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*J Immunol* 2011; 187:919-931; Prepublished online 13 June 2011;
doi: 10.4049/jimmunol.1100690
http://www.jimmunol.org/content/187/2/919

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/06/13/jimmunol.1100690.DC1

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Endoplasmic Reticulum Calcium Depletion Impacts Chaperone Secretion, Innate Immunity, and Phagocytic Uptake of Cells

Larry Robert Peters* and Malini Raghavan†

A number of immunological functions are ascribed to cell-surface-expressed forms of the endoplasmic reticulum (ER) chaperone calreticulin (CRT). In this study, we examined the impact of ER stress-inducing drugs upon cell surface CRT induction and the resulting immunological consequences. We showed that cell surface expression of CRT and secretion of CRT, BiP, gp96, and PDI were induced by thapsigargin (THP) treatment, which depletes ER calcium, but not by tunicamycin treatment, which inhibits protein glycosylation. Surface expression of CRT in viable, THP-treated fibroblasts correlated with their enhanced phagocytic uptake by bone marrow-derived dendritic cells. Incubation of bone marrow-derived dendritic cells with THP-treated fibroblasts enhanced sterile IL-6 production and LPS-induced generation of IL-1β, IL-12, IL-23, and TNF-α. However, extracellular CRT is not required for enhanced proinflammatory responses. Furthermore, the pattern of proinflammatory cytokine induction by THP-treated cells and cell supernatants resembled that induced by THP itself and indicated that other ER chaperones present in supernatants of THP-treated cells also do not contribute to induction of the innate immune response. Thus, secretion of various ER chaperones, including CRT, is induced by ER calcium depletion. CRT, previously suggested as an eat-me signal in dead and dying cellular contexts, can also promote phagocytic uptake of cells subject to ER calcium depletion. Finally, there is a strong synergy between calcium depletion in the ER and sterile IL-6, as well as LPS-dependent IL-1β, IL-12, IL-23, and TNF-α innate responses, findings that have implications for understanding inflammatory diseases that originate in the ER. The Journal of Immunology, 2011, 187: 919–931.

The endoplasmic reticulum (ER) is an important site of protein folding, calcium storage, and intracellular signaling (1). A number of ER chaperones are important in the functions of the ER. Calreticulin (CRT) is a chaperone that maintains quality control of glycoprotein folding by binding monoglucosylated proteins in the ER. CRT also contributes to calcium storage in the ER (reviewed in Ref. 2). Several recent studies showed that the cell surface expression of CRT can be induced in different cell types by different cell treatments (reviewed in Refs. 3, 4). Cell death stimuli suggested to induce cell surface CRT on dying cells include UV light, gamma irradiation, anthracycin chemotherapeutics, such as mitoxantrone (MTX), and platinum-based chemotherapeutics, such as oxaliplatin (5–8). The presence of CRT on the surface of dead and dying tumor cells was suggested to stimulate therapeutic and protective antitumor immune responses in mice (6–8). CRT on the surface of apoptotic cells and dying tumor cells is also suggested to function as an eat-me signal in the phagocytosis of these cells (5, 7, 9); by this mechanism, CRT could promote the presentation of Ags derived from dying cells to T cells to induce antitumor immunity. Other mechanisms could also account for the immunostimulatory effects of cell surface CRT. Purified ER chaperones, such as heat shock protein (HSP) 90, CRT, (10) and gp96 (HSPC4), have been implicated in induction of costimulatory molecule expression and cytokine production by dendritic cells (DCs) (reviewed in Ref. 11). Although it was suggested that TLR ligand contamination could account for the reported immunogenicity of these and other soluble HSPs, studies demonstrating the immunogenicity of cell-associated HSPs, including HSP90 and gp96, suggested that HSPs can be immunomodulatory independently of microbial contaminants (12–14).

A number of studies also suggested links between ER calcium depletion and detection of ER-resident proteins, including CRT, in the extracellular space. Treatment of NIH3T3 cells with the calcium ionophore A23167, which depletes intracellular calcium stores (15), resulted in secretion of gp96, an ER-resident chaperone, and reduced intracellular levels of CRT (referred to in that article as CRP55) (16). Furthermore, treatment of NIH3T3 cells with the sarco-ER Ca²⁺-ATPase inhibitor thapsigargin (THP) (17) resulted in a decrease in CRT ER staining intensity and an increase in the colocalization of CRT with wheat germ agglutinin in a non-ER cellular compartment suggested to be the Golgi (16). Another study showed that THP increased levels of surface CRT in the SH-SYSY neuroblastoma cell line (18). In HeLa cells, a direct correlation between an agent’s ability to deplete ER

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Received for publication March 9, 2011. Accepted for publication May 13, 2011.

This work was supported by NIH Grant AI066131 (to M.R.), University of Michigan Rheumatic Diseases Core Center Grant NIH P30 AR048310, and Immunology and Cellular Biotechnology training program fellowships (to L.R.P.). This work utilized the Hybridoma Core(s) of the Michigan Diabetes Research and Training Center funded by Grant NIH P60 DK20572 from the National Institute of Diabetes and Digestive and Kidney Diseases.

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The online version of this article contains supplemental material.

Abbreviations used in this article: 7AAD, 7-aminoactinomycin D; AmaV, Annexin V; BFA, brefeldin A; BMDC, bone marrow-derived dendritic cell; CM, conditioned media; CMFDA, 5-chloromethylfluorescein diacetate; CRT, calreticulin; DC, dendritic cell; ER, endoplasmic reticulum; HSP, heat shock protein; MEF, mouse embryonic fibroblast; MTX, mitoxantrone; THP, thapsigargin; TUN, tunicamycin; UPR, unfolded protein response; WT, wild-type.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1100690
calcium and its ability to induce surface CRT expression was also shown (18). Furthermore, two other groups showed that ER calcium depletion by THP resulted in secretion and surface expression of BIP (19, 20). Finally, we recently showed that amino acid residues that contribute to the polypeptide-specific chaperone activity of CRT also influence its surface expression in THP-treated mouse embryonic fibroblasts (MEFs) (21).

