Published in final edited form as:

Mol Immunol. 2008 March ; 45(5): 1414–1423.

CALRETICULIN REQUIRES AN ANCILLARY ADJUVANT FOR THE INDUCTION OF EFFICIENT CYTOTOXIC T CELL RESPONSES

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Abstract

Molecular chaperones stimulate the immune system to induce both protective immune responses and therapeutic tumor rejection. However, the underlying basis for this immunogenic activity is not well understood. A variety of chaperones, including calreticulin, hsp70 and grp94, function as vehicles to efficiently traffic associated peptides into professional antigen presenting cells. Importantly, these chaperones have also been proposed to function as adjuvants by stimulating the dendritic cell activation and co-stimulatory responses required to elicit peptide-specific CD8⁺ T cell cytolytic activity. The efficacy of chaperone-mediated tumor rejection has been attributed to the ability of chaperones to function in both of these capacities. However, purified calreticulin has not previously been assessed for its ability to elicit DC maturation and, moreover, recent data indicates that it is not efficient at inducing Nf-κB activity which often accompanies or stimulates DC maturation. Here we use two complementary methods to produce endotoxin-free calreticulin and demonstrate that it does not measurably mature or activate dendritic cells both *in vitro* and *in vivo*. Additionally, a calreticulin/peptide complex required the addition of an exogenous adjuvant to elicit *in vivo* cytotoxic CD8⁺ T cell responses. These data are discussed with respect to current models for chaperone-derived immune responses and in regard to rational vaccine design.

1. Introduction

Molecular chaperones are ubiquitous intracellular proteins that have potent immunological activity. Chaperones were originally identified as immunogens in a screen for tumor-rejection antigens. Subsequent studies have led to proposals that they may also endogenously function as 'danger signals' when released from dying cells and may exacerbate autoimmune diseases (Eggleton and Llewellyn, 1999; Routsias and Tzioufas, 2006; Srivastava, 2002). A variety of chaperones, including calreticulin (CRT), gp96, hsp70 and hsp60, elicit prophylactic and therapeutic anti-tumor responses (Basu and Srivastava, 1999; Sato et al., 2001; Tamura et al., 1997; Yedavelli et al., 1999) which has resulted in ongoing clinical trials for their evaluation as autologous cancer therapies (Belli et al., 2002; Misra et al., 2006). However, the underlying basis for the immunological activity of the chaperones is not well understood, nor is it known if all immunogenic chaperones activate the same immunological pathways. Chaperones were originally proposed to shuttle associated peptides into antigen presenting cells (APCs) while concomitantly eliciting the requisite co-stimulatory responses, including dendritic cell (DC)

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activation and cytokine production, to induce peptide-specific adaptive immune responses (Srivastava, 2002). Recent reports indicate that, under some experimental conditions, the ability of chaperones to activate the innate immune system, likely through DCs, is sufficient to elicit tumor rejection (Baker-LePain et al., 2002). The ability to activate DCs has likewise been proposed as an underlying basis for their role as endogenous danger signals (Gallucci et al., 1999). Therefore, a current emphasis is to understand how chaperones function as immunological adjuvants.

The mechanisms by which chaperones activate DCs are not well understood and are, indeed, contentious. Early studies evaluating the abilities of molecular chaperones to function as molecular adjuvants and to induce the maturation of dendritic cells (DCs) were potentially compromised by endotoxin contamination. However, subsequent investigations of several endotoxin-free preparations of chaperones indicated that they can function as adjuvants, with DC activation and maturation used as a common experimental endpoint (Flohe et al., 2003; Ramirez et al., 2005). In contrast, others have reported that hsp60, hsp70 and hsp105 prepared under endotoxin-free conditions are unable to activate DCs (Bausinger et al., 2002; Gao and Tsan, 2003a; Gao and Tsan, 2003b), while gp96 and CRT were surprisingly unable to activate the pro-inflammatory NF- κ B cellular signaling pathway when prepared under endotoxin-free conditions (Reed et al., 2003). Clearly one continuing complication is that TLRs (Toll-like receptors) 2 and 4 have been reported to mediate DC activation by chaperones (Asea et al., 2002; Vabulas et al., 2001; Vabulas et al., 2002b), however both TLRs 2 and 4 are also established DC receptors for microbial products that frequently contaminate protein preparations. Interestingly, both gp96 and hsp60 have LPS binding activity which augments the activity of LPS to stimulate DCs (Osterloh et al., 2007; Warger et al., 2006); relevant to our present studies, CRT harbors no endogenous LPS binding activity (Reed et al., 2003). DC activation and maturation is invoked as an underlying basis for the CRT contribution to autoimmune disorders (Eggleton and Llewellyn, 1999), to the use of CRT as an adjuvant to elicit peptide-specific responses (Nair et al., 1999), and to its tumor-rejection activity (Basu and Srivastava, 1999; Cheng et al., 2005; Hsieh et al., 2004). However, the ability of purified CRT to activate and mature DCs has not been directly tested. Therefore, we endeavored to assess the efficacy of purified, endotoxin-free, CRT to activate DCs.

Here we report the surprising failure of CRT to induce DC maturation and activation. Multiple sources of endotoxin-free CRT did not induce phenotypic DC activation as assessed by upregulation of the cell surface molecules MHC-II and CD86. This result was confirmed both *in vitro* and *in vivo*. Additionally, CRT did not elicit a pro-inflammatory cytokine response from DCs. We then extended these functional studies to test the ability of CRT to induce a cytotoxic T cell (CTL) response, canonically mediated by activated DCs (Adams et al., 2005). Purified CRT/peptide complexes injected into mice did not elicit a measurable peptide-specific *in vivo* CTL response. However, the presence of an additional exogenous adjuvant demonstrated that CRT-associated peptides indeed accessed antigen presenting cells and were available to elicit a CTL response. These data contrast with previous reports that other chaperones stimulate DC maturation, and indicate that CRT may not be an optimal chaperone to use to elicit adaptive immune responses. Moreover, it calls into question the role of CRT-induced DC maturation during both chaperone-mediated tumor rejection and autoimmunity.

2. Materials and Methods

2.1. Mice and Cell Lines

C57Bl/6 mice from the National Cancer Institute were used in all experiments. Animal experiments were approved by the Dartmouth Medical School Institutional Animal Care and Use Committee. Mouse embryonic fibroblasts (MEF-1 cells) were obtained from ATCC (Manassas, VA) and were cultured according to accompanying protocol. Bone marrow derived

dendritic cells were generated using a modification of the protocol by Inaba et al. (Berwin et al., 2004; Inaba et al., 1993). Briefly, bone marrow-derived cells were resuspended at 10^6 cells/ml in DC culture media (RPMI 1640 medium, 10% heat-inactivated fetal/bovine serum, 100 units/ml penicillin/streptomycin, 50 mM β -mercaptoethanol, 5% cell culture supernatant from X63 cells secreting GM-CSF) and plated at 1 ml/well in a 24-well tissue culture plate. On days 2 and 4, the cells were washed and re-fed, and non-adherent cells removed. On day 6, wells were vigorously washed with culture medium to collect semi-adherent cells, which were phenotypically confirmed to be immature DCs.

2.2. Generation of CRT Δ KDEL Construct and Recombinant Calreticulin

Calreticulin without its endogenous KDEL sequence was cloned from the murine CRT cDNA (a generous gift of Dr. David Williams, University of Toronto) in the pcDNA3.1/Zeo vector using PCR (5' Primer: CCTAAGCTTAAGGCCTGTGTGCCGCCG; 3' Primer: GGCTTGGCCAGGGGATTCTTC). The PCR reaction was run on a 0.8% agarose gel and the resulting 1.2Kbp band corresponding to CRT Δ KDEL was excised, purified, and ligated into the pcDNA3.1-V5-His-Topo (Invitrogen, Carlsbad, CA) vector according to the manufacturer's protocol; this construct is referred to as CRT Δ KDEL. MEF cells were transfected with CRT Δ KDEL, or empty vector for mock transfection control, using the Lipofectamine Reagent (Invitrogen, Carlsbad, CA). Two days later supernatants were collected. Supernatant samples were split and either assayed for the presence of CRT Δ KDEL or used for DC maturation assays. Presence of the protein in the supernatant used for DC assays was confirmed by Western analysis: 500 μ l of the CRT Δ KDEL transfected MEF supernatant or mock transfected MEF supernatant were collected and added to 20 μ l of TALON Metal Affinity Resin (BD Biosciences, San Jose, CA) and incubated at 4°C for 1h. Protein-resin complexes were washed twice with PBS, resuspended in Laemmli buffer, and analyzed by Western blot using polyclonal anti-V5 antibody (Chemicon International Temecula, CA). Additionally, recombinant CRT was produced and purified from bacterial extracts as previously described (Baksh and Michalak, 1991). Recombinant CRT was depleted of endotoxin using the method of Reed et al. using polymixin B agarose (Sigma, St. Louis, MO) (Reed et al., 2003), and subsequent endotoxin levels were assessed using the LAL QCL-1000 assay (Cambrex, Walkersville, MD).

2.3. DC Maturation and Activation Assays

Day 5 cultured BMDCs were washed once with serum-free media and assayed for the ability of recombinant CRT and CRT Δ KDEL to induce phenotypic maturation. As a positive control 1 μ g of LPS (Sigma, St. Louis, MO, USA) was added to the cells. To assay for recombinant CRT-mediated maturation, BMDCs were incubated with the indicated concentration of recombinant CRT in 1 ml of medium containing GM-CSF. To test the ability of the CRT Δ KDEL protein to mature DCs, 300 μ l GM-CSF-containing medium was supplemented with 700 μ l of supernatants from untransfected MEFs, CRT Δ KDEL transfected MEFs, or mock transfected MEFs. DCs were then incubated for 40 h, harvested, stained with PE-conjugated CD11c antibody (clone N418, eBiosciences, San Diego, CA) and either FITC-conjugated CD86 (clone GL1, eBiosciences, San Diego, CA) or FITC-conjugated MHC-II (obtained from Corey Ahonen, Dartmouth Medical School) antibodies, and analyzed on a FACS Calibur (BD Biosciences, San Jose, CA) using CellQuest software. Supernatants from the DC maturation assay (DCs cultured in the presence of LPS, untreated media, CRT Δ KDEL-conditioned media, and media from mock transfection) were also subsequently screened for the presence of pro-inflammatory cytokines. Aliquots of the media were analyzed by Luminex Assay (Immune Monitoring Lab, Dartmouth Medical School) for the indicated cytokines.

2.4. Recombinant CRT Peptide Binding

Endotoxin-depleted recombinant CRT in PBS containing 0.1mM CaCl₂ and 1 mM MgCl₂ was incubated in the presence or absence of 50ng of FITC-labeled SIINFEKL peptide (GenScript Co., Scotch Plains, NJ) for 20 min at 50°. Laemmli sample buffer with out β-mercaptoethanol was added to each sample and samples were run on a 12% SDS gel. The gel was exposed on a Typhoon 9410 Variable Mode Imager (GE Healthcare, Piscataway, NJ). The scanned image was analyzed using ImageQuant v5.2 software (GE Healthcare, Piscataway, NJ). The gel was then stained for total protein content with coomassie blue (Sigma, St. Louis, MO).

2.5. BMDC Uptake of CRT

Recombinant CRT preparations were labeled with Alexa647 fluorescent dye as previously described (Berwin et al., 2004). Day 5 BMDCs were incubated for 20 minutes at 37°C with the indicated concentration of Alexa647-CRT in the presence or absence of 75 µg/ml carrageenan, a competitive scavenger receptor ligand. Relative cellular binding and uptake of CRT was determined by FACS analysis.

2.6. In vivo DC Maturation Assay

The *in vivo* DC maturation is a modification of a protocol described by Wilson et al. (Wilson et al., 2006). Mice were injected i.v. with either 3 µg LPS (Sigma) or 25 µg CRT in 200 µl PBS, or with PBS alone. 24 h later spleens were harvested, digested with collagenase/DNase, and passed through a 70 µm cell strainer (BD Biosciences) to achieve a single cell suspension. The cells were stained with APC-conjugated anti-CD11c (clone N418, BioLegend, San Diego, CA) and either FITC-conjugated anti-MHC-II or anti-CD86, followed by FACS analysis.

2.7. In vivo CTL Assay

C57Bl/6 mice were injected with the indicated concentrations of endotoxin-free calreticulin/SIINFEKL (CRT/SIINFEKL) complexes with and without 10 µg of LPS (Sigma, St. Louis, MO). As a positive control, mice were immunized with 2 µg SIINFEKL peptide/10 µg LPS/100 µg of agonistic αCD40 antibody (gift of the Noelle Lab, Dartmouth Medical School, Lebanon, NH). The *in vivo* CTL assay was performed as per Ahonen et al. (Ahonen et al., 2004). Mouse splenocytes were harvested and washed in serum-free HBSS. Half of the splenocytes were labeled with 2.5 µM CFSE (Sigma, St. Louis, MO) and the other half were labeled with 0.25 µM CFSE to achieve “high” and “low” CFSE-labeled splenocyte populations. Cells were then washed with HBSS and the high-CFSE population was pulsed with 25 µg/ml SIINFEKL peptide for 1 hour at 37°C. Peptide pulsed high-CFSE and unpulsed low-CFSE splenocytes were mixed at a 1:1 cell ratio (confirmed by FACS analysis) and this 1:1 mixture was injected peri-orbitally 4 days after immunization. 24 hours later, splenocytes from recipient mice were harvested and analyzed by FACS for specific killing of the peptide-pulsed high-CFSE target cells. Peptide-specific cytotoxicity was calculated as the percent of peptide-pulsed target cells eliminated in comparison to unpulsed control cells (peptide-specific cytotoxicity = [1-(cells in target peptide pulsed peak/cells in unpulsed peak)]). Statistical significance was calculated using an unpaired t-test comparing experimental values to the negative control immunization (PBS).

3. Results

3.1 Testing endotoxin-free calreticulin for induction of phenotypic DC maturation

The molecular chaperone calreticulin (CRT) is predicted to be an adjuvant and thereby to elicit murine tumor rejection (Basu and Srivastava, 1999; Cheng et al., 2005; Hsieh et al., 2004; Nair et al., 1999), however the purified protein has neither been directly tested for its ability to induce the maturation of DCs nor is the basis for its immunogenicity delineated. In order to

assess the adjuvant activity of endotoxin-free CRT, we engineered a system for expression of recombinant CRT in a mammalian cell line. We constructed a CRT expression vector referred to as CRT Δ KDEL that expresses CRT with its endogenous signal sequence and with His₆ and V5 purification and epitope tags. However, this construct lacks the C-terminal KDEL endoplasmic reticulum (ER) retention sequence and is thereby predicted to be secreted from the cell (Fig. 1A). Fibroblasts (MEFs) were transfected with the CRT Δ KDEL construct, and the media was subsequently assessed for the presence of CRT Δ KDEL protein. 48 hours following transfection the presence of CRT Δ KDEL protein was robustly detected by western analysis in the media of cells transfected with the CRT Δ KDEL vector but not in media from untransfected cells (Fig. 1B). The secretion of CRT into the culture medium allowed us to use conditioned media as a source of endotoxin-free CRT with which to test the ability of CRT to induce DC maturation. Surprisingly, CRT Δ KDEL-conditioned medium did not induce murine bone marrow-derived dendritic cell (BMDC) maturation, as assessed by levels of cell surface expression of MHC class-II and CD86 (Fig. 1C and D, respectively). CRT Δ KDEL-containing media did not differ in their ability to induce DC maturation from media that did not contain CRT Δ KDEL, while LPS was used as a positive control and it accordingly substantially up-regulated the BMDC cell surface expression of both CD86 and MHC class-II (Fig. 1C and D, respectively).

3.2 Cytokine profile of DCs exposed to endotoxin-free calreticulin

Although CRT Δ KDEL was not observed to induce phenotypic DC maturation, we hypothesized it may induce a pro-inflammatory cytokine response from the DCs that would induce or enable a cytotoxic T cell response. To test this, BMDCs were incubated with CRT Δ KDEL-containing medium and the elicited cytokine response was subsequently assayed by Luminex analysis. BMDCs incubated with CRT Δ KDEL-containing medium for 40 hours were not induced to measurably secrete the pro-inflammatory and CTL-activating cytokines TNF α , IL-1 α , IL-6, IL-12, IL-13 or GM-CSF, nor was the BMDC response to CRT Δ KDEL-containing medium different from that of BMDCs incubated with medium from mock-transfected MEF cells (Fig. 2 A-F). In contrast, LPS (a positive control) strongly elicited a measurable response of all of the aforementioned pro-inflammatory cytokines (Fig. 2 A-F).

3.3 Purified calreticulin fails to mature DCs

Since these findings differed from our predicted experimental outcome we assessed a second source of CRT to confirm these results. Bacterially-expressed recombinant CRT (rCRT) was purified to identify if there were source-specific differences in the adjuvant activity of CRT, to confirm that the KDEL portion of the protein is not required to induce DC maturation, and for subsequent use *in vivo*. Using the method of Wright et al (and as used for CRT in Reed et al.) (Reed et al., 2003; Wright et al., 1999), purified rCRT protein was depleted of endotoxin with polymyxin B and the resulting endotoxin levels were confirmed with the LAL assay to be <0.1 EU/mg protein. Endotoxin-depleted rCRT also did not elicit the phenotypic maturation of BMDCs, as assayed by CD86 and MHC Class II expression, over a range of rCRT concentrations (Fig. 3A and B). These data are consistent with those derived from the use of CRT Δ KDEL (Fig. 1) and demonstrate that purified CRT is inefficient at eliciting *in vitro* DC maturation. Moreover, these data are consistent with previous findings that CRT does not stimulate cellular NF- κ B activation or nitric oxide release that, respectively, induces and accompanies DC activation and maturation (Reed et al., 2003).

3.4 Recombinant calreticulin binds peptide and DCs

The ability of CRT to elicit antigen specific immune responses has been attributed to its ability to bind peptides, to traffic them into DCs, and to elicit peptide-independent maturation of DCs. As a control that purification and subsequent endotoxin decontamination of CRT did not

compromise its integrity, rCRT was tested for its ability to bind peptide and to traffic into DCs. rCRT was incubated with FITC-labeled SIINFEKL peptide under previously-established peptide-binding conditions (Basu and Srivastava, 1999), whereupon the resulting complexes were isolated from unbound peptide and were analyzed by native gel electrophoresis. rCRT retained the ability to bind the fluorescently-labeled peptide (Fig. 4A, lane 2) as assessed by FITC-SIINFEKL co-migration with the rCRT; rCRT was required to supershift the fluorescence (Fig. 4A, lane 1). As an additional control, we tested whether LPS-depleted rCRT still bound to DCs, since previous data has demonstrated that native CRT is efficiently bound and endocytosed by DCs (Berwin et al., 2004). Labeled CRT bound to DCs dose-dependently and, as a control for specificity, was competed by the addition of carrageenan, a previously described competitive ligand for the binding of CRT to endocytic receptors present on APC (Fig. 4B) (Berwin et al., 2004; Berwin et al., 2003). These data demonstrate that purified and endotoxin-decontaminated rCRT retains structural integrity and peptide-binding activity.

3.5 Assessment of *in vivo* DC maturation by calreticulin

To extend our *in vitro* findings, and to reconcile possible discrepancies between BMDCs and primary DCs, we tested the ability of rCRT to mature DCs *in vivo*. Following the protocol of Wilson et al. (Wilson et al., 2006), mice were injected i.v. with PBS (carrier), LPS, or LPS-free rCRT. After 24 h spleens were harvested and stained for CD11c (DC cell-surface marker) and either MHC class-II or CD86 to assess phenotypic DC maturation. As predicted, *in vivo* LPS treatment increased cell-surface MHC class-II and CD86 expression on the CD11c⁺ cells, while *in vivo* PBS injection had little effect (Fig. 5A-C, compare bold to black lines). However, consistent with our *in vitro* data, rCRT did not measurably induce maturation of DCs *in vivo* (Fig. 5D and E).

3.6 Calreticulin-peptide complexes require an additional stimulus to induce peptide-specific CTL responses

Despite a lack of measurable DC maturation or activation by CRT, it is conceivable that CRT influences DCs to induce CD8⁺ CTL responses through an alternate activation pattern that is not reflected in changes in the canonical cell-surface maturation markers MHC class-II and CD86. To directly test the ability of CRT to initiate a peptide-specific CTL response, we conducted an *in vivo* CTL assay. As a positive control for the assay, mice were primed with SIINFEKL peptide, LPS, and agonistic anti-CD40 antibody (as per (Ahonen et al., 2004)); as negative controls mice were primed with no peptide or peptide without adjuvant. Primed mice were then challenged with equal numbers of CFSE^{lo} un-pulsed and CFSE^{hi} SIINFEKL-pulsed splenocytes. After 24h the spleens were harvested and the SIINFEKL-specific CTL response against the target cells analyzed by flow cytometry (Fig. 6A). Peptide priming in conjunction with a potent adjuvant (LPS and α CD40) resulted in a robust SIINFEKL-specific CTL response, while the negative controls resulted in little if any specific killing (Fig. 6A). We then proceeded to test in parallel with our controls mice that were primed with varying quantities of rCRT/SIINFEKL complexes (purified of free peptide, as previously done (Berwin et al., 2002; Berwin et al., 2003)). Consistent with previous data (Ahonen et al., 2004) and with Fig. 6A, priming with peptide in the absence of adjuvant or in the presence of LPS resulted in minimal specific killing, while peptide in conjunction with both LPS and agonistic anti-CD40 antibody resulted in robust specific killing (Fig. 6B). However, CRT/SIINFEKL complexes were consistently inefficient at eliciting a CTL response across the indicated range of immunogen doses and were not significantly different from the negative control (up to 20 μ g of CRT/SIINFEKL complex; Fig. 6B). We note that the concentrations of CRT used in Fig. 6B spanned those previously reported to induce tumor-rejection (Basu and Srivastava, 1999; Cheng et al., 2005; Hsieh et al., 2004; Nair et al., 1999). Molecular chaperones, including CRT, are well-characterized in their capacity to traffic associated peptides into the MHC class-I antigen cross-presentation pathway of APCs (Berwin et al., 2004; Berwin et al., 2003; Walters

and Berwin, 2005). Therefore, as a control that 1) derivation of CRT/peptide complexes occurred, 2) the quantity of CRT/peptide complex used was theoretically sufficient to derive a measurable CTL response, and 3) that the CRT-associated peptide could access APCs, an equal quantity of the CRT/SIINFEKL preparation that failed to efficiently induce a CTL response was injected into mice in conjunction with 10 µg LPS. The addition of the auxiliary adjuvant resulted in significant CTL activity against the peptide-specific target cells (Fig. 6B). This latter result confirms that sufficient peptide was present to derive a CTL response, and we speculate that the heightened CTL response observed may reflect the efficiency of CRT-associated peptides to access the antigen cross-presentation pathway of APC (in comparison to the inefficiency of free peptide and LPS to elicit a CTL response, as in Fig. 6B and (Ahonen et al., 2004)) or, as an alternative explanation, may reflect a synergy between CRT and LPS similar to that observed between gp96 and LPS (Warger et al., 2006). However, endotoxin-free CRT, by itself, is not a potent adjuvant for inducing DC maturation and subsequent CTL responses. As will be discussed, these data support an alternative mechanism from the current elicited-CTL dogma of CRT-mediated tumor rejection, and additionally these data suggest that CRT, in the absence of additional adjuvants, may not be an optimal choice of adjuvant for CTL-based vaccine design.

4. Discussion

A broad array of molecular chaperones, including calreticulin, have been reported to elicit host immune responses that result in tumor rejection or, in some cases, autoimmunity (Berwin and Nicchitta, 2001; Eggleton and Llewellyn, 1999). Current dogma invokes the ability of chaperones to traffic associated peptides into APCs and concomitantly to induce the maturation of DCs, thereby propagating peptide-specific immune responses that result in the generation of anti-tumor CTL response. Indeed, several cell-surface endocytic receptors have been identified that traffic chaperones, and their associated peptides, into APCs (Basu et al., 2001; Berwin et al., 2004; Berwin et al., 2002; Delneste et al., 2002). However, the mechanisms by which the numerous chaperones activate the innate immune system are still unclear; in the case of CRT, the basis for this activity has not been directly tested.

Here we directly test the efficacy of endotoxin-free CRT to induce DC maturation and activation and to elicit peptide-specific CTL. Endotoxin-free CRT expressed by a mammalian cell culture system failed to mature DCs *in vitro* and moreover, did not elicit a measurable pro-inflammatory cytokine response from DCs. We then tested endotoxin-depleted recombinant CRT which similarly failed to elicit DC maturation *in vitro* and, additionally, did not induce measurable DC maturation *in vivo*. Finally, when bound to an immunogenic peptide, CRT was insufficient to elicit an *in vivo* CTL response. However, the addition of a supplementary adjuvant to the CRT/peptide complexes induced potent peptide-specific CTL responses. These data are consistent with the ability of CRT to traffic peptides into the APC MHC class-I antigen presentation pathway, but contrast with the notion that purified chaperones are efficient and sufficient adjuvants to elicit CTL responses.

These studies raise a number of questions regarding the immunogenic mechanisms of CRT and whether the various chaperones employ shared or disparate immunological pathways. Of particular relevance is the role of Toll-like receptors (TLR) in mediating the activation of DCs by chaperones. TLR ligands are well-established potent inducers of DC maturation; this is the basis for our use of LPS (a TLR-4 ligand) as a positive control. Several of the immunogenic chaperones, including hsp70, hsp60 and gp96 have been proposed as TLR ligands and, thus, to mediate DC activation and maturation via their cognate TLR receptors (Ohashi et al., 2000; Vabulas et al., 2002a; Vabulas et al., 2002b). However, Gao et al. reported that some of the pro-inflammatory effects prescribed to hsp60 and hsp70 were likely due to endotoxin contamination (Gao and Tsan, 2003a; Gao and Tsan, 2003b; Gao and Tsan, 2004); likewise,

flagellin (a TLR-2 ligand) has also been cited as a contaminant of hsp70 responsible for some of the observed stimulatory effects (Ye and Gan, 2007). Despite these reports, recent studies indicate that hsp70 produced in cell free systems, or expressed ectopically on the cell surface, can lead to activation of APCs, with CCR5 recently reported to be a candidate receptor (Korbelik et al., 2005; Pido-Lopez et al., 2007; Quintana and Cohen, 2005). Of note is that previous studies showed that endotoxin-depleted CRT and gp96 (up to 40 $\mu\text{g/ml}$) did not stimulate NF- κB activation nor did they induce nitric oxide synthesis by APCs: these are characteristic of TLR-mediated APC activation (Reed et al., 2003). In following up these observations, higher concentrations of gp96 (≥ 50 $\mu\text{g/ml}$) were subsequently shown to activate BMDCs and, intriguingly, gp96 potentiated LPS-stimulated responses by APCs (Warger et al., 2006). The mechanism for the synthetic activity of gp96 and LPS is not yet understood, but it may have a basis in the high-affinity interaction between these molecules. In contrast, here we show that up to 20 $\mu\text{g/ml}$ of CRT (the molar equivalent of ~ 64 $\mu\text{g/ml}$ gp96) does not induce measurable DC maturation *in vitro* or *in vivo*. Since these quantities of CRT (and gp96 in Warger et al. (Warger et al., 2006)) span and exceed the amount necessary to induce murine tumor rejection, and the results both derived herein and elsewhere (Bausinger et al., 2002; Gao and Tsan, 2003a; Gao and Tsan, 2003b; Ye and Gan, 2007) indicate that pure preparations of several chaperones are ineffective at eliciting responses from DCs, these results raise the broader issue of how chaperones mediate tumor rejection.

Many chaperones, including CRT, have repeatedly been shown to elicit tumor rejection (Basu and Srivastava, 1999; Segal et al., 2006; Tamura et al., 1997; Uono et al., 1994; Yokomine et al., 2006). This is particularly evident with the recent use of cells engineered to secrete chaperones or express them on their surface: these systems likely avoid endotoxin contamination and are reported to be efficient at inducing tumor suppression, but the quantities of chaperone expressed are relatively minute and not likely to exceed the quantities of purified protein used here and elsewhere. We note that many of these effective endotoxin-free systems have intentional or incidental cell death that is concomitant with chaperone expression, including cell death from cellular irradiation (Baker-LePain et al., 2002), that which accompanies gene-gun use (Cheng et al., 2001), and elicited drug-induced cell death (Melcher et al., 1998). This leads us to speculate that although purified gp96 and CRT may be relatively ineffective at inducing DC activation, these chaperones may synthetically interact with additional factors contained within necrotic cell lysates to induce DC stimulation. This would be in accord with the ‘danger model’, supported by previous observations that cellular lysate preparations mature DCs (Gallucci et al., 1999; Somersan et al., 2001) and, moreover, that cell lysates enriched in chaperones are particularly effective in this respect (Li et al., 2007; Zeng et al., 2003). It is also intriguing to speculate that chaperones suppress tumor progression through alteration of regulatory T (T_{reg}) cell activity. However, both gp96 and hsp60 are reported to enhance T_{reg} activity (Dai et al., 2007; Zanin-Zhorov et al., 2006), which is counterintuitive to induction of immunological tumor suppression, while CRT has yet to be tested in this regard.

In summary, many chaperones including CRT are being explored for their use as adjuvants (Segal et al., 2006). Our study indicates that purified CRT is not an optimal chaperone for use as a vaccine adjuvant in which the immunological response is dependent upon efficient induction of CTL. Purified, LPS-free, CRT from two distinct sources does not function as an adjuvant *in vivo* or *in vitro*. This data does substantiate previous investigations demonstrating that CRT is efficient in ferrying associated peptides into APCs. However, to raise an efficient CTL response against the CRT bound peptide, additional immune stimulus is needed. Further studies will be necessary to identify the basis for CRT modulation of the immune system that results in tumor suppression.

Acknowledgements

We thank the Norris Cotton Cancer Center Engert Cell Analysis Laboratory for help with FACS analysis and the Immune Monitoring Lab (Dartmouth) for Luminex analysis. This research was supported by NIH COBRE P20RR016437, NIH R01 AI067405 and a Hitchcock Foundation Research Fellowship (BB) and NIH training grant T32 AI07363 (EA).

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FIG. 1. Endotoxin-free calreticulin secreted by mammalian cells does not mature DCs

A, CRT Δ KDEL was engineered to be secreted from cells, since it retains the N-terminal signal sequence (SS) of native CRT (CRT) but the C-terminal ER retention signal, KDEL, was deleted and replaced with V5 and His₆ epitope and affinity-purification tags. **B**, Western blot analysis of culture medium from fibroblasts (MEF-1 cells) transfected as indicated demonstrates the presence of CRT Δ KDEL in the medium, as assessed by α V5 antibody reactivity. *Lane 1*, untransfected; *Lane 2*, mock transfected; *Lane 3*, CRT Δ KDEL transfected. (**C** and **D**) Maturation of BMDCs after 40 h of culture with media alone (GM-CSF), medium supplemented with 1 μ g LPS (LPS), conditioned medium from cultured MEF-1 cells (Medium), mock transfected MEF-1 cells (Mock Tf), or conditioned medium from CRT Δ KDEL-transfected MEF-1 cells (CRT Δ KDEL Media). BMDC maturation was assessed by FACS analysis of cell surface expression of MHC Class-II and CD86 (**C** and **D**, respectively). Error bars show the standard deviation of triplicate samples.

FIG. 2. Analysis of pro-inflammatory cytokines elicited from DCs by CRT Δ KDEL

Immature BMDCs were incubated for 40 hours with conditioned medium from CRT Δ KDEL-transfected MEF cells (CRT Δ KDEL), conditioned medium from mock-transfected MEF cells (Mock Tf), or with fresh medium (medium). In parallel, as a positive control, BMDCs were incubated with 1 μ g/ml LPS. Following the incubation of the BMDCs under these conditions, the pro-inflammatory cytokines TNF α , IL-1 α , IL-6, IL-12, IL-13 and GM-CSF were then quantitatively assayed in the media by Luminex analysis. The standard deviation of triplicate samples is shown.

FIG. 3. Full-length calreticulin does not induce *in vitro* DC maturation

Bacterially-expressed full length CRT was depleted of LPS and used to assess the ability of CRT to mature BMDCs. As performed in Figure 1, the CRT was added to cultures of BMDCs at the concentrations indicated for 40h. Subsequently, the cells were assayed for cell surface CD86 and MHC Class-II expression (A and B, respectively) by FACS analysis. 1 µg LPS was used as a positive control for induction of BMDC maturation. Each bar represents a minimum of three experiments and the standard deviation is shown.

FIG. 4. Purified and endotoxin-decontaminated calreticulin retains the ability to bind peptide and to be bound by dendritic cells

(A) Calreticulin preparations were assayed for the ability to bind fluorescently-labeled peptide *in vitro*. LPS-free, recombinant CRT was incubated in the presence or absence of 50 ng FITC-labeled SIINFEKL peptide for 20 min at 50°C. The CRT/FITC-SIINFEKL and CRT (no peptide) samples (lanes 2 and 3, respectively), along with 50 ng of FITC-SIINFEKL alone (lane 1), were run on a SDS gel under non-reducing conditions and subsequently exposed on a Typhoon imager to visualize the peptide-fluorescence that co-migrated with the CRT (upper panel). The same gel was then stained with coomassie blue as a protein loading control (lower panel). (B) BMDCs were incubated with the indicated concentrations of fluorescently-labeled CRT for 20 min at 37°C in the presence or absence of carrageenan. The cells were then washed and CRT uptake into the DCs was assessed by FACS analysis. CRT exhibited a dose-dependent uptake by the DCs (squares) and this uptake was partially blocked in the presence of carrageenan (triangles), a known competitive binding inhibitor of CRT to DCs. Standard deviation is shown.

FIG. 5. Calreticulin does not induce DC maturation *in vivo*

(A) Identification of the CD11c⁺ dendritic cell population in splenocytes of naïve mice. (B and C) LPS induces *in vivo* maturation of splenic DCs. Mice were injected with 3 µg of LPS in 200 µl of PBS, or with PBS alone. 24 hours later, splenic DC (gated on CD11c⁺ cells, as in A) maturation was assessed by staining for MHC class-II and CD86. LPS-treated mice exhibited increased cell-surface expression of (B) MHC-II and (C) CD86 (bold lines) on their CD11c⁺ DCs relative to the DCs of mock-treated mice (solid lines). Antibody-unstained DCs are also shown (dotted lines). (D and E) CRT is not effective at inducing *in vivo* DC maturation. As in Figures 4 B and C, mice were injected with PBS (negative control) that, in some cases as indicated, contained 3 µg LPS (positive control) or 25 µg CRT. As assessed by (D) MHC-II and (E) CD86 expression on the CD11c⁺ DCs following the injection, LPS was effective at maturing the splenic DCs while CRT did not induce MHC-II or CD86 expression beyond background. Each bar represents the average of a minimum of 4 mice.

FIG. 6. Calreticulin is not an effective adjuvant for eliciting *in vivo* CTL responses in the absence of an ancillary adjuvant

(A) Mice were injected i.p. with either PBS (negative control) or with 2 μg SIINFEKL peptide in the presence of LPS and agonistic αCD40 antibody (positive control). 4 days later equal amounts of SIINFEKL-pulsed CFSE^{hi} splenocytes and unpulsed CFSE^{lo} splenocytes were injected i.v. into the mice. The spleens were subsequently harvested and the CFSE^{hi} and CFSE^{lo} cell populations were assessed by FACS in order to determine peptide-specific cytotoxicity. The CFSE profiles of spleens from mice receiving PBS (left panel) or SIINFEKL plus stimulus (right panel) were used to determine % maximal killing of peptide-pulsed splenocytes in primed mice. (B) Mice were primed as indicated and *in vivo* CTL responses, as a function of percent peptide-specific killing (Y axis), were assayed (as in 6A) with CFSE-labeled splenocyte targets. CRT/SIINFEKL complexes, over the indicated range of doses, fail to increase peptide-specific killing significantly above mock-immunized mice (PBS), while CRT/SIINFEKL complexes co-injected with LPS induce significant peptide-specific CTL activity. As a positive control, SIINFEKL peptide in the presence of LPS and agonistic αCD40 antibody (denoted as αCD40 in the LPS legend) was used (as in 6A). Each bar represents a minimum of three experiments. Significance ($p \leq 0.001$) in comparison to the PBS negative control was determined using an unpaired t-test and is indicated with an asterisk (*).