS; a shift that promotes phagocytosis (20). Of note, the balance between pro-phagocytic calreticulin and anti-phagocytic CD47 has also been described in multiple human cancer cells, with cancer cells often being characterized by CD47 overexpression to elude phagocytic removal (26, 27). Similarly, our experiments indicate that calreticulin dissociates from CD47 upon TRAIL treatment of A375M cells (**Figure 1C**). Together with literature, this dissociation suggests that the interaction of calreticulin with TRAIL and TRAILR2 may facilitate phagocytic clearance (**Figure 2B**).

CALRETICULIN IS RECRUITED TO ACTIVE SIGNALING COMPLEXES OF THE TNFR SUPERFAMILY

In an earlier study on TNFR-signaling, surface expressed calreticulin was reported to be recruited to TNF receptor 1 (TNFR1) and its adaptor TNFR-associated death domain (TRADD) upon treatment with bacterial peptidoglycan *N*-acetylmuramyl-L-alanyl-D-isoglutamine (L,D-MDP) (28). This interaction was independent of TNF and triggered apoptosis in RK_{13} kidney cells that could be blocked by anti-calreticulin antibodies. Similarly, calreticulin associated with TRAIL, TRAILR2, as well as adaptor protein FADD and pro-caspase-8 in A375M melanoma cells (**Figures 1D,E**). Of note, caspase-8 was predominantly present in its pro-form after 15 min of TRAIL treatment, but was in its processed p41/43 form after 180 min. Further, caspase-8 processing occurred mainly at 37°C, although treatment on ice also induced DISC formation (**Figures 1D,E**). The interaction of calreticulin with TRAIL was necessary for its association with TRAILR2 as calreticulin was not co-immunoprecipitated with TRAILR2 upon treatment with agonistic TRAILR2 antibody, despite DISC formation (**Figure 1F**). Thus, physical interaction of TRAIL with calreticulin mediates its recruitment to the TRAILR2 DISC.

CALRETICULIN ASSOCIATION WITH THE TRAILR2–DISC DEPENDS ON RAFT REORGANIZATION BUT IS INDEPENDENT OF APOPTOTIC SIGNALING

During ICD with mitoxantrone, the maximum level of surface exposed calreticulin was observed within 15 min, and remained at stable levels for at least 24 h (7). In line with this, surface calreticulin became visible after 30 min on neutrophils and continued to increase as neutrophils became more apoptotic (20). In contrast, induction of ICD upon treatment of Hela cells with TRAIL only induced detectable levels of calreticulin on the surface after 24 h of incubation (11). On A375M cells, calreticulin translocated to and associated with calreticulin with TRAIL already after 15 min, with maximal association after 1 h (**Figure 1G**), which corresponded to the maximum level of processed caspase-8 (p43/41) (**Figure 1H**). Further, the maximum association of calreticulin with TRAIL and the DISC occurs before the execution of apoptosis, as determined by activation of effector caspases-3 and -7 (**Figure 1H**). This dynamic corresponds to the pre-apoptotic exposure of calreticulin during ICD (7). Interestingly, induction of apoptosis was required to induce cell surface calreticulin exposure upon treatment with mitoxantrone, with depletion of caspase-8 completely abolishing

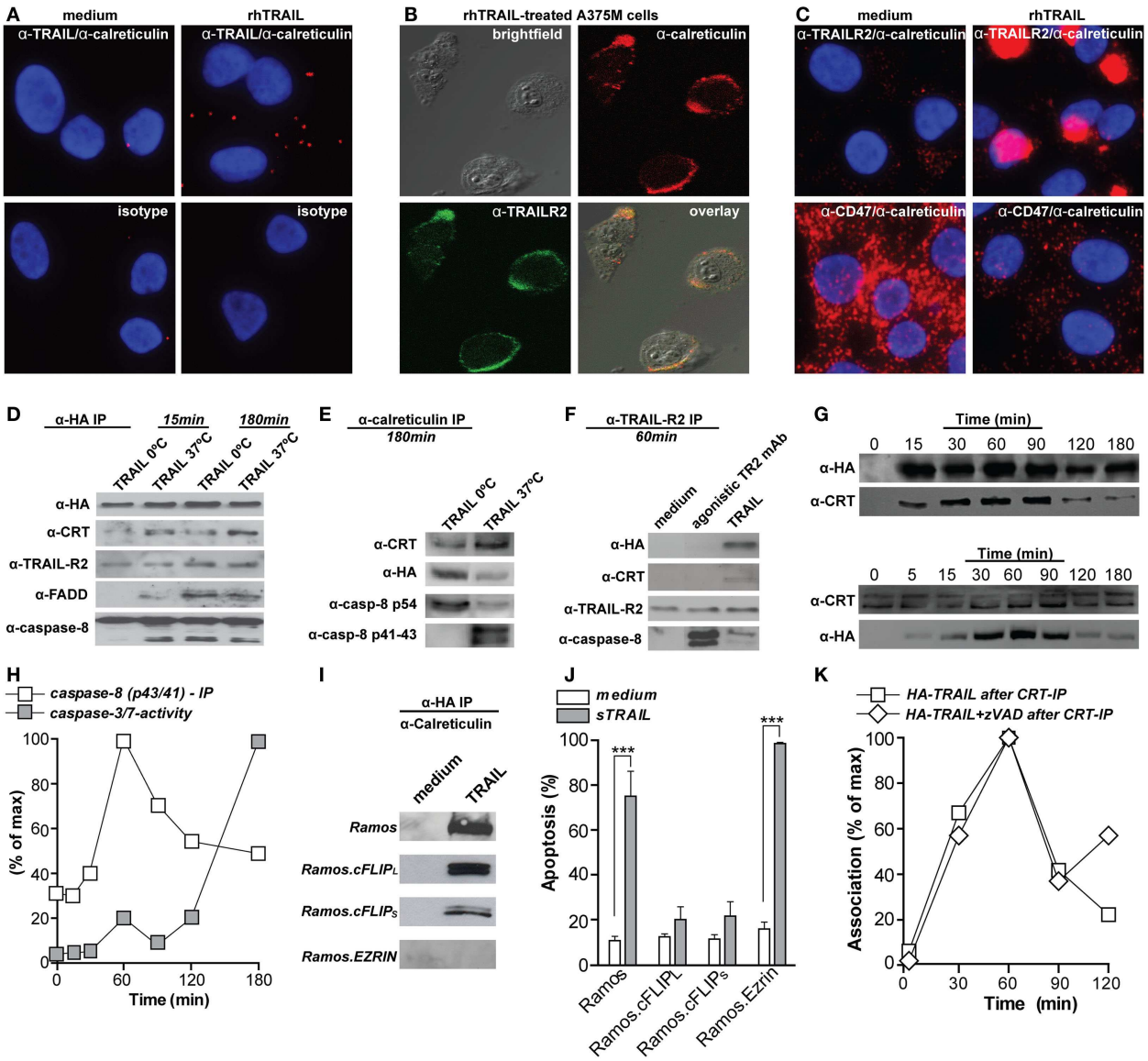

FIGURE 1 | Calreticulin binds to TRAIL and associates with the TRAILR2–DISC. **(A)** A375M cells were treated with 100 ng/ml rhTRAIL for 1 h, incubated with mAbs directed against calreticulin (Ab2907, Abcam) and TRAIL (B-S23, Diaclone), or isotype controls, and subsequently analyzed by proximity ligation assay (Duolink II, Olink Bioscienscse) (distance between both proteins; <40 nM or ~4–12 proteins). **(B)** A375M cells were treated with 100 ng/ml rhTRAIL for 1 h, double stained for calreticulin (red; Ab2907, Abcam) and TRAILR2 (green; HS-201, Alexis) and analyzed using confocal fluorescent microscopy. **(C)** As in **(A)**, but the proximity ligation assay were performed using mAbs directed against calreticulin (Ab2907, Abcam), TRAILR2 (HS-201, Alexis), and CD47 (ab3283, Abcam). **(D)** A375M cells were treated with HA-TRAIL [constructed by cloning the cDNA of HA-tagged TRAIL in frame into previously described vector pEE14 (23), yielding pEE14-HA-TRAIL] for 15 or 180 min at the indicated temperatures. Cells were subsequently lysed and HA-TRAIL was precipitated via affinity tag purification (HA-sepharose beads; 3F10, Roche). Precipitates were subsequently probed for the presence of HA (3F10, Roche), calreticulin (Ab39818, Abcam), TRAILR2 (HS-201, Alexis), FADD (272S, cell signaling), and caspase-8 (18C8, cell signaling). **(E)** As in **(D)**, but precipitated using anti-calreticulin. Precipitates were subsequently probed for the presence of calreticulin, HA, and caspase-8. **(F)** A375M melanoma cells were treated with HA-TRAIL or agonistic TRAILR2 mAb for 60 min at 37°C. Cells were subsequently lysed and TRAILR2 was precipitated. The precipitates were probed for the presence of HA, calreticulin, TRAILR2, and caspase-8. **(G)** A375M cells were treated with HA-TRAIL at 37°C for the indicated time-points. Cells were subsequently lysed and HA-TRAIL (upper panel) or calreticulin (lower panel) were precipitated. Precipitates were subsequently probed for the presence of calreticulin or HA-TRAIL, respectively. **(H)** A375M cells were treated with HA-TRAIL at 37°C for the indicated time-points. □ Quantification of caspase-8 (p42/41) association with TRAIL as determined by immunoprecipitation ■ caspase-3/-7 activity as determined by flow cytometry (CellEvent caspase-3/-7 probe, Invitrogen). **(I)** Ramos, Ramos.cFLIP$_L$, Ramos.cFLIP$_S$, and Ramos.Ezrin cells were treated with HA-TRAIL for 1 h at 37°C. Cells were subsequently lysed and HA-TRAIL was precipitated via affinity tag purification. Precipitates were subsequently probed for the presence of calreticulin. **(J)** Ramos, Ramos.cFLIP$_L$, Ramos.cFLIP$_S$, and Ramos.Ezrin cells were treated with rhTRAIL for 16 h and apoptosis was assessed (DioC6 staining, flow cytometry using BD Accuri Flow cytometer). ***$p < 0.001$. **(K)** A375M cells were treated with HA-TRAIL in the presence or absence of 20 μM zVAD-fmk (CalBiochem). Subsequently, calreticulin was precipitated and the precipitates were stained for HA-TRAIL. Association of calreticulin and TRAIL is depicted as percentage of maximum association.

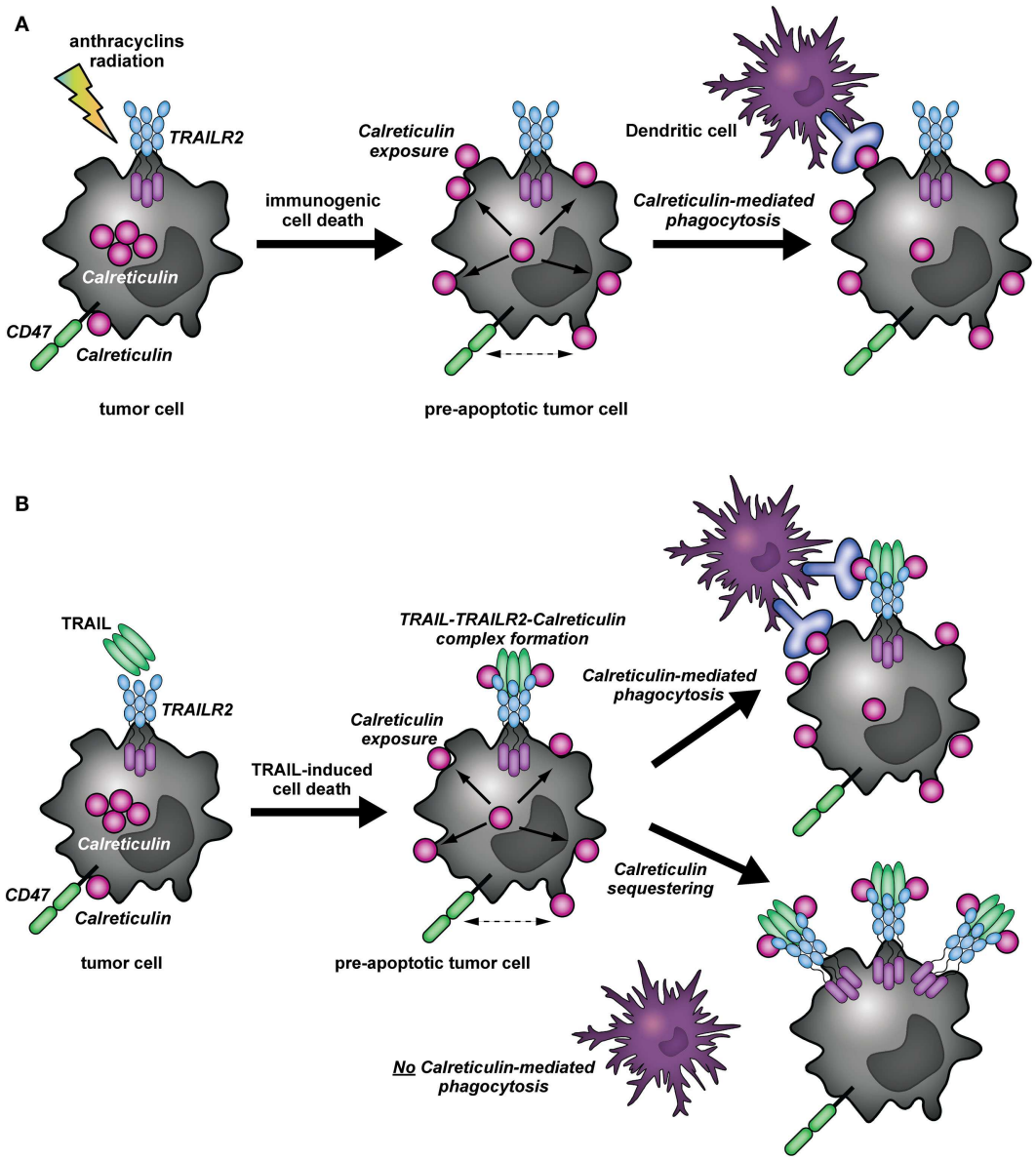

FIGURE 2 | Immunomodulatory role of calreticulin in cancer immunity (A). In (tumor) cells, calreticulin predominantly resides in the ER. Upon induction of immunogenic cell death (ICD) by, for instance, anthracycline or radiation therapy, calreticulin translocates to the surface of pre-apoptotic cells. In addition, surface exposed calreticulin (eat me signal) dissociates from CD47 (don't eat me signal), whereupon the apoptotic cancer cells can be recognized and taken up by DCs. **(B)** The binding of TRAIL to its receptor (TRAILR2) results in the translocation of calreticulin to the cell surface of cancer cells, whereby a complex is formed between TRAIL, TRAILR2–DISC, and calreticulin. Simultaneously, calreticulin dissociates from CD47. The formation of the TRAIL/TRAILR2/Calreticulin-complex may have different outcomes: (1) As described for the concept of ICD, cell surface exposure of calreticulin and dissociation from CD47 induced by TRAIL treatment may facilitate phagocytic uptake by DCs. (2) the binding of calreticulin to TRAIL and TRAILR2 may impair phagocytic clearance by DCs, as calreticulin may be segregated away from the membrane microdomains in which it can partake in the phagocytic uptake by DCs.

the translocation of calreticulin (11). In contrast, inhibition of caspase-8 did not inhibit calreticulin exposure upon photodynamic therapy with hypericin (9). In line with the latter report, calreticulin was still co-precipitated with TRAIL in Ramos B-cells that are resistant to TRAIL-mediated apoptosis by overexpression of the anti-apoptotic DISC component cFLIP (Ramos.cFLIP$_L$ and Ramos.cFLIP$_S$) (**Figures 1I,J**). Correspondingly, co-treatment of A375M cells with TRAIL and pan-caspase-inhibitor zVAD-fmk did not abrogate the association of calreticulin with TRAIL (**Figure 1K**).

During ICD, the actin cytoskeleton was required for translocation of calreticulin to the pre-apoptotic cell surface (11). Similarly, calreticulin–TRAIL association was dependent on cytoskeletal rearrangements and was abolished in Ramos cell overexpressing

the double negative Ezrin mutant (DN-Ezrin), which is incapable of interacting with actin (**Figure 1I**). Thus, calreticulin association with the DISC is independent of apoptosis, but dependent on cytoskeletal reorganization.

CONCLUSION/FUTURE DIRECTIONS

It is clear from literature that treatment of cancer cells with TRAIL can induce calreticulin relocalization to the cell surface. Further, as evidenced by the data in the current manuscript, surface calreticulin is recruited to the TRAIL/TRAILR/DISC complex and dissociates from CD47. Together, these observations suggest that calreticulin could promote phagocytosis of apoptotic cells upon TRAIL-mediated killing (**Figure 2B**). On the other hand, its association with the TRAILR2–DISC might render calreticulin unable to effectuate pro-immunogenic removal of target cells by DCs. In this respect, it is tempting to speculate that by binding to the TRAILR2–DISC, calreticulin is segregated away from the membrane microdomains in which it can partake in the phagocytic uptake of target cells by DCs or other phagocytes (**Figure 2B**). These findings would be consistent with the non-immunogenic clearance of cells undergoing TRAIL-mediated programed cell death (29). To gain insight in the possible immunomodulatory role of calreticulin during TRAIL-induced apoptosis, it will be imperative to characterize the particular complex or membrane microdomain in which calreticulin resides in the context of ICD. Comparison of calreticulin localization upon treatment with typical immunogenic inducers, and how this compares to its localization during TRAIL-mediated cell death may shed light on its particular role.

The inhibition of FasL-mediated T-cell death by calreticulin, the direct induction of apoptosis by L,D-MDP-bound calreticulin via TNFR1 and the here described association of calreticulin to TRAILR2 during TRAIL-mediated cell death suggest a much broader role for calreticulin in the antitumor immune response than as a mediator of ICD. By directly modulating apoptosis and associating with key apoptotic players, calreticulin could function as a bridge between innate and adaptive immunity. In this paradigm, induction of programed cell death by immune effector molecules, such as TRAIL and FasL, induces a rapid pre-apoptotic translocation of calreticulin to the cell surface. On the cell surface, calreticulin can then either modulate induction of cell death as described for FasL (19) or translocate to specific cell surface microdomains (with or without receptors of the TNF superfamily), thereby modulating phagocytosis and pro-immunogenic DC-mediated removal. In its soluble state, secretion of calreticulin in response to apoptotic stimuli can directly affect the immediate early innate immune response by modulating cytokine secretion of macrophages. Interestingly, agonistic TRAIL-receptors have been reported as negative regulators of innate immune responses (30). An intriguing possibility currently being investigated in our laboratory is that sequestration of calreticulin by TRAIL-receptors could contribute to limiting activation of innate immunity.

Calreticulin exposure can further trigger activation of the adaptive immune response by promoting immunogenic DC-uptake of apoptotic cells during ICD. In this respect, it is also of interest that of all the TNF ligands it was found to interact with in a catching-type ELISA, calreticulin most strongly associated with CD40L. Thus, recruitment of calreticulin to CD40L/CD40 signaling complexes may impact DC activity. Whether calreticulin directly associates with TNF family members beyond CD40, FasL, and TRAIL has not been identified, to date. However, the reported interaction of calreticulin with several TNF-ligand family members points to the possibility that calreticulin may have an as yet unrecognized wide(r) impact on immunity, with the TNF ligand family modulating virtually all aspects of the immune response.

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Phage display engineered T cell receptors as tools for the study of tumor peptide–MHC interactions

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Cancer immunotherapy has finally come of age, demonstrated by recent progress in strategies that engage the endogenous adaptive immune response in tumor killing. Occasionally, significant and durable tumor regression has been achieved. A giant leap forward was the demonstration that the pre-existing polyclonal T cell repertoire could be re-directed by use of cloned T cell receptors (TCRs), to obtain a defined tumor-specific pool of T cells. However, the procedure must be performed with caution to avoid deleterious cross-reactivity. Here, the use of engineered soluble TCRs may represent a safer, yet powerful, alternative. There is also a need for deeper understanding of the processes that underlie antigen presentation in disease and homeostasis, how tumor-specific peptides are generated, and how epitope spreading evolves during tumor development. Due to its plasticity, the pivotal interaction where a TCR engages a peptide/MHC (pMHC) also requires closer attention. For this purpose, phage display as a tool to evolve cloned TCRs represents an attractive avenue to generate suitable reagents allowing the study of defined pMHC presentation, TCR engagement, as well as for the discovery of novel therapeutic leads. Here, we highlight important aspects of the current status in this field.

Keywords: phage display, tumor immunity, antigen presentation, T cell receptor, immunotherapy

INTRODUCTION

T cells initiate and regulate adaptive immune responses to infections, are major components of allergic and autoimmune responses as well as transplant rejection, and play a pivotal role in cancer immune surveillance (1). The cancer-prone phenotypes of mice that lack components of the adaptive immune system strongly points to lymphocytes as critical factors in the anti-tumor activity (2). That the T cells represent the critical lymphocyte population is further underscored by a correlation between the presence of tumor infiltrating lymphocytes (TILs) and ability to control tumor growth. The $CD45RO^+$ memory sub-group of the CD3 T cell compartment appears responsible of this activity (3, 4), and the $CD8^+$ and $CD4^+$ T cells probably act in concert (5). Furthermore, the observation that selective $CD4^+$ T_H cell silencing may abrogate the anti-tumor response points to the $CD4^+$ T_H cells as crucial (6, 7). It is also clear that adoptive cell therapy (ACT) through the use of $CD8^+$ cytotoxic T lymphocyte (CTL) or $CD4^+$ T_H cells may both result in durable anti-tumor activity (8–10). This is not merely a consequence of specific T cell target recognition, nor the affinity by which the T cell receptor (TCR) recognizes the target (11–13). Thus, to further delineate the mechanisms that lead to successful anti-tumor responses and how these can be exploited, it becomes imperative to further characterize the TCR–peptides bound to MHC molecules (pMHC) interaction, both at the cellular and the molecular level. The latter has posed a challenge to the field, since recombinant soluble TCRs have proven difficult to manufacture and work with. Consequently, our ability to study this pivotal interaction still depends on technology development (14). As such, protein engineering using combinatorial technologies is a powerful tool (15). Here, we focus on examples derived from the most prevalent combinatorial platform technology, namely phage display (16).

T CELL SPECIFICITY AT THE MOLECULAR LEVEL

T cell function relies on productive binding between TCRs and antigens, which are proteolytically derived pMHC displayed on the surface of a variety of antigen presenting cells (APCs). Most TCRs bind pMHC ligands in a semi-conserved diagonal orientation with the somatically derived CDR3 loops located centrally atop the bound peptide, and the germ-line encoded variable CDR1 and CDR2 loops positioned over the MHC α helices (17). Upon activation, T cells may proliferate, differentiate, release cytokines, kill target cells, or carry out other effector functions. Thus, the ability of T cells to orchestrate the adaptive anti-tumor response depends on the TCR–pMHC interaction and downstream signaling events (18, 19). Productive interactions between TCRs and pMHCs are among the weakest known to initiate a biological response (20–22). Thus, a T cell needs to discriminate between foreign and self-peptides bound to MHC molecules even though the differences in affinity and binding kinetics may be minute (21, 22). Nonetheless, the earliest events in TCR signaling are characterized by high sensitivity and selectivity toward agonist pMHC (19). This is remarkable considering the apparent promiscuity of TCR binding, which in extreme cases have been suggested to be

in the range of 10^6 different peptides, yet still in a HLA restricted context (23). Such scaffold-dependent ligand binding promiscuity may partly be attributed to germ-line encoded HLA interaction signatures that ensure preservation of HLA restriction (24–27). It could also be an important feature explaining how a limited number of TCR germ line segments in combination with somatically generated CDR3 loops serve as versatile building blocks that generate a supply of TCRs able to promptly respond to a universe of pathogens (28–30). Clearly, multi-epitope specificity can also be a characteristic of tumor-specific TCRs, as shown in the study of Chinnasamy et al. focusing on HLA-A2/MAGE-A3 targeting (31). However, during ACT, such lack of mono-specificity may translate into fatal toxicity, underscoring the need for improved procedures for pre-clinical testing (32). Also, there is a need for a very precise delineation of how a TCR actually sees its cognate pMHC target, since minute structural changes may translate into very different cellular responses (33). Here, elucidating the underlying thermodynamic parameters governing the interaction may give clues to the rules that dictate TCR specificity (34, 35). Such biophysical insight may be further complemented by precise delineation of docking modes and binding studies that mimic the cellular topology (36, 37). In either case, one will need access to sufficient amounts of pure and stable soluble TCR and pMHC proteins.

REDUCTIONIST APPROACH TO UNDERSTANDING THE pMHC–TCR INTERACTION – THE TCR EXPRESSION PROBLEM

T cell receptors are membrane anchored proteins, and it is challenging to obtain sufficient quantities of recombinant soluble TCRs for molecular studies. A variety of approaches have therefore been adopted, including formation of single chain (sc) TCR, an analog to scFv antibody (Ab) fragments, and fusion of the extracellular TCR domains to other proteins; i.e., maltose binding protein, thioredoxin, human constant kappa domain, or leucine zippers (38–42). However, all of these strategies have had limited success due to low production yield and poor functionality. The most widely applied format as of today is the disulfide-bond linked TCRs (dsTCRs), which have a non-native disulfide bridge between the TCR constant domains (43). The method has significantly increased the stability and improved the folding characteristics of several human TCRs (44) when refolded from inclusion bodies, whereas direct soluble expression appears of limited utility (44, 45). An alternative approach is periplasmic expression with simultaneous over-expression of the chaperone FkpA, which has a huge impact on both the yield and functionality of the TCRs expressed (46). However, despite the optimized and improved methods, all are laborious and the expression levels vary extensively between individual clones. Thus, in many cases engineering of the TCR scaffold for higher stability, solubility and clone independent expression levels appears needed to obtain high quality protein.

TCR STABILITY CAN BE ENGINEERED BY USE OF PHAGE DISPLAY

Evolution of recombinant proteins by random mutagenesis and subsequent *in vitro* selection has been successfully applied to a wide range of protein classes (47), and in particular antibodies (48). One such strategy has utilized selection of mutated heavy chain variable domains in combination with thermal challenge to obtain aggregation-resistant domains (49). Recently, guided by the study of Jespers et al., molecular evolution of a TCR for increased stability and expression was carried out by use of phage display (50). Libraries of randomly mutated scTCRs were produced as fusion to protein III on the surface of M13 phage. High valence display allowed stress-induced aggregation after thermal challenge (**Figure 1**). Variants characterized by markedly increased soluble expression levels and reduced aggregation propensity were obtained after rapid heating and cooling, followed by capture of aggregation-resistant scTCRs (**Figures 1A,D**). Importantly, over-expression of the periplasmic chaperone FkpA resulted in even display levels among the TCR library members, which proved imperative for successful selection. Thus, the previously identified folding assistance to soluble and phage displayed scTCRs offered by FkpA now allows for extended engineering opportunities to TCRs in conjunction with high-throughput soluble screening (**Figures 1A,B,D**). The list of strategies used for engineering of increased protein biophysical stability employing destabilization challenges in combination with multivalent phage display selection has been further extended. Famm et al. reported selection of Ig domains resistant to e.g., acidic pH induced aggregation with increased thermodynamic stability (51, 52). Furthermore, Christ et al. have reported a method for generation of Ab sub repertoires, based on combinatorial assembly of CDRs from an aggregation-resistant repertoire (53). Repeated cycles of selection and thermal denaturation generated domains with remarkable aggregation-resistant properties. Similar strategies may well be employed to obtain soluble TCR scaffolds with even higher expression levels and increased stability than reported to date (50, 54).

TCR AFFINITY CAN BE ENGINEERED BY USE OF PHAGE DISPLAY

To overcome the intrinsically low binding affinity of the TCR–pMHC interaction, two approaches have been utilized, namely multimerization and affinity maturation. Tetrameric forms of soluble TCRs have been produced by capturing biotinylated TCRs onto avidin, which have four binding sites for biotin (60, 61). The overall increased avidity greatly increases the half-life of the TCR–pMHC interaction. Such reagents are used in cellular binding assays, as they stably adhere to the cell surface. Crucial information may be collected that allows for deduction of biologically relevant information (61). However, for example direct assessment of peptide presentation at stoichiometric levels requires stronger binding between the TCR and pMHC than what is possible to reach with native TCRs (60). Therefore, affinity maturation of TCRs for increased binding has been performed. Again, phage display technology has been efficient (15, 57), and selection from mutant TCR display libraries can yield TCRs with dramatically increased affinities toward the cognate pMHCs without concomitant increase in cross-reactivity (**Figures 1B–D**). Crystallographic data show that this can be explained by a loss of flexibility in the otherwise entropically unfavorable TCR–pMHC interaction interphase, as well as an overall increase in shape complementarity (62). The degenerate pMHC interaction mode of TCRs could suggest that engineering must be restricted to the somatically derived CDR3 loops to preserve MHC restriction (26). However,

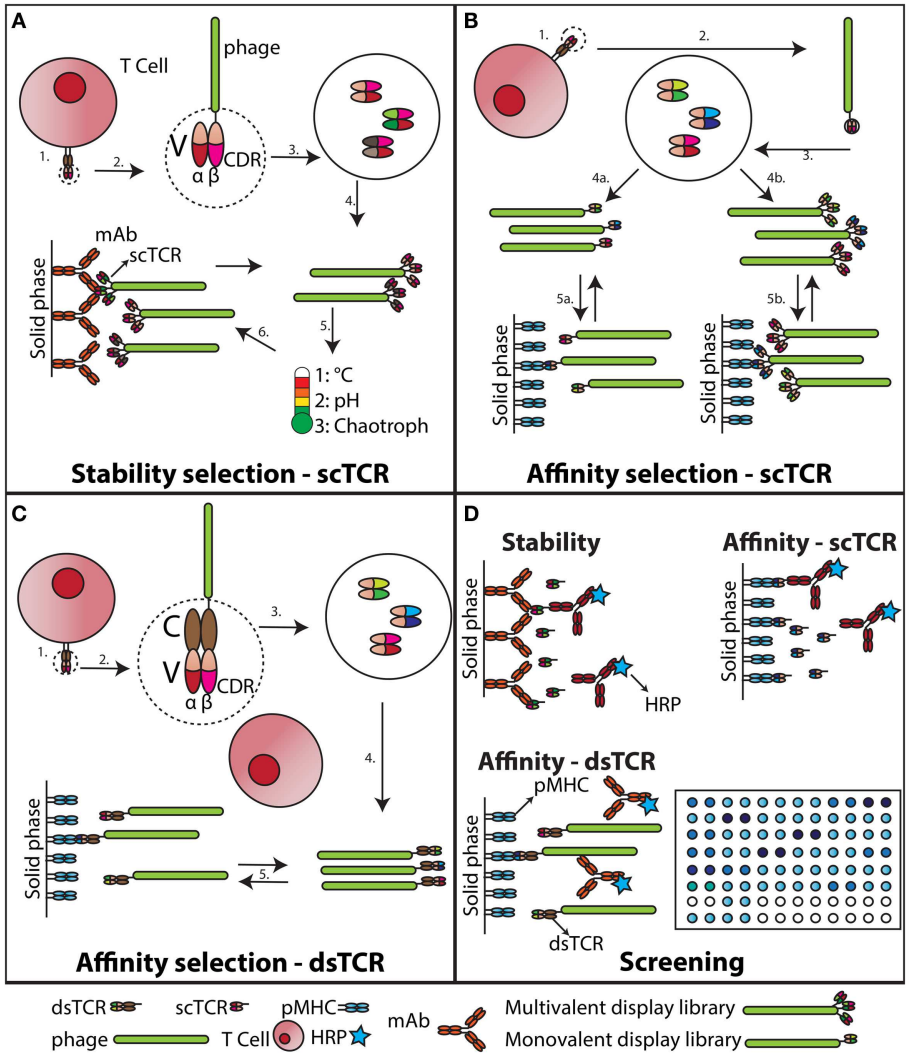

FIGURE 1 | Stability engineering and affinity maturation of soluble TCRs. **(A)** Stability engineering of scTCR. The variable (V) regions of an individual TCR (1) are cloned and connected via a synthetic linker (55). This scTCR is expressed as fusion to the M13 pIII capsid protein (2). The scTCR is then diversified by *in vitro* mutagenesis (3). This collection of mutagenized scTCRs are expressed as a high valence display phage library (4), which is challenged with increased temperature, unfavorable acid/base, or chaotropic conditions (5). Stabilized scTCR resisting aggregation despite the challenge is retrieved by capture on a conformation-specific ligand, such as an Ab (6). **(B)** Affinity maturation of scTCR. The V regions of an individual TCR (1) are cloned and expressed as a scTCR fusion to either the M13 pIII (55), or pIX capsid protein (56) (2). Individual TCR α- and β-chain CDR loops of the scTCR are randomized to create diversity (3). This collection of mutagenized scTCRs is then expressed as a low (4a) or high (4b) valence display phage library, which is selected against pMHC (5a and b). **(C)** Affinity maturation of dsTCR. The V regions of an individual TCR (1) are cloned and expressed as fusions to prototypic constant (C) domains stabilized by an artificial disulfide bridge, hence reconstituting the complete TCR ectodomain architecture (43). This recombinant dsTCR is then expressed as fusion to the M13 pIII capsid protein (2). Individual TCR α- and β-chain CDR loops of the dsTCR are randomized to create diversity (3). Usually this process is confined to the *in vivo* pMHC specificity-determining CDR3 loops (57), but has also been successfully applied to the germ-line encoded CDR2 only (58, 59). This collection of mutagenized dsTCRs is then expressed as a low valence display phage library (57), which is selected against pMHC (5). **(D)** Screening of engineered dsTCR and scTCR. The stability engineered **(A)**, or affinity matured **(B)** scTCR is reformatted to soluble, periplasmic expression (46), and individual mutated scTCRs screened for functionality against target immobilized on solid phase. The screening for desired binders following dsTCR selection is done on phage due to incompatibility with high-throughput soluble dsTCR screening (45).

this appears not to be the case as also the germ-line encoded CDR2 loop has been targeted by mutagenesis resulting in increased affinity (58, 63). Such engineered high-affinity TCRs have been used to study low level tumor associated pMHC presentation at physiological levels to obtain information that has previously been unattainable (64–66).

LESSONS LEARNT – TRANSLATION TO THE TUMOR pMHC COMPLEXES AND CANCER THERAPY

Conformational plasticity in the CDR loops upon pMHC binding appears to be a driving mechanism upon TCR–pMHC complex formation, whereas, rigid "lock and key" interaction modes also have been reported (67). This energetic diversity reflects

the multiple binding strategies implemented by the TCR during pMHC engagement. However, in spite of the described diversity (68), step by step, we are unveiling the mechanism by which a TCR deciphers a pMHC complex.

The low level of molecular shape complementarity in the TCR–pMHC complex gives rich opportunities for *in vitro* affinity maturation (57, 58, 62). This feature is likely to be generic to most TCRs (26). In the case of the affinity maturation of a HLA-A2/MART-1 specific TCR, the increase in shape complementarity was focused primarily onto the MHC portion of the complex essentially without affecting the peptide interaction (69). Thus, loss of peptide specificity could potentially be expected. However, structural and thermodynamic investigations suggested that this was not the case. In stark contrast, the opposite pMHC interaction strategy was employed by a different TCR recently reported, which was evolved toward high affinity against the HLA-A2/Tax complex. Here, a peptide-focused mechanism was found to underlie the enhanced affinity (59). Thus, the authors suggest an alternative interaction mode to the generally accepted two-step TCR–pMHC binding model (19). Here, instead of first docking the CDR1 and CDR2 onto the MHC, followed by kinetic proofreading of the peptide by the CDR3s, the opposite order of interaction is suggested. This scan-clamp model fits well with how weak, but specific protein–protein interactions have been stabilized by affinity clamping in other trimeric complexes analogous to TCR–pMHC (70, 71). It also explains how exquisite peptide specificity can be preserved both in natural and engineered systems. In either case, the picture is not consistent, and the observation that complementary structural fluctuations of both the antigenic peptide and the CDR3s of the TCR prevail even after final complex formation, underscores the remarkable flexibility of the interaction (35). Thus, at present it is challenging to validate both naturally and artificially evolved TCRs e.g., for safe use in therapy. Despite rigorous classical pre-clinical validation, a human clinical pilot study resulted in fatal cardiac toxicity due to unforeseen cross-reactivity when an affinity matured TCR against HLA-A1/MAGE-A3 was employed in specificity redirected ACT (32).

Also, there are still many questions to be answered regarding the difference between MHC class I and class II restricted TCRs. In particular, it is important to understand the significance of co-receptors in creating a fully functional immunological synapse (72). For instance, it has been shown that CD8 plays a stabilizing role in the TCR–pMHC class I interaction (73), whereas, CD4 does not appear to play a role in the corresponding TCR–pMHC class II interaction (36). Notably, these two co-receptor interactions differ significantly in MHC binding strength, which may possibly elude to their differential importance (72). Thus, an affinity threshold has been observed for the CD8 T cell compartment that limits the benefits of very high intrinsic affinity between TCR–pMHC class I (11, 74–77). So far, this has not been observed for TCR–pMHC class II. Even though fewer examples have been reported with respect to TCRs reactive toward pMHC class II, it appears that different functional rules govern this interaction (74, 77, 78), and pilot trials have shown promising results in pMHC class II restricted ACT (10, 79).

CONCLUDING REMARKS

The ability to engineer stable and high-affinity TCRs offers a unique ability to harness the immune system with an improved ability to respond to a given pMHC. However, our current understanding is still incomplete with respect to how this can safely be translated into durable cancer immunotherapy (9). One would expect improved affinity to translate into improved killing ability, but the empirical data suggest otherwise. Rather, an affinity threshold limiting any additional benefit in cellular responses above a certain TCR–pMHC binding strength has been reported, as outlined above. Moreover, the affinity threshold appears to be largely confined to the CD8 T cell compartment, as nearly all high-affinity engineered CD4 T cells have responded with both improved peptide sensitivity and preserved specificity. This gives clues as to how one might differentially exploit TCRs derived from the two distinct T cell compartments. On one hand, engineered high-affinity MHC class I and II restricted TCRs may both serve as very potent cytotoxic drugs in a soluble format (80). On the other hand, the most potent avenue for redirected cell therapy might in some cases be limited to the MHC class II restricted compartment due to the CD8 T cell affinity threshold (10, 74).

A final question is whether or not one actually needs to apply ACT to achieve optimal clinical benefit. ACT is demanding as it relies on massive *ex vivo* autologous cell expansion, which will be difficult in major patient groups for example due to cellular senescence (81–83). Epitope spreading appears to be the signature of successful anti-tumor immune responses (10, 13, 84, 85). Now it appears that this can also be achieved by the use of soluble TCRs harnessed with the ability to recruit the endogenous adaptive effector apparatus (80, 86). Such soluble TCRs appear attractive compared to the cellular approaches in light of patient convenience and safety issues (32). The use of a soluble TCR obviates the need for *ex vivo* cell expansion and a single drug may be used by a genetically heterogeneous patient population sharing the target MHC allele only. Putative off target toxicity may also be better controlled, and quenched if needed, due to tunable dosing and limited drug half-life. A soluble drug would also be less prone to efficacy variation due to *in vivo* regulatory mechanisms than ACT. How well the soluble TCR approach is reduced to clinical practice is currently under investigation through a first in man phase I/II clinical trial in late-stage malignant melanoma targeting a HLA-A2/gp100 complex (http://www.clinicaltrials.gov/ and IMCgp100).

Undoubtedly, improved phage display technology will continue to be a driver in providing engineered TCRs, which will be powerful tools to monitor and elucidate specific pMHC complexes, as well as creating novel specificities suitable for safe use in the clinic.

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Endoplasmic reticulum chaperones and their roles in the immunogenicity of cancer vaccines

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The endoplasmic reticulum (ER) is a major site of passage for proteins en route to other organelles, to the cell surface, and to the extracellular space. It is also the transport route for peptides generated in the cytosol by the proteasome into the ER for loading onto major histocompatibility complex class I (MHC I) molecules for eventual antigen presentation at the cell surface. Chaperones within the ER are critical for many of these processes; however, outside the ER certain of those chaperones may play important and direct roles in immune responses. In some cases, particular ER chaperones have been utilized as vaccines against tumors or infectious disease pathogens when purified from tumor tissue or recombinantly generated and loaded with antigen. In other cases, the cell surface location of ER chaperones has implications for immune responses as well as possible tumor resistance. We have produced heat-shock protein/chaperone protein-based cancer vaccines called "chaperone-rich cell lysate" (CRCL) that are conglomerates of chaperones enriched from solid tumors by an isoelectric focusing technique. These preparations have been effective against numerous murine tumors, as well as in a canine with an advanced lung carcinoma treated with autologous CRCL. We also published extensive proteomic analyses of CRCL prepared from human surgically resected tumor samples. Of note, these preparations contained at least 10 ER chaperones and a number of other residents, along with many other chaperones/heat-shock proteins. Gene ontology and network analyses utilizing these proteins essentially recapitulate the antigen presentation pathways and interconnections. In conjunction with our current knowledge of cell surface/extracellular ER chaperones, these data collectively suggest that a systems-level view may provide insight into the potent immune stimulatory activities of CRCL with an emphasis on the roles of ER components in those processes.

Keywords: endoplasmic reticulum, cancer vaccine, chaperones, CRCL, immunotherapy

INTRODUCTION

The endoplasmic reticulum (ER) is an organelle of new beginnings, sudden endings, twists, turns and connections, major changes, and passage to new places. During protein translation, nascent proteins destined for the ER or other locations along the secretory route protrude an appropriate "signal sequence" from the ribosome that the signal recognition particle (SRP) distinguishes as an ER address label (1). After the SRP binds to the peptide, it tethers the ribosome near the SRP receptor on the ER membrane. The ribosome docks with the SEC61 complex for co-translation of the rest of the protein across the ER membrane (2, 3). Once in the ER, chaperone-based folding occurs, along with glycosylation, disulfide bond formation, and transport out of the ER into the Golgi if such address labels are found in the newly minted protein (4). These activities require calcium, and the ER (along with mitochondria) is the major calcium storage compartment in a typical cell. Many of the chaperones are calcium-binding proteins with extensive capacity; this plays into their functions, as well as to other calcium-essential units in the cell (5). The oxidizing environment of the ER lumen promotes disulfide bridge formation, largely via protein disulfide isomerase (PDI/PDIA) family members, and these bonds are likely critical in the proper folding of individual proteins and in formation of multi-subunit complexes (6). The ER has numerous quality control (ERQC) mechanisms to assure properly folded proteins exit the ER for other destinations, but may essentially end in ER-associated degradation (ERAD) (7). Proteins that do not achieve the appropriate tertiary or quaternary confirmations are considered terminally misfolded and are poly-ubiquitinated (in a complex fashion) with retrotranslocation to the cytosol for proteasomal degradation (8). The efficiency of entry, exit, and arrival at the final destination varies dramatically for different proteins and ranges from nearly 100% "success" (i.e., amount of a given protein entering the ER compared to the amount of that protein reaching its final localization, such as the cell surface) to as low as 25% (9). The lumenal environment of the ER is most akin to the cell's exterior, and the ER is a portal connecting the cytosol to the cell surface and beyond.

The chaperones of the ER are critical to many aspects of ER function, whether in protein folding modes, as calcium binders, as sensors of stress such as the unfolded protein response (10), or due to cell-surface localization or extracellular release, as immune modulators (11–18). These latter characteristics combine with the protein- and peptide-binding/carrying capacity of chaperones to allow for their utilization as vaccines, particularly in oncology

Table 1 | Chaperone proteins described herein and their subcellular localizations.

Protein common name	Gene name	Subcellular localization					
		Endoplasmic reticulum/Golgi	Cytosol	Nucleus	Mitochondria	Lysosome	Cell surface[a]
HSP27[b]	HSPB1		X	X			X
HSP47 (serpin H1)	SERPINH1	X					X
HSP60	HSPD1		X		X		X
HSP70	HSPA1A/B		X	X		X	X
HSC70	HSPA8		X	X		X	X
GRP78 (BiP)	HSPA5	X	X	X	X		X
HSP90	HSP90AA/B1		X	X			X
HSP110	HSPH1		X	X			X
GRP94 (gp96)	HSP90B1	X		X			X
GRP170 (ORP150)	HYOU1	X					X
PDI/PDIA[c]	P4HB	X					X
CRT/CALR	CALR	X	X				X

Chaperones in this article and their subcellular localizations. This is a list of the chaperone proteins and their gene names mentioned in this article annotating their known subcellular localizations.

[a] *Cell-surface localization is most often associated with tumor cell surfaces.*

[b] *Murine version is often called HSP25.*

[c] *There are muliple PDI (protein disulfide isomerase) family members too numerous to include here.*

(19–22). This review will highlight the multifaceted roles of the ER in immunity, and will then focus on how chaperones from the ER may contribute to immune responses under "exogenous" circumstances, e.g., once outside the cell. We will further discuss how such chaperones may contribute to anti-cancer immunity in a complex vaccine like chaperone-rich cell lysate (CRCL). As we will discuss a number of chaperone proteins from various subcellular locations beyond those of the ER, we have prepared **Table 1** to aid in keeping track of these proteins.

THE ENDOPLASMIC RETICULUM AS A CONDUIT TO IMMUNITY: T CELLS "SEE INTO THE SOUL" OF A CELL

The mammalian immune system has developed a largely "non-invasive" means of assessing the immune status of most of the host organism's cells. Immune effector cells of both the adaptive arm (i.e., $CD4^+$ and $CD8^+$ T cells) and the innate arm [e.g., natural killer (NK) cells] monitor cell surfaces by engaging major histocompatibility complex class I and II (MHC I and II) molecules in the case of T cells (23–25) and damage-associated molecular patterns (DAMPs) (26), as well as stress ligands such as MICA/B and ULBP families (27) in the case of NK cells. NK cells also balance activating and inhibitory receptor stimulation that may be present on normal cells, or downregulated or absent on abnormal cells, such as loss of MHC I (28). Perturbations that occur in the cytosol such as pathogenic infection or the genetic, proteomic, and metabolic disarray of neoplasia may lead to the expression of non-self proteins or of mutated self proteins. These, along with other "normal" but obsolete proteins are poly-ubiquitinated and are targeted for degradation into short peptides by the proteasome. With additional trimming (or outright proteasome-independent generation) possible by cytosolic peptidases, peptides enter the ER through the TAP transporters (transporters associated with antigen processing; ABC family members). There, the peptides may be further pruned before chaperone-assisted loading onto MHC I molecules, which are then packaged for transit to the cell surface. Display of peptides in the context of MHC I molecules provides the reading frame for $CD8^+$ T cells that determine the normal or abnormal status of the presenting cell.

MHC II display and presentation generally only occurs in specialized immune cells known as professional antigen-presenting cells (APCs), such as macrophage, dendritic cells (DCs), and B cells (29). However, MHC II expression can occur on other cells such as endothelial cells following IFNγ exposure (30) or on neuronal cells in peripheral neuropathies (31), turning such cells into APCs. Exogenous antigens are engulfed at the cell surface into endosomal/phagosomal vesicles (32) where denaturation and degradation of proteins begins. Meanwhile, MHC II molecules are assembled in the ER with a "placeholder" in the peptide-binding cleft, the chaperone invariant chain (Ii). The MHC II molecules enter vesicles and are released into the cytosol's endocytic pathway where the Ii is cleaved to class II-associated invariant chain peptide (CLIP). These vesicles encounter the late endocytic/phagocytic vesicles with lysosomal characteristics where CLIP is displaced by peptides with higher affinity for the MHC binding pocket. These are often called MHC II compartments (MIICs), and the vesicles eventually deliver MHC II to the cell surface for presentation to $CD4^+$ T cells (33) for the latters' assessments of the immune status of the presenting cell. Recent work with the MHC II process describes more complicated routes and alternatives, and some of this information will re-appear in our discussion of antigen cross-presentation (34, 35).

Thus, $CD4^+$ and $CD8^+$ T cells scan cell surfaces for the MHC-displayed peptides that may indicate a pathologic state within those cells; however, the T cells require activation and "education"

concerning the nature of the problem. Professional APCs serving as scavenger cells may have confronted a situation with cell/tissue damage that resulted in the APCs engulfing extracellular material. If this occurred in an inflammatory environment in the presence of released "danger signals" (36, 37), the APCs become stimulated to provide "signal one" and "signal two" to T cells (38, 39). The first signal is the MHC-restricted peptide that is capable of triggering a T-cell receptor (TCR) specific for that particular peptide in that particular MHC peptide-binding cleft; the assumption is that the peptide is indicative of the distress (infection, mutation) in the donor cell before acquisition by the APC. The second signal comes from the expression of co-stimulatory molecules by the APC, such as CD80/CD86, that provide activation impetus for T cell. The interface between APC and T cell in this scenario is called the "immunological synapse" (40). After recognition of antigen, activation, and stimulation, the T cells exit the lymph node and enter the periphery to search for affected cells that display the antigens that indicate disease (i.e., the same ones that triggered the TCRs originally).

In the scenario described, APCs acquire exogenous antigens that are displayed to T cells; the classical mode of antigen uptake and display by MHC in this trafficking pathway is via MHC II, which would induce only a $CD4^+$ T-cell response. However, it is clear that APCs also display foreign and self peptides on MHC I molecules, called "cross-presentation" (41). The endosomal trafficking of endocytosed proteins, particularly in professional APCs, can direct such vesicles away from lysosomal degradation; peptide generation within endosomes may allow for direct loading of vesicle-bound MHC I molecules (42). On the other hand, the proteins or peptides could passage out of the vesicles and into the cytosol for proteasomal processing and entry into the classical MHC I pathway. These peptides could also passage back into endosomes via endosomal TAP transporters; if MHC I molecules are in those vesicles, the peptides may be loaded onto the presentation proteins (42).

Through these various mechanisms, which start with the assembly of MHC molecules in the ER, T cells may be stimulated to respond to a pathogenic state, and during surveillance are able to determine the internal stasis or possible malcontented nature of the MHC-presenting cell. The "outside-looking-in" format does not require destruction of the presenting cell, unless that cell displays antigens indicative of a pathogenic state.

CHAPERONES ON THE ROOF: CELL-SURFACE CHAPERONES IN IMMUNITY

Roles of chaperones in the antigenicity of foreign, and possibly self peptides and proteins, are usually associated with extracellular chaperones as danger signals (36, 37, 43–45). The strong sequence and structural relationships between chaperones from primitive and more advanced organisms (46) suggest that immune reactivity that evolved against bacterial chaperones may lead to cross-reactivity with mammalian chaperones [e.g., Ref. (47)], but those outcomes are varied (48). Binding to pattern recognition receptors such as Toll-like receptors (TLRs) is a characteristic of extracellular chaperones, both mammalian and bacterial (49, 50) but TLR stimulation may, at least in some cases, result from bacterial PAMPs associated with the chaperones (51). Nonetheless, the innate immune signaling aspects of chaperones outside the cell are likely the key initial mediator steps in promoting an immune response.

Cell-surface display of chaperones represents a special case of re-localized chaperones capable of provoking immune responses. In oncology, membrane HSP70 is one of the most heavily studied (52), where a 14-mer region of the chaperone is recognized as a target for NK cells (53). The mechanism for the HSP70's membrane association remains unclear, although its interactions with negatively charged phospholipids may play a role (54), with involvement of particular domains of the protein (55). The "large" relative of HSP70, HSP110 (22), has been noted on the surfaces of brain tumor cell lines (15, 16), but the implications of this localization are unknown.

The small heat-shock protein HSP27 (HSP25 in mice) was one of the number of chaperone proteins found on tumor cell surfaces by proteomic analyses (56), as well as by flow cytometry (15). The immune responses to surface HSP27 remain unclear, but murine mammary cancer cells selected for cell-surface expression of HSP25 proliferated faster and exhibited more frequent lung metastatic lesions than cells with lower or minimal surface HSP25 display (57). Interestingly, in those immune-competent animals, heat-shock-driven inducible HSP70 surface expression on those cells resulted in reduced metastatic growth and overall increased survival compared to implantation of cells with low surface HSP70 expression, suggesting that immune responses may play a role, perhaps via NK cells (53).

HSP90 was one of the original chaperones found to be a "tumor-specific transplantation antigen" (TSTA) potentially useful as a vaccine when purified from tumors (58), and was shown to be present on murine MethA tumor cell surfaces. The surface expression was discovered on other tumor cell lines as well (59). Surface HSP90 interacts with HER2 and mediates tumor cell invasiveness on breast cancer cells (60), and blocking surface HSP90 activity with a cell-impermeant inhibitor or antibodies validates this in other tumor types (61–63). The chaperone was also identified on the surfaces of CNS/neuronal-derived tumors (15, 64), where expression on spheroid lines was higher (64). Since the growth of CNS tumors in "stem cell-like" cultures (that frequently form spheroids) is a relatively new phenomenon, HSP90 surface expression may need to be re-examined for those tumors. As mentioned above, tumor-surface HSP70 is a known NK cell target, but both surface HSP70 and HSP90 are also gamma–delta T-cell targets, at least in EBV-transformed B cells (65, 66).

While the mechanisms of cell-surface display for chaperones considered to be canonically localized to the cytosol (or nucleus, in some cases) remain puzzling, one can imagine a simpler route to the cell surface for chaperones originally localized to the ER. As they are residents of the compartment of origin for proteins destined for cell-surface expression or extracellular release, their passage out of the ER requires bypassing KDEL receptors. These are proteins in pre- or *cis*-Golgi compartments that recognize the lys–asp–glu–leu (KDEL) motif present on most ER resident proteins. Those proteins that progress from the ER into the Golgi compartments are recognized and bound by the family of KDEL receptors that then engage in retrograde transport to return the KDEL-containing proteins to the ER (67).

Of the cell surface-expressed ER chaperones, GRP78 (BiP) is one of the best characterized and was noted on the surfaces of a hybrid neuroblastoma cell line in the late 1990s (68) (and has been found on other CNS/neurologic tumors) (15, 16). GRP78 was also one of the chaperone proteins identified on tumor cell surfaces in a proteomic study (56) (along with other HSP70 family members, and HSPs 27, 47, and 60, and PDI members). GRP78's chaperone capacity, apparently still intact on the cell surface, was used to target pro-apoptotic peptides fused to consensus GRP78 binding motifs resulting in cell death and reduced model tumor growth (69) [and further reviewed here (70)]. At the cell surface, GRP78 acts as a receptor or in complexes with numerous partners that may promote cell survival or engage in apoptosis (71). Surface GRP78 is a therapeutic antibody target (72, 73), but in some cases antibodies in patient sera bind to activated α2-macroglobulin's agonist site on its receptor GRP78. This leads to tumor cell growth stimulation and apoptosis prevention (74). In general, tumor-surface GRP78 is indicative of enhanced malignant tumor phenotypes (71).

GRP94 (also called gp96, endoplasmin, ERp99), is the ER HSP90 paralog. Like HSP90, it was identified as a "tumor rejection antigen" (TRA) purified from MethA and CMS5 murine sarcomas (75), and was found in plasma membrane fractions and on murine and human tumor cell surfaces (76–78). The function of surface GRP94 is not entirely clear, but it appears to play a role in the processing of surface metalloproteinases (79). Immunologically, surface GRP94 can activate DCs, inducing a pro-inflammatory state with activation of tumor-specific T cells (80).

Despite its prominent role as an ER chaperone cancer vaccine (81), GRP170/ORP150 has only rarely been cited as a cancer cell-surface protein (15, 82), but it has been identified on mouse egg oolemma (83) as well as human sperm surfaces (84, 85). Other ER residents such as ERp5/PDI6 (PDI family) are present on tumor cell surfaces; in this case, the chaperone is involved in the release of the NK cell activating receptor MICA from tumor cell surfaces, presumably as a protective measure to avoid NK attack. PDI also functions in the shedding of tumor endothelial maker 5 (TEM5) with potential impacts on cell adhesion and migration (86). PDI and calreticulin were among the KDEL-containing proteins previously identified as surface components of the NG108-15 cell line (68). PDI family members had been identified as localized to platelet surfaces as early as 1995 (87) and were later shown to be on B-CLL cells (88). PDI plays a role in glioma xenograft tumor invasiveness (89). Roles for surface PDIs include transnitrosation and nitric oxide metabolism (86) and formation of thiols on cell-surface proteins (90).

Calreticulin (CRT; CALR) is considered as an ER chaperone, but with very divergent intracellular, cell-surface, and extracellular localizations (91). It was identified with cell surfaces as early as 1995 (92, 93), and is regarded as a major immunologic player whose surface exposure promotes the immunogenicity of tumor cells dying by particular chemotherapy agents (94). CRT was already known as a tumor peptide-carrying cancer vaccine candidate (95–97), but in these scenarios of (normally immune-silent) apoptotic cell death, it is viewed as an engulfment signal for phagocytic cells such as macrophage and DCs (98, 99). While CRT is clearly present on numerous cell types, including cancers (15, 91), those cells may resist APC interactions and phagocytosis via CD47 (98, 100). These studies strongly suggest that how tumor cells die matters greatly to the immune system, and delineate potential avenues of improved therapy.

Thus, cell-surface localization of various chaperone proteins, while originally quite controversial, is now accepted, and seems to associate with cancer pathology. The roles of surface chaperones in anti-tumor immunity may be complicated in terms of putative function favoring the tumor's growth versus serving as immune attractants; perhaps, this balance can somehow be shifted toward effective immune responses.

CHAPERONES OUTSIDE: EXTRACELLULAR CHAPERONES IN IMMUNITY

As mentioned above, we have few well-understood mechanisms for the localization of cytosolic chaperones/heat-shock proteins to the cell surface. Similarly, we know little about the release of such chaperones outside the cell (101, 102), despite nearly three decades of research. ER chaperones are already in the secretory pathway, so bypassing KDEL receptors could explain that release. Another mechanism from the cytosol or the ER could involve vesicular release via endolysosomes (103, 104) or by extracellular vesicles (exosomes, microvesicles) (15, 105). While there may be a number of functional roles for extracellular chaperones such as extracellular signaling (106), chaperoning extracellular matrix components (107, 108), and general cytoprotection during injury (109) or in proteostasis (110), much of the research on extracellular chaperones concerns their roles in immunity.

We noted above that the immune properties of extracellular chaperones are intrinsically related to those proteins acting as danger signals when they interact with innate immune cells (111, 112). This stimulatory capacity at a distance is reminiscent of cytokines, and thus led to the term "chaperokine" (113), with particular involvement of cellular TLRs. Extracellular chaperones such as HSP27 (114), HSP60 (115), HSP70 (14), GRP94 (116), HSP90, and GRP170 (117) have all been shown to bind TLRs. Other chaperone receptors include molecules such as CD14, CD36, CD40, LOX1, scavenger receptors SR-A and SREC-1, and CD91 (also called LRP1 and A2MR, the α2-macroglobulin receptor) (118). Thus, innate immune cells, APCs, and a number of other cell types possess receptors implicated in binding extracellular chaperones presumably released by cells under stressful circumstances.

In the area of cancer immunotherapy, the concept of cancer cells producing and releasing chaperones as a form of "auto-vaccination" is an attractive one, and there have been a number of attempts to generate tumor cell lines producing secretable versions of immunogenic chaperones. An example of this is the ER resident vaccine candidate GRP170 (also called ORP150) (22), which has demonstrated danger signal capacity if secreted outside the cell (45) and has been shown to chaperone whole proteins in that secretable form that are antigenic (119). This links the innate immune stimulation by chaperone proteins with the adaptive (targeted) response and demonstrates how chaperones released by or derived from pathogenic tissues may possess both adjuvant and antigen. Numerous other chaperones have been engineered or designed for secretion from tumor cells [reviewed here (120)], including GRP78, which was previously regarded as ineffective as

a cancer vaccine (121). The use of an allogeneic tumor cell vaccine with secretable GRP94 (AD100-gp96-Ig) in clinical trials has been reported (122). Putative benefits of this latter form of a vaccine include the "off-the-shelf" utility (i.e., the vaccine may be used on essentially any patient and does not need to come from autologous tumor), the "host versus graft" immune cross-reactivity with the allogeneic cells, and the "continuous-release format" of the GRP94 as an advantage in stimulating immune responses in contrast to the bolus effect from an injectable vaccine. One disadvantage would be the lack of true personal, individual patient tumor-specific antigens available from an autologous preparation, and the constant need for reassurance that the tumor cells were not proliferating.

The presence of chaperones in the extracellular milieu, by intent, or by stress, or damage, offers insight into the biology of the sensation of danger by the immune system, as well as potential practical applications from a vaccine perspective. Our next section will discuss chaperone protein-based cancer vaccines, with an emphasis on ER proteins as components of those vaccines.

CHAPERONES AS VACCINES: LONE WOLVES AND GETTING THE WAGONS IN A CRCL

The release of chaperones extracellularly, whether by bioengineering, induced stress, or immune-noticeable forms of cell death, may be reenacted in vaccine scenarios where chaperones are purposefully extracted from tumor cells/tissues and re-introduced to patients, typically by parenteral administration. Benefits in this situation include known dosages, ability to monitor local reactions [e.g., delayed-type hypersensitivity (DTH) responses], and the ability to directly enhance APC activation and migration with topical applicants such as imiquimod (123). Depending on the type of vaccine generated, if the source is a tumor sample, that may become the limiting reagent (124, 125), the heterogeneity of tumors may make accurate "dosing" (i.e., how much of the chaperone is actually from the tumor) more difficult. Nonetheless, patient tumor-derived GRP94/gp96 as an autologous therapeutic vaccine has progressed the farthest in various clinical trials, starting in 2000 [reviewed here (126)] and has since included trials for patients with colorectal and pancreatic cancers, melanoma, non-Hodgkin's lymphoma, renal cell carcinoma, and continues with Phase II trials for patients with high grade gliomas. The product is currently owned by Agenus[1], and has gone by HSPPC-96, Oncophage, Vitespen, and currently, Prophage. A major attractive feature has been the low incidence of deleterious side effects, and it has received regulatory approval in Russia for patients at intermediate risk for disease recurrence of renal cell carcinoma, the first such cancer vaccine approved anywhere (127). However, further European Union approval was unsuccessful, where the agency cited, among other issues, a lack of identified antigenic peptides associated with the vaccine preparations[2]. Other heat-shock protein vaccines are also at clinical trial stages (e.g., the HSP110–gp100 complex, NCT01744171)[3], including one that induces HSPs by inflicting cryoablation or radiofrequency ablation on tumors (NCT00568763) rather than the direct use of individual HSPs as vaccines.

A question that frequently arises in these cancer vaccine scenarios regards the generation of autoimmunity. To some extent, that is indeed the goal of cancer immunotherapy, targeting a tissue that is largely "self." However, the immune suppressive activities of most cancers likely prevent anti-tumor activity as well as true autoimmune activity. Current immune "checkpoint inhibitors" such as antibodies against CTLA-4 (ipilimumab) that prevent T-cell repression have driven potent anti-tumor responses, but also occasional significant autoimmunity (128). However, such autoimmune responses have not been noted in chaperone-based anti-cancer vaccines (129, 130), but as combination therapies will start utilizing such checkpoint inhibitors (131), vigilance will be essential.

The concept of a multiple-chaperone vaccine arose from the thought that dying cells release entire cohorts of proteins rather than purified batches of individual ones. CRCL is such a multi-chaperone vaccine preparation that initially was shown to contain the four known immunogenic chaperones of that time: HSP70, HSP90, GRP94, and calreticulin (132). The rationale was that these four chaperones, from the cytoplasmic and ER compartments, would likely contain a broader repertoire of tumor antigens from an autologous source, and possibly provide greater APC stimulation than single chaperone vaccines. Rather than purification, CRCL preparation utilized a free solution-isoelectric focusing (FS-IEF) technique that resulted in a large, highly cohesive complex of hundreds of proteins (97, 133). This complex activated DCs yielding high expression of CD40 and MHC I and release of IL-12, resulting in highly stimulated T cells (134, 135). This APC stimulation remained effective even in the face of regulatory T cells (Tregs) (136). Additional studies showed that CRCL-stimulated APCs upregulated CD70, NFκB, and iNOS, along with NO, TNFα, and RANTES production, and enhanced phosphorylation of STAT1 and STAT5, and activation of the AKT and MAPK pathways (137). Depletion of chaperones diminished CRCL's immune properties (134). Immunological testing demonstrated the presence of the BCR–ABL fusion peptide in CRCL derived from BCR–ABL positive tumors that resulted in peptide-specific responding T cells (138), and further biochemical and proteomic work identified nearly 60 peptides associated with CRCL (139). When used pre-clinically as a single agent in prophylactic and therapeutic vaccination schemes, or as an antigen source for DC vaccines, or in combination with other treatment regimens, CRCL was found effective against numerous murine hematologic malignancies, and against melanoma, fibrosarcoma, breast cancer, and brain tumor models (15, 97, 134, 140–144). CRCL was also shown to drive NK cell pro-inflammatory cytokine and chemokine release (145) as well as bioactive anti-tumor antibody production (143). CRCL, combined initially with the topical TLR stimulant imiquimod, was used as the sole post-surgical therapeutic agent to treat an aggressive metastatic lung cancer in a canine patient (146); the dog's prognosis was <4 weeks survival, but she survived for 11 months with CRCL treatment. Finally, CRCL is a component of an immunotherapy regimen currently in clinical trials (NCT01998542, NCT01995227).

[1]http://www.agenusbio.com/

[2]http://www.ema.europa.eu/docs/en_GB/document_library/Application_withdrawal_assessment_report/2010/03/WC500075459.pdf

[3]www.clinicaltrials.gov

Table 2 | ER/ER-associated proteins identified in a previous proteomic study of human CRCL.

ID	Symbol	Entrez gene name
Q6DD88	ATL3	Atlastin gtpase 3
P27797	CALR	Calreticulin
P27824	CANX	Calnexin
O14735	CDIPT	CDP-diacylglycerol – inositol 3-phosphatidyltransferase
Q9UKY3	CES1P1	Carboxylesterase 1 pseudogene 1
Q99653	CHP1	Calcineurin-like EF-hand protein 1
Q9BUN8	DERL1	Derlin 1
Q7Z2K6	ERMP1	Endoplasmic reticulum metallopeptidase 1
P30040	ERP29	Endoplasmic reticulum protein 29
Q9BS26	ERP44	Endoplasmic reticulum protein 44
P30443	HLA-A	Major histocompatibility complex, class I, A
D3U3L9	HLA-B	Major histocompatibility complex, class I, B
A5D8 × 1	HLA-C	Major histocompatibility complex, class I, C
P14625	HSP90B1	Heat-shock protein 90 kDa beta (grp94), member 1
P11021	HSPA5	Heat-shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)
Q9Y4L1	HYOU1	Hypoxia upregulated 1
P13674	P4HA1	Prolyl 4-hydroxylase, alpha polypeptide I
P07237	P4HB	Prolyl 4-hydroxylase, beta polypeptide
O75340	PDCD6	Programed cell death 6
P30101	PDIA3	Protein disulfide isomerase family A, member 3
P13667	PDIA4	Protein disulfide isomerase family A, member 4
Q15084	PDIA6	Protein disulfide isomerase family A, member 6
O60240	PLIN1	Perilipin 1
Q96Q06	PLIN4	Perilipin 4
P28066	PSMA5	Proteasome (prosome, macropain) subunit, alpha type, 5
Q99436	PSMB7	Proteasome (prosome, macropain) subunit, beta type, 7
Q06323	PSME1	Proteasome (prosome, macropain) activator subunit 1 (pa28 alpha)
Q9UL46	PSME2	Proteasome (prosome, macropain) activator subunit 2 (pa28 beta)
O75396	SEC22B	SEC22 vesicle trafficking protein homolog B
Q15437	SEC23B	Sec23 homolog B (*S. cerevisiae*)
P61619	SEC61A1	Sec61 alpha 1 subunit (*S. cerevisiae*)
Q03518	TAP1	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)
Q03519	TAP2	Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)
Q04323	UBXN1	UBX domain protein 1
P09936	UCHL1	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)
P55072	VCP	Valosin containing protein

ER and ER-associated proteins identified in a previous proteomic study of human CRCL. Proteins were identified by gel separation, excision, digestion, and mass spectrometry. These proteins were originally found in separate locations in this publication (133), but were extracted and organized into this table.

Previous proteomic work to better biochemically characterize human CRCL preparations from various tumor types (133) identified at least 10 known ER chaperones; re-evaluating all of the data in that publication provided us with 36 proteins that are from the ER or have close associations with that organelle, such as proteasome components (**Table 2**). Gene ontology (GO) assessment of those proteins using Ingenuity Pathway Analysis (IPA) revealed canonical pathways with clear immunological relevance, including antigen presentation, dendritic cell maturation and communications, and T-cell signaling; the top 20 significantly scoring Pathways are shown in **Figure 1**. There is also a high overlap among two-thirds of the pathways (not shown).

One striking outcome from previous IPA applications was a networks/associated functions interactome generated that showed connectivity among various chaperones (both ER and cytosolic), immune-related molecules, nuclear factors, and metabolic enzymes (133). Focusing here on the ER components and associated proteins, we have generated a similar interactome by combining two networks with very high scores (derived from Fisher's exact test) that seemingly recapitulate the antigen processing pathway for MHC Class I molecules, as well as portions of the ERAD pathways (**Figure 2**). The selective entries of ER and ER-related proteins may serve to skew the readouts from IPA, but it also suggests that the ER contributions to these interactomes in particular may play heavily into CRCL functionality.

Of the molecules included in this list but not discussed previously (133) from an immune perspective, DERL1 is a member of the ER quality control/ERAD system, where it mediates MHC degradation (147). It is also upregulated in tumors and in epithelial cells exposed to tumors, where it may be involved in angiogenesis (148). Its presence on tumor cell surfaces makes it amenable to antibody targeting (149). ERP44, a PDI family member, plays a controlling role in IgM assembly in B cells (150). The perilipins play roles in the formation and transport of lipid bodies/lipid droplets such as those formed in leukocyte inflammatory responses (151); such lipid bodies are involved in

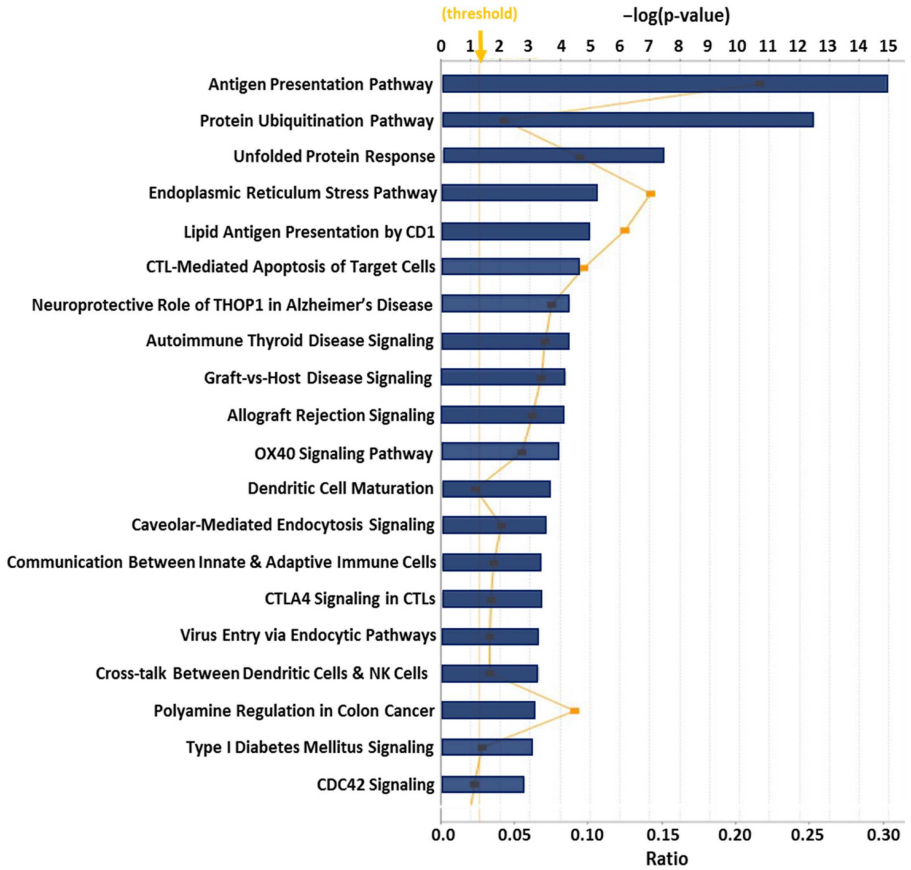

FIGURE 1 | Top 20 canonical pathways derived from ingenuity pathway analysis (IPA) gene ontology algorithms for the 36 ER and ER-associated proteins from Table 2. These pathways emerged following IPA "Core Analysis." Graph shows category scores; "threshold" indicates the minimum significance level [scored as −log(*p*-value) from Fisher's exact test, set here to 1.25]. "Ratio" (differential yellow line and markers) refers to the number of molecules from the dataset that map to the pathway listed divided by the total number of molecules that map to the canonical pathway from within the IPA knowledgebase.

phagocytosed antigen cross-presentation in DCs (152). SEC22B, a SNARE (soluble *N*-ethylmaleimide sensitive fusion attachment protein receptor) protein, is another molecule clearly involved in antigen cross-presentation via maturation of phagosomes (153). SEC23B, while having no clear immune function, is a required gene for cells with high secretory outputs (154), and not surprisingly is overexpressed in hepatocellular carcinomas (155), and perhaps could be regarded as an immune target. UCHL1, also called protein gene product 9.5 (PGP9.5) has been identified as an autoantigen in lung cancer patients (156). Thus, CRCL may contain ER proteins besides the chaperones that may play roles in immune cells or may act as targets of immune responses.

The extraordinary connectivity found in the interactome of these proteins (**Figure 2**) suggests that there may be structural relationships involved, and indeed bizarre structures were seen in electron microscopy, and large particles were identified by nanoparticle tracking analysis, in the aforementioned publication (133). Prior to that, CRCL was shown to exist biochemically as a large entity of virus-sized proportions by size-exclusion chromatography (97). Which proteins are involved, and what roles they may play, are currently matters of speculation, but conceptually a model for a "relay line" of chaperones sequentially transferring peptides during antigen processing and presentation has been proposed (157). There has even been validation of the peptide transfer (158, 159), suggesting that at least close physical proximity, if not protein–protein contact, is necessary. Nanoparticles for immune stimulation, such as pathogen-like particles, are gaining headway in vaccine research (160, 161). Perhaps, CRCL inadvertently retains some form of particulate assembly due to its cytoskeletal content, and carries antigens within this "cage" due to its chaperone content. The calreticulin component of CRCL may be an especially potent "eat me" signal for APCs, which then view CRCL as an object with viral-like physical properties, and upon engulfment, have endocytosed numerous antigens via the chaperones, including antigens carried by the ER chaperones abundant in CRCL.

Our "peptidomics" work with CRCL-associated peptides implied that the protein origins of those putative antigens came from all cellular compartments, and were high-value targets for immune responses (139). Coupled with the proteomics work

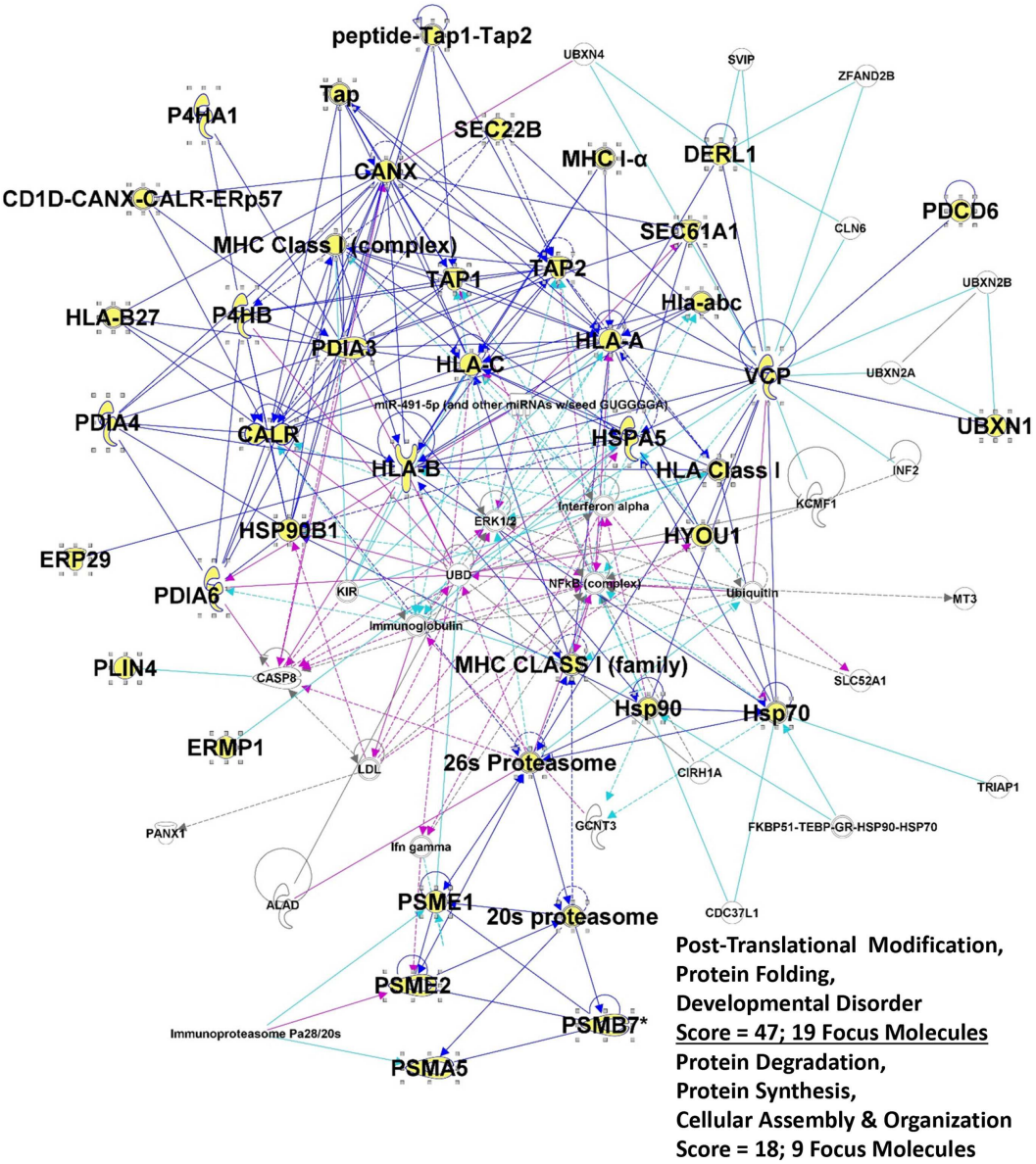

FIGURE 2 | Intersection of the top 2 IPA interactomes derived from the top networks/associated functions for the 36 proteins listed in Table 2. Proteins from table are shown in large bold font, and the protein symbols are shown in yellow fill. "Edges" (lines) show connections between or among molecules; solid lines indicated known direct interactions. Dotted lines indicated indirect interactions. Dark blue lines connect proteins from within the entry group; turquoise lines connect proteins that were in the network but not found in our proteomic study. Cranberry colored lines show the intersection of proteins between the two interactomes. "Score" refers to the $-\log(p\text{-value})$ from Fisher's exact score, and "Focus Molecules" are "seeds" for generation of focal points or nodes within the network.

mentioned here (133), the GO analyses provide a basis for a systems biology approach to understanding the biochemical (and perhaps structural) mechanisms for the success of the vaccine. The intrinsic roles the ER-derived and -associated components of CRCL are undoubtedly critical to the vaccine's utility. Further research is required to truly understand the biophysical structure of the vaccine and to determine what impact that has on the immunological responses driven by the vaccine. The ER proteins, representing the connection between the antigen-generating cytosol, the antigen-presenting cell surface, and danger signal activities extracellularly, are undoubtedly vital to the inherent adjuvant/antigen formulation that is CRCL.

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