Calcium depletion in the ER impairs protein folding (1, 22) as a result of its impact on the functional activities of calcium-dependent chaperones (23). In calcium-depleted cells, the unfolded protein response (UPR) pathway, a cellular stress response pathway activated by the presence of misfolded proteins in the ER, is induced (24). Different arms of the UPR pathway function to stop protein translation, increase syntheses of molecular chaperones, and induce other cellular changes toward the restoration of the folding capacity of the ER (reviewed in Ref. 24). THP is a commonly used drug to induce the UPR pathway and, as noted above, THP induces calcium depletion (17). Another drug that is commonly used to induce the UPR pathway is tunicamycin (TUN), which inhibits protein glycosylation, thereby causing protein misfolding in the ER (25). In the present study, we compared the impact of these two ER stress-inducing drugs, as well as the chemotherapeutic MTX, on cell surface expression of CRT in different cell types. We showed that cell surface expression of CRT and secretion of other ER proteins, including CRT, are specific to the ER calcium depletion-induced form of ER stress. Cell surface expression of CRT in THP-treated cells involves a failure in effective ER retention mechanisms for a number of ER-resident proteins. This mode of CRT release from the ER is distinct from that described for anthracyclin-induced surface CRT expression, for which cotranslocation with ERP57 is suggested (26, 27). Nonetheless, surface CRT induced by THP treatment does enhance the phagocytic uptake of cells. The presence of various extracellular chaperones, including CRT, allowed us to examine functions of the extracellular forms of CRT and other ER chaperones in inducing an innate immune response. We found that incubation of bone marrow-derived DCs (BMDCs) with THP-treated fibroblasts or conditioned media (CM) from the treated cells enhanced production of IL-6 under sterile conditions but not the production of IL-1β, IL-12, or TNF-α, cytokines previously shown to be induced by macrophages following exposure to extracellular gp96 (14) or a CRT fragment (10). Furthermore, direct THP treatment of BMDCs enhanced IL-6 production under sterile conditions, as previously shown in macrophages (28), and enhanced various proinflammatory responses to LPS more broadly and significantly than did BMDC treatment with TUN. Together, the findings pointed to strong synergies between ER calcium depletion and innate immune responses and suggested that the combined presence of several ER chaperones in the extracellular environment per se is not strongly proinflammatory.

**Materials and Methods**

**Animals**

Mice were maintained in a specific pathogen-free facility and cared for according to the University Committee on Use and Care of Animals-approved protocols. Mice were euthanized by CO₂ asphyxiation.

**Drugs and Abs**

TUN and MTX were purchased from Calbiochem or Sigma and used at 10 μg/ml or 1 μM, respectively, unless indicated otherwise. THP was purchased from both Sigma and Enzo Life Sciences and used at 5 μM, unless indicated otherwise. MTX, THP, and TUN were dissolved in DMSO and stored in single-use aliquots at −20°C at stock concentrations of 1 mM, 5 mM, and 10 mg/ml, respectively. Frozen aliquots and freshly dissolved drugs did not show differences in measured activities. Chicken anti-CRT and rabbit anti-CRT were purchased from Affinity Bioreagents, now part of Thermo Scientific (PA-902A), and Abcam (ab2007), respectively, and were used for flow cytometry at a concentration of 2.5 μg/ml and a dilution of 1:200, respectively. Annexin V (Ann-V)-FITC was purchased from Biovision and BD Biosciences. 7-Aminoactinomycin D (7AAD) was purchased from Sigma.

**Immunoprecipitations**

The day before drug treatment, 1–2 × 10⁶ cells were seeded in a 10-cm dish and allowed to grow overnight. The next day, the media were removed, and cells were treated with different drugs for 5 h, as described above. Supernatants from each treatment were centrifuged to remove cell debris transferred to fresh tubes, and incubated overnight with the indicated Abs at 4°C, with gentle agitation. The following day, samples were centrifuged to remove precipitated proteins, transferred to new tubes, and incubated for ≥5 h with Protein G beads (GE Healthcare). The beads were

Flow cytometry

For CRT surface analysis by flow cytometry, cell lines were detached with PBS + 8 mM EDTA, washed in PBS, and resuspended in PBS containing 2% FBS (flow cytometry buffer). A total of 1.5–2 × 10⁶ cells was stained in 70 μl flow cytometry buffer containing the respective Ab. For CRT + block stains, the same dilution of chicken anti-CRT was preincubated for 15–30 min at room temperature with a saturating concentration of peptide (KEQELPDG-DAWTNRWE/ESHKH) corresponding to the sequence in CRT that the Ab was generated against and affinity purified with. Cells were washed twice with flow cytometry buffer, resuspended in 70 μl buffer containing donkey anti-chicken Ig conjugated to PE (1:250), and incubated for 30–60 min with rocking at 4°C. Cells were then washed twice. Finally, cells were resuspended in 1 × dilution of Ann-V-FITC and 7AAD in Annexin V binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, and 2.5 mM CaCl₂) and data for each sample were collected on a FACSCanto flow cytometer (BD Biosciences). Analysis was performed using FlowJo 8.8.6 (TreeStar).
washed three times with 1 ml 1% Igepal CA-630 in PBS and then boiled in the presence of SDS and DTT. The samples were analyzed by SDS-PAGE and immunoblotting with the indicated Abs. In parallel, cells from each plate were harvested and lysed in Triton X-100 lysis buffer (10 mM Na2HPO4, 10 mM Tris, 130 mM NaCl, 1% Triton X-100, complete EDTA-free protease inhibitors [pH 7.5]) on ice for ≥1 h, followed by a 30-min centrifugation at 4°C to remove cell debris. Protein concentrations of cleared lysates were determined with Pierce bicinchoninic acid assay, and these lysates were included in the immunoblotting analysis of the immunoprecipitated proteins.

**Cytokine profiling of BMDCs co-incubated with drug, drug-treated MEFs, or MEF-derived CM**

For data in Fig. 5, MEFs were drug treated as indicated for 5–6 h; subsequently, cells were harvested, washed two or three times (first in 25 ml media, then either twice in 10 ml or once in 25–40 ml media), and incubated alone or with BMDCs, as described below. For data shown in Fig. 6, MEFs were treated with indicated drugs for 5–6 h, supernatants were collected (CM1), cells were washed three times in 7 ml PBS, and 4 ml BMDC media was added to the cells. After a 5–6h collection period, CM2 were harvested. CM1 and CM2 were centrifuged to clear any floating cells or cellular debris. For Fig. 6A, a portion of CM2 was added to BMDCs (MEF CM2 + DC), and another portion was directly tested for the presence of specific cytokines (MEF CM2). For Fig. 6B, CM1 or CM2 were added directly to BMDCs (MEF CM2 + DC). For Supplementary Fig. 3, CM1 and CM2 were dialyzed using 3- or 10-kDa Centricon filters, as indicated (using at least three successive concentrations and dilutions), before adding retentate or flow-through to BMDCs. Separate wells of DCs were directly treated with the indicated concentrations of the described drugs (direct treatment) or left untreated. In parallel analyses, a second set of MEF plates were treated as described above for generation of CM2. MEFs were then harvested and stained with AnnV and 7AAD (to measure cell viability at the end of the MEF CM2 collection). Protocols for BMDC incubation with treated cells, CM, or drug were adapted from Torchinsky et al. (30). Day 5 or -6 BMDCs (5 × 10^6) were mixed with 1 × 10^5 washed target cells, cosedimented, and incubated for 19–23.5 h (Fig. 5) or were treated separately with drugs for 21–23 h (Fig. 6B) or with MEF CM for 15–23 h (Fig. 6) in 24-well plates in the presence or absence of described amounts of LPS before centrifuging the plates for 5 min at 1200 rpm and harvesting the resulting supernatants. Supernatants were submitted to the University of Michigan Cancer Center Immunology Core to detect the indicated cytokines by ELISA. Duoset ELISA development systems were purchased from R&D Systems (Minneapolis, MN).

**Phagocytosis assays**

Target cells were labeled with CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA) (Invitrogen) in 10-cm plastic plates and seeded with 2–2.5 × 10^5 target MEFs/plate 12–20 h before labeling. Each plate was labeled as directed with 0.1–0.35 μM CMFDA diluted in OptiMEM serum-free media for 20–40 min at 37°C. Media were removed, replaced with previously described tissue culture media (RPMI 1640 with 10% serum), and incubated at 37°C for 30–60 min. After this, the media were removed and replaced with fresh media. Next, the plates were exposed to 3 min of light on a UV light box or left untreated, and all plates were incubated overnight in a tissue culture incubator. The following morning, the plates were treated with 5 μM THP or 10 μg/ml TUN for 5–6 h or left untreated as indicated. Cells were finally harvested (UV) or washed twice in 10–12 ml PBS (untreated and drug treated), harvested with 8 mM EDTA in PBS, and diluted in RPMI 1640 for counting (a third wash) before pelleting and resuspending at the appropriate concentration. A total of 1 × 10^6 or 5 × 10^6 targets in 96-well plates, and the plates were incubated at 37 or 4°C for 60 min. Cells were then fixed for 10 min with formalin (fishel PROTOCOL), diluted 1:10 in flow cytometry buffer (PBS + 2% BSA), washed twice, and stained with anti-CD11c-allophycocyanin (1:220; BD Biosciences) for 20–40 min at 4°C. Cells were washed twice, and data for each sample were collected on the FACSCanto flow cytometer (BD Biosciences). Analysis was performed using FlowJo 8.8.6 (TreeStar). CMFDA was read on the FITC channel.

**Results**

**Differential impact of ER stress-inducing drugs on cell surface CRT**

THP inhibits sarco-endoplasmic reticulum Ca²⁺-ATPase pumps and causes a reduction in levels of ER calcium (17). We recently showed that THP treatment of MEFs for short times (5–6 h) induced cell surface expression of CRT (21), as previously shown in neuroblastoma cells (18), and that THP treatment of MEFs also induced CRT secretion (21). In the present study, we asked whether treatment of cells with another drug commonly used to induce ER stress, TUN, would also result in cell surface and secreted CRT. Additionally, the effect of treating cells with the anthracyclin chemotherapeutic MTX was tested, because previous studies suggested that MTX is a strong inducer of cell surface CRT in preapoptotic and apoptotic cells (7, 8, 26).

We first examined cells subjected to short drug exposures, which left the large majority of cells intact. AnnV was used to distinguish the apoptotic population that had exposed phosphatidylserine on the outer leaflet of the plasma membrane (31). Furthermore, 7AAD was used, which is impermeable to live and early apoptotic cells but stains late apoptotic/secondary necrotic cells that have lost membrane integrity. The analyses were initially conducted with WT MEFs or CRT−/− MEFs to control for the specificity of Ab binding. Untreated cells or cells exposed to TUN, THP, or MTX for 5–7 h were triple stained with AnnV-FITC, chicken anti-CRT followed by anti–chicken-PE, and 7AAD and analyzed by flow cytometry. As previously shown (21), in the AnnV−, 7AAD− gate, which makes up 80–90% of cells in all treatment groups (Fig. 1A), THP treatment significantly enhanced surface CRT relative to untreated cells (Fig. 1B). A THP-induced increase in CRT surface staining of treated cells relative to untreated cells was observed in WT MEFs but not in CRT-deficient MEFs, demonstrating specific staining for CRT (Fig. 1B). The THP-induced increase in CRT staining was also inhibited by pretreatment of the anti-CRT Ab with the CRT-derived peptide epitope (peptide block) that had been used as an immunogen for generating the anti-CRT Ab (anti-CRT + block), demonstrating that the staining was specific to the Fab region of the Ab. Consistent with a study by Obeid et al. (7), TUN did not induce surface CRT expression in viable cells. However, in contrast with the same study, we saw little preapoptotic induction of surface CRT in response to short MTX treatments relative to the CRT upregulation induced by short THP treatments of cells (Fig. 1B).

Because of the contrast of our data with the findings presented by Obeid et al. (7), we measured changes in surface CRT levels in the CT26 mouse colon cancer cell line used in that study. The strong induction of cell surface CRT seen in THP-treated WT MEFs was also seen in CT26 cells. In contrast, MTX treatment did not expose surface CRT in CT26 cells, similar to the results obtained with WT MEFs (Fig. 1B). It is possible that expression of cell surface CRT in CTX-treated cells occurs with different kinetics than in THP-treated cells or in cells that are at a different stage of cell death. To examine the impact of longer-duration drug treatment on surface CRT induction, we next examined cells treated with each drug for 17 h, which induced ~2–5 times more apoptotic cells (Fig. 1C). This allowed us to compare surface CRT levels on AnnV+ apoptotic cells and AnnV− preapoptotic cells with those on viable, AnnV− untreated cells (Fig. 1D). None of the long drug treatments induced a significant increase in CRT staining in apoptotic or preapoptotic MEFs, although the MTX-treated, apoptotic cells had a trend suggesting a slight upregulation of surface CRT relative to viable, untreated cells (Fig. 1D).

However, interpretation of some of the data presented in Fig. 1D may be complicated by the fact that apoptotic cells are smaller than viable cells (Fig. 2A). To further examine this point, we compared the level of surface CRT staining in the presence or absence of the anti-CRT peptide block within a given viable or apoptotic cell population (Fig. 2B, 2C). Fig. 2B and 2C include some of the same data shown in Fig. 1B and 1D, but analyzed differently as described, and they include analyses of additional
AnnV and 7AAD values of anti-CRT staining of treated cells were measured for the AnnV and 7AAD and analyzed by flow cytometry. The median fluorescence +block), or secondary Ab alone (control). All cells were also stained with anti-CRT, anti-CRT preincubated with the CRT-derived peptide used as the immunogen and used to affinity purify the Ab (anti-CRT +block), or secondary Ab alone (control). All cells were also stained with AnnV and 7AAD and analyzed by flow cytometry. The median fluorescence values of anti-CRT staining of treated cells were measured for theAnnV 7AAD (B, D, as indicated) or the AnnV 7AAD populations (D, as indicated) and plotted as a ratio relative to the median fluorescence of AnnV 7AAD, untreated cells stained under the same condition (B, D). Inset in B shows representative overlays of untreated (UNT) or THP-treated (dashed) CT26 cells stained with anti-CRT Ab. THP-treated cells were stained in the presence (block [line]) or absence (THP [dashed]) of the peptide block. A and C. Percentage of cells in the AnnV 7AAD (viable/preapoptotic), AnnV 7AAD (early apoptotic), and AnnV 7AAD (late apoptotic/secondary necrotic) populations are shown. Graphs show averages of 5–10 (A), 5–7 (B), 3–5 (C), or 2–3 (D) experiments. The p values from two-tailed, paired t tests are indicated.

Cell death profiles (A, C) and surface CRT-expression profiles (B, D) of different treatment conditions were analyzed by flow cytometry. WT or CRT−/− MEFs were incubated in 5 μM THP, 1 μM MTX, 10 μg/ml TUN, or media alone (UNT) for 5–7 h (A, B) or 17 h (C, D). Cells were then stained with anti-CRT, anti-CRT preincubated with the CRT-derived peptide used as the immunogen and used to affinity purify the Ab (anti-CRT +block), or secondary Ab alone (control). All cells were also stained with AnnV and 7AAD and analyzed by flow cytometry. The median fluorescence values of anti-CRT staining of treated cells were measured for the AnnV 7AAD− (B, D, as indicated) or the AnnV 7AAD populations (D, as indicated) and plotted as a ratio relative to the median fluorescence of AnnV 7AAD−, untreated cells stained under the same condition (B, D). Inset in B shows representative overlays of untreated (UNT) or THP-treated (dashed) CT26 cells stained with anti-CRT Ab. THP-treated cells were stained in the presence (block [line]) or absence (THP [dashed]) of the peptide block. A and C. Percentage of cells in the AnnV 7AAD− (viable/preapoptotic), AnnV 7AAD− (early apoptotic), and AnnV 7AAD− (late apoptotic/secondary necrotic) populations are shown. Graphs show averages of 5–10 (A) 5–7 (B), 3–5 (C), or 2–3 (D) experiments. The p values from two-tailed, paired t tests are indicated.

Several ER-resident proteins are released in THP- but not TUN-treated cells, and CRT surface expression is independent of ERp57 cotranslocation

We examined secretion of ER-resident proteins in response to 5-h treatments of WT MEFs with different drugs. Supernatants from the different drug-treated cells were separated by SDS-PAGE, and immunoblotting analyses were performed with Abs directed against gp96 and PDI. In addition, because neither CRT nor BiP was detectable by direct immunoblotting analyses, proteins present in supernatants were immunoprecipitated with Abs against CRT and BiP, followed by immunoblotting analyses to detect those proteins. THP, but not TUN or MTX, induced secretion of at least four proteins that are normally retained within the ER: CRT, gp96, PDI, and BiP (Fig. 3A). Cells treated with 200 nM or 5 μM THP secreted similar levels of gp96 and PDI (Fig. 3B) and induced similar elevations in surface CRT expression (data not shown). Although depletion of ER calcium is expected to occur almost immediately following THP treatment (17), THP-induced secretion of ER chaperones was not detectable at 1.25 or 2.5 h following THP treatment (Fig. 3C). These findings suggested that ER calcium depletion alone is insufficient to induce a detectable loss of ER retention of chaperones. Previous findings from other cell types indicated that BiP (33, 34) and CRT (35, 36) mRNA levels were upregulated following 2–5 h of TUN and THP treatment and that BiP protein levels were increased within 5 h of TUN treatment (34). Consistent with these kinetics of UPR induction, an examination of changes in BiP and CRT protein levels revealed small increases in CRT and BiP expression at 3 h and a stronger induction 5 h post-TUN treatment (Fig. 3D). Over the same time frame, THP treatment decreased the levels of cellular CRT and BiP, with a greater reduction seen at 5 h compared with 3 h (Fig. 3D), consistent with the observation of increases in ER chaperone secretion over this time frame (Fig. 3C). Taken together, these findings suggested that both depletion of ER calcium and increases in ER chaperone expression are required for chaperones to escape ER retention in THP-treated cells.

The data in Fig. 3A indicate that THP treatment does not result in a specific release of CRT but rather in a generalized loss of ER retention of various ER proteins. This mechanism of CRT release from the ER is distinct from that suggested for anthracyclinduced CRT surface expression, for which the specific cotranslocation of CRT–ERp57 complexes was indicated (26, 27). In the ER, CRT and ERp57 interact via the P-domain of CRT, and amino acid W244 within the P-domain of CRT is important for binding to ERp57 (29, 37, 38). To test the requirement for CRT–ERp57 interaction for CRT surface expression in THP-treated cells, WT CRT or CRT mutants unable to bind to ERp57 (29) (a W244A point mutant and a CRT truncation mutant lacking the P domain

FIGURE 1. A specific form of ER stress induces cell surface CRT. Cell death profiles (A, C) and surface CRT-expression profiles (B, D) of different treatment conditions were analyzed by flow cytometry. WT or CRT−/− MEFs were incubated in 5 μM THP, 1 μM MTX, 10 μg/ml TUN, or media alone (UNT) for 5–7 h (A, B) or 17 h (C, D). Cells were then stained with anti-CRT, anti-CRT preincubated with the CRT-derived peptide used as the immunogen and used to affinity purify the Ab (anti-CRT +block), or secondary Ab alone (control). All cells were also stained with AnnV and 7AAD and analyzed by flow cytometry. The median fluorescence values of anti-CRT staining of treated cells were measured for the AnnV 7AAD− (B, D, as indicated) or the AnnV 7AAD populations (D, as indicated) and plotted as a ratio relative to the median fluorescence of AnnV 7AAD−, untreated cells stained under the same condition (B, D). Inset in B shows representative overlays of untreated (UNT) or THP-treated (dashed) CT26 cells stained with anti-CRT Ab. THP-treated cells were stained in the presence (block [line]) or absence (THP [dashed]) of the peptide block. A and C. Percentage of cells in the AnnV 7AAD− (viable/preapoptotic), AnnV 7AAD− (early apoptotic), and AnnV 7AAD− (late apoptotic/secondary necrotic) populations are shown. Graphs show averages of 5–10 (A), 5–7 (B), 3–5 (C), or 2–3 (D) experiments. The p values from two-tailed, paired t tests are indicated.
and adherent cells, and short drug treatments analyzed adherent cells only. Otherwise, long drug treatments analyzed the combination of nonadherent cells and Annexin V−/− Annobin−/−, which is present in supernatants of THP-treated cells, because it was not detectable in cell supernatants by immunoblotting analyses (data not shown); however, Erp57 migrates in close proximity to background bands present in the cell supernatants, and low levels of secreted Erp57 may be masked by the background signals.

In THP-treated cells, ER chaperones follow the secretory route to gain access to the extracellular compartment

As previously described (39), analysis of cell lysate-derived CRT by immunoblotting revealed that THP treatment of cells induced CRT glycosylation (Fig. 3A, CRT blot, lane 4) in a subset of CRT molecules present in the cell. The glycosylation site of CRT seems to be buried under normal conditions, but it becomes partly exposed under calcium-depleting conditions (21). It was of note that the glycosylated CRT species was absent from cell supernatants, suggesting that it was either ER retained or preferentially retained on the cell surface (Fig. 3A) (21).

Because CRT glycosylation is partial and induced by specific cellular conditions, we assessed the glycosylation status of gp96, a constitutively glycosylated protein, to further understand the cellular exit route for ER chaperones in THP-treated cells. A cytosolic route was recently described for CRT release from apoptotic cells (40). Because cytosolic localization of proteins induces their deglycosylation, we assessed the glycosylation status of extracellular gp96 to further understand whether a secretory or cytosolic route was used in the trafficking of gp96 (and by inference, other ER proteins) to the extracellular space in THP-treated cells. Previous findings indicated that gp96 secreted in response to cellular calcium perturbation exits the cell through the secretory pathway and becomes differentially glycosylated as it travels through the Golgi (16). Consistent with these findings, gp96 from cell supernatants migrated more slowly than did gp96 in cell lysates (Fig. 4A). When supernatants from THP-treated cells were digested with peptide:N-glycosidase F, which cleaves carbohydrate residues from glycoproteins, and analyzed by immunoblotting with an anti-gp96 Ab, a decrease in m.w. was observed, indicating that secreted gp96 is indeed glycosylated (Fig. 4B, gp96 blot, lanes 8, 9). Finally, the induction of cell surface CRT in response to THP was reduced when the cells were pretreated with brefeldin A (BFA), which blocks ER–Golgi trafficking (Fig. 4C). Together, these findings indicated that THP treatment of cells perturbs normal ER retention mechanisms and that ER proteins exit the cell via a secretory route in response to THP treatment.

In a CRT-independent manner, THP-treated cells, cell supernatants, and THP itself induce and enhance innate immune responses more broadly and significantly than corresponding TUN treatments

Using THP-treated WT or CRT-deficient MEFs, we investigated whether cell surface and/or extracellular CRT was linked to enhanced proinflammatory cytokine production. We first examined
production of IL-6, a cytokine previously shown to be induced by THP (28) and TUN (41) treatments of murine macrophages. WT or CRT-deficient MEFs were treated with TUN, MTX, and THP for short (5–6.5 h) or long (20.5 h) times, as described in Fig. 1, harvested, washed extensively with PBS, and coincubated with BMDCs or alone, and cytokines in the supernatants of these cultures were quantified by ELISA. BMDC coincubations with MEFs treated with THP resulted in higher levels of IL-6 compared with BMDC coincubations with other target cells (Fig. 5A); however, these effects were independent of CRT expression. A THP-induced increase in IL-6 production was also seen with MEFs subjected to longer drug treatments (Supplemental Fig. 1B), although the levels of IL-6 measured were lower (Supplemental Fig. 1B compared with Fig. 5A). These findings are consistent with higher levels of MEF apoptosis following long drug treatments (Supplemental Fig. 1A compared with Fig. 1A) and the expected immunosuppressive effects of apoptotic cells (42). It is noteworthy that MTX-treated, preapoptotic (Fig. 5) or apoptotic (Supplemental Fig. 1) cells did not induce significant proinflammatory cytokine production by BMDCs, despite being reported to induce strong antitumor immune responses in mice (7).

We also examined production of IL-12, IL-1β, and TNF-α, cytokines that were shown to be induced by a membrane-linked extracellular form of gp96 (14) or a bacterially expressed CRT fragment (10). Additionally, we compared induction of IL-23, because both THP and TUN were recently reported to enhance expression of this cytokine in the presence of TLR agonists (43). Coincubating THP-treated MEFs with BMDCs strongly enhanced TLR-dependent (0.5–2 ng/ml LPS) production of IL-1β, IL-12p70, and IL-23 compared with coincubating other drug-treated or untreated MEFs with BMDCs (Fig. 5B–D). CRT was not required for enhanced cytokine production in response to THP-treated cells; in fact, CRT deficiency in the THP-treated MEFs seemed to correlate with higher levels of cytokine production. However, observed differences between WT and CRT-deficient cells were not statistically significant (Fig. 5B–D). Compared with other drug treatments, THP-treated cells subjected to long (20.5 h) drug treatments also stimulated IL-1β and IL-23 (Supplemental Fig. 1C, 1D), although at reduced levels compared with coincubations with MEFs subjected to short drug treatments (Fig. 5). Immunostimulatory effects of TUN-treated apoptotic cells upon IL-23 production were also measurable following longer drug treatments but at reduced levels compared with THP-treated cells (Supplemental Fig. 1C).

To better understand the molecular basis for the immunostimulatory effects of THP-treated cells (Fig. 5), we next examined the impact of CM from various drug-treated cells, as well as direct drug treatments of BMDCs, on induction or enhancement of proinflammatory cytokine production (Fig. 6). We were particularly interested in determining whether secreted ER chaperones

FIGURE 3. Various ER chaperones are released from THP-treated MEFs, and THP-induced surface CRT expression is independent of CRT–ERp57 binding. A, Immunoblotting of cell supernatants and lysates for the presence of various ER chaperones. Supernatants (CM1) from cells treated with TUN, MTX, or THP or untreated cells were harvested in parallel with the corresponding cell lysates. Direct immunoblotting analyses of each fraction were performed for PDI and gp96. For CRT and BiP, proteins in cell supernatants were immunoprecipitated (IP) with anti-CRT or anti-BiP prior to immunoblotting analyses. Approximately 8500 cells/lane contributed to the CM1 used to blot for PDI and gp96. The blots of corresponding lysates show ≈30,000 cells’ worth of lysate. B, Comparison of PDI and gp96 release in response to low (200 nM) or high (5 μM) THP dose. C, Kinetics of PDI and gp96 secretion. Analyses were performed as in A, but immunoblotting analyses were undertaken at the indicated time points. D, Kinetics and magnitude of BiP and CRT induction in response to TUN and THP. Indicated microgram amounts of total cell lysates were analyzed as in A at the indicated timepoints postdrug treatment. E, Flow cytometric assessments of ERp57 binding dependence of CRT surface expression. Cell surface CRT, measured as in Fig. 1, before and after THP (5–7 h of 5 μM THP) treatment of CRT−/− MEFs infected with retroviruses encoding WT CRT (WT), a CRT construct lacking the P-domain (ΔP), a CRT point mutant that is unable to interact with ERp57 (W244A), or a virus lacking CRT (vec). Representative blots of three or four experiments are shown in A. Data in B and C are based on one experiment Data in D are from one experiment, representative of two total experiments. Data in E are based on three to five experiments. A–D, Vertical black lines in blots indicate places where lanes of the blot were rearranged to match the labeling scheme used in adjacent immunoblots or to digitally remove lanes containing size standard (relevant bands from size standard are shown digitally for each blot). The p values from two-tailed, paired t tests are indicated.
present in supernatants of THP-treated cells or residual THP itself might be responsible for the observed proinflammatory effects of THP-treated cells. For these analyses, following each drug treatment of 5–6.5 h, media containing drugs (CM1) were removed, and cells were washed three times with ≥7 ml PBS to remove soluble drug present in the media. Following these wash steps, fresh media were collected from the washed cells over a 5–6-h period to generate different MEF CM (CM2). We noted that secretion of PDI and gp96 was detectable for several hours following THP treatment. A, SDS-PAGE-based protein separation was undertaken on an 8% polyacrylamide gel, and separation was optimized compared with that shown in Fig. 3A, to allow for resolution of different forms of gp96. The supernatant sample (CM1) was also run adjacent to the lysate sample to better resolve alterations in electrophoretic mobility resulting from posttranslational modifications. The vertical black line denotes lanes that were cut and pasted from the same blot. B, Indicated cell supernatant samples were digested with peptide-N-glycosidase F (+) or left undigested (−) prior to the immunoblotting analyses. The blot is representative of three experiments. C, BFA blockade of the secretory pathway inhibits THP-induced surface CRT expression. Cells were pretreated with BFA for 45 min, followed by the addition of THP for 6–7 h. Cells were harvested, and surface CRT expression was measured in the AnnexV and 7AAD population by flow cytometry, as described. Bar graph shows ratio of surface CRT median fluorescence of BFA+THP-treated/untreated cells. The data are the average of two experiments.
that when CM1 (cell supernatants from drug-treated cells) and CM2 (cell supernatants from drug-treated, washed cells) were extensively dialyzed in Centricon (3 or 10 kDa) to remove free soluble drug that was present, the majority of cytokine-inducing activity was present in the retentates rather than in the flow through, even though THP has a molecular mass of 650 Da (Supplemental Fig. 3). Based on these findings, it is likely that the activity of THP-treated cell supernatants (CM1 and CM2) (Fig. 6, Supplemental Fig. 3) and the resemblance of the stimulatory capacities of the supernatants to direct THP treatment of BMDCs (Fig. 6B) result from sequestration of THP within cell membranes or intracellularly during drug treatment of MEFs, as well as subsequent partitioning of active drug from MEFs into the CM within higher m.w. membrane vesicles or protein complexes. This would allow THP to directly stimulate BMDCs. THP is hydrophobic and requires organic solvents (such as DMSO) for its solubilization; thus, its partitioning into membranes is perhaps not unexpected.

If residual drug in THP-treated cells was responsible for the BMDC-stimulating activity, we expected that reducing the concentration of THP used to treat MEFs would reduce the immunostimulatory capacity of THP-treated cells. Thus, we compared abilities of 5 μM or 200 nM THP CM1 and CM2 to induce production of various proinflammatory cytokines. Although treating BMDCs with 5 μM THP CM1 or CM2 induced IL-6 production, treatment of BMDCs with 200 nM THP CM1 or CM2 did not induce sterile IL-6 production (Fig. 6B). Furthermore, reduced levels of LPS-induced IL-1β and IL-23 (Fig. 6B) were observed when 200 nM THP CM1 or CM2 was compared with 5 μM THP CM1 or CM2. Notably, levels of chaperone secretion were very similar in 200 nM and 5 μM THP CM1 (Fig. 3B).

Together, these findings indicated that production of proinflammatory cytokines induced by THP-treated cells or CM was not enhanced by CRT expression by target cells (Figs. 5, 6, Supplemental Fig. 2C, 2D). Notably, the profiles of proinflammatory cytokine induction by THP-treated cells and dialyzed CM closely resembled those induced by THP itself, suggesting that residual THP within protein/lipid complexes was responsible for enhanced cytokine production (Fig. 6B). Significantly, the effects of direct BMDC treatment with THP and TUN were different, with direct THP treatment being more strongly proinflammatory.

**CRT expression enhances phagocytic clearance of THP-treated cells but not TUN- or UV-treated cells**

Because cell surface CRT is an eat-me signal in the context of apoptotic cells (5), preapoptotic tumor cells (7), and live tumor cells subject to CD47 blockade (32), we asked whether THP-treated cells would also be phagocytosed in a CRT-dependent manner. A previously published flow cytometry-based assay (7) was used to measure phagocytosis. WT or CRT-deficient MEFs were labeled with a fluorophore, treated with drugs or UV light, washed, and incubated with BMDCs. The cocultures were then fixed and stained previously published flow cytometry-based assay (7) was used to measure phagocytosis. WT or CRT-deficient MEFs were labeled with a fluorophore, treated with drugs or UV light, washed, and incubated with BMDCs. The cocultures were then fixed and stained with an Ab specific for the DC-specific marker CD-11c. The percentage of CD-11c+ events that were also positive for the target cell marker was recorded as a measure of target cell/DC association. The ratio of DC/target cell association in cocultures incubated at 37°C relative to that of cocultures incubated at 4°C is reported as the phagocytic index. The 37°C readings quantify adhesion and phagocytosis, whereas the 4°C readings quantify adhesion alone.
Uptake of UV-treated MEFs was significantly greater than untreated MEFs, but it was independent of CRT expression by the MEFs (Fig. 7A). The latter finding was surprising in light of a previously suggested role for CRT in the phagocytic uptake of apoptotic UV-treated fibroblasts using fibroblasts or a macrophage cell line as the phagocyte (5). Therefore, surface CRT expression was tested in the UV-treated fibroblasts, which were triple stained for CRT, as well as markers of apoptosis and plasma membrane integrity, as defined in the analyses of Fig. 1. Our analyses indicated that CRT is not present or significantly induced on the surface of AnnV+PI+ (Fig. 7B) or AnnV−PI− (data not shown) MEFs in response to UV treatment, consistent with the absence of significant, CRT-dependent uptake of UV-treated cells. Several differences in protocol may explain these discrepancies. These include the use of a different phagocyte [BMDCs (Fig. 7) rather than macrophages or fibroblast (5)], a different phagocytosis assay [flow cytometry (Fig. 7) rather than microscopy (5)], and the use of both adherent and nonadherent UV-treated target cells (Fig. 7), rather than nonadherent UV-treated target cells (5).

In contrast to UV-treated MEFs, THP-treated WT MEFs were phagocytosed with significantly higher efficiency than were THP-treated CRT-deficient MEFs and untreated MEFs (Fig. 7A).
levels of target cell death were similar between WT and CRT-deficient MEF target cells within a treatment group (Fig. 7C).

Additionally, the level of dead target cells in the untreated group was least as high as that in the THP-treated group (Fig. 7C), a result that indicated that cell death in the THP-treated group was insufficient to account for the level of phagocytic uptake observed. Finally, although TUN treatment enhanced phagocytic uptake of target cells relative to untreated cells, there was no CRT dependence to this effect, consistent with the inability to observe cell surface CRT exposure in some cell types (Figs. 1, 2); that THP treatment of target cells increases proinflammatory cytokine production by BMDCs in a manner independent of target cell-derived CRT (Fig. 5); that extracellular CRT, either in a cell surface-bound (Fig. 5, Supplemental Fig. 2D) or soluble form (Fig. 6, Supplemental Fig. 2C), is unable to stimulate production of IL-6, IL-1β, TNF-α, IL-23, or IL-12p70; and that THP treatment of cells impacts their phagocytic uptake in a CRT-dependent manner (Fig. 7).

Two mechanisms were previously described for cell surface CRT exposure (8, 40). The first pathway is dependent on the pancreatic ER kinase arm of the UPR and results in the specific cotranslocation of CRT–ERp57 complexes to the cell surface (8). The second suggested pathway, relevant to apoptotic cells, involves the binding of cytosolic CRT to phosphatidylserine on the inner leaflet of the plasma membrane prior to apoptotic exposure of phosphatidylserine–CRT complexes on the cell surface (40). Neither of these pathways seems to be related to the mechanisms of surface exposure of CRT in viable cells subjected to ER calcium depletion, which display a general loss of ER retention of various ER chaperones. The THP-induced mode of CRT surface expression is independent of CRT–ERp57 binding (Fig. 3E). Rather, we recently showed that induction of a generic polypeptide binding site on CRT facilitates its cell surface expression under calcium-depleting conditions (21).

**Discussion**

Cell surface CRT has been characterized as an important factor in inducing antitumor immune responses (7, 8, 26, 47). Anticancer regimens that induce an antitumor immune response are likely to be more effective than are nonimmunogenic regimens with similar cancer cell cytotoxicity (4). Identifying conditions that induce cell surface CRT, as well as understanding mechanisms and functional consequences of cell surface CRT expression, could help in the design of more effective antitumor therapies. Our findings indicated that THP induces transient, preapoptotic, cell surface CRT exposure in some cell types (Figs. 1, 2); that THP treatment of target cells increases proinflammatory cytokine production by BMDCs in a manner independent of target cell-derived CRT (Fig. 5); that extracellular CRT, either in a cell surface-bound (Fig. 5, Supplemental Fig. 2D) or soluble form (Fig. 6, Supplemental Fig. 2C), is unable to stimulate production of IL-6, IL-1β, TNF-α, IL-23, or IL-12p70; and that THP treatment of cells impacts their phagocytic uptake in a CRT-dependent manner (Fig. 7).
Our data indicated a secretory route for ER proteins (Fig. 4), as also suggested for the CRT–ERp57 cotranslocation mechanism (8). It is noteworthy that several of the ER-resident proteins detectable in supernatants of THP-treated cells (Fig. 3) are known calcium-binding proteins of the ER (48). As is the case with CRT (49), ER retention of Bip, PDI, and gp96 may, in part, involve calcium-dependent processes. However, calcium-depletion alone seems to be insufficient to induce secretion of the ER chaperones (Fig. 3C, 3D). Rather, the combined effects of KDEL receptor saturation and calcium-depletion seem to be the mechanism that drives secretion of the ER chaperones in THP-treated cells. Similar mechanisms may be relevant to the extracellular localization of ER chaperones under physiological protein-misfolding conditions known to induce UPR and alter ER calcium homeostasis. To investigate this possibility, we compared chaperone secretion in WT and cog/cog mice thyroids, which express a misfolded, mutant thyroglobulin that accumulates in the ER (50). In preliminary findings, we observed enhanced secretion of CRT, PDI, and gp96 into the apical follicular lumen of cog/cog mice thyroids compared with their WT counterparts, correlating with significant enhancement of total chaperone levels in the cog/cog thyroid lysates (E. Jeffery, A.P. Kellogg, P. Arvan, and M. Raghavan, unpublished observations).

Previous studies in HeLa cells showed that NF-κB activation in response to agents that induce ER stress involves the release of calcium from the ER and the subsequent generation of reactive oxygen species (reviewed in Ref. 51). This mechanism likely accounts for sterile IL-6 production in response to direct THP treatment of BMDCs (Fig. 6B). THP is a stronger activator of the sterile IL-6 response compared with TUN (Fig. 6B), which likely results from the rapid effect of THP on cytosolic calcium elevation, whereas elevation of cytosolic calcium in response to TUN treatment may be more modest, as well as kinetically delayed. Many recent studies showed synergies between TLR-derived signals and transcription factors induced by UPR, including XBP-1 and C/EBP homologous protein. Of the cytokines measured in Figs. 5 and 6, XBP-1 was shown to synergize with TLR signals in enhancing IL-6 production by murine macrophages (41). In human monocye-derived DCs, C/EBP homologous protein binding to the IL-23p19 promoter enhanced IL-23 production in response to TLR ligation (43). Similar mechanisms could account for the synergy of ER stress signals and LPS in IL-23 production by BMDCs (Fig. 6B). We found that THP also strongly synergized with LPS in the generation of IL-1β and IL-12p70. Recent findings showed that high-avidity ligation of ITAM-containing receptors, such as DAP12, can synergize with TLR signals to activate proinflammatory gene expression (reviewed in Refs. 52, 53). High-avidity ligation of DAP12 triggers an acute, transient calcium increase (reviewed in Ref. 52). In this article, we showed that, compared with TUN, THP treatment strongly enhanced various proinflammatory responses to LPS (Fig. 6B), consistent with similar findings from previous studies (45, 46). These results point toward direct synergies between cytosolic calcium and TLR responses, as well as broader synergy between intracellular calcium and TLR signaling compared with those between the UPR and TLR signaling. Interestingly, it was suggested that ER calcium depletion can occur under certain conditions of ER overload and protein polymerization, even in the absence of classical UPR induction (54). Thus, the findings described in this article may be relevant to a better understanding of inflammatory protein-folding disorders that do not induce a classical UPR pathway.

A truncated bacterially expressed CRT construct was shown to induce TNF-α production by murine macrophages and human PBMCs (10). N-terminal truncation of CRT induced self-association, likely via the exposure of hydrophobic surfaces (10, 29), and it is possible that protein truncation induces enhanced binding to bacterial pathogen-associated molecular patterns. We showed in this study that full-length extracellular CRT derived from an endogenous source does not enhance the production of various proinflammatory cytokines in a THP-treated BMDC context (Figs. 5, 6, Supplemental Fig. 2C, 2D). These findings are consistent with a previous report that a secreted form of calreticulin does not induce proinflammatory cytokine production by BMDC (55). CRT is known to be present in the extracellular environment under a number of physiological conditions, and a number of extracellular functions are described for CRT (reviewed in Ref. 3). Given these extracellular roles of the protein, the lack of innate immune induction by endogenously derived, extracellular CRT is perhaps not surprising.

CRT-high, THP-treated, target cells were phagocytosed more efficiently than were untreated cells, and enhanced phagocytosis was dependent, in part, on CRT expression by the target cells (Fig. 7). Correlating with undetectable levels of surface CRT in UV-treated cells, we did not observe an effect of CRT on the phagocytosis of UV-treated cells (Fig. 7). In the context of UV-treated apoptotic cells, other changes are expected that promote phagocytic uptake (reviewed in Ref. 3), including the exposure of phosphatidylserine, which likely explain why significant uptake was observed in the absence of any CRT expression by the target cells (Fig. 7). Correspondingly, UV-treated target cells had a higher level of total association (adhesion and phagocytosis, indicated by the 37°C measurements) with BMDCs than did any other target cell group (Supplemental Fig. 4). This is likely the result of molecules that are upregulated on the surface of apoptotic cells and responsible for bridging apoptotic phagocytic cargo to potential phagocytes (reviewed in Ref. 56). It is important to consider that increases in surface calreticulin by a magnitude measurable by flow cytometry were, in general, difficult to detect in apoptotic cells (Figs. 1, 2). Stronger nonspecific protein binding to apoptotic cells could render a specific signal more difficult to detect by flow cytometry. Microscopic observations could allow for calreticulin redistribution or upregulation to be more readily visualized on apoptotic cells, as described in other studies (5, 9, 57). It is also important to note that cell surface CRT functions in the context of several other key eat-me and don’t-eat-me signals that are described for CRT (reviewed in Ref. 3). Given these extracellular roles of the protein, the lack of innate immune induction by endogenously derived, extracellular CRT is perhaps not surprising.

In contrast to some previous findings (7), we showed in this article that THP induces cell surface CRT and that THP promotes phagocytic uptake of cells via mechanisms that are, in part, CRT dependent. Thus, the inability of THP-treated cells to induce antitumor immunity with sufficient potency (7, 47) is likely not related to an absence of surface CRT per se. Other immunogenic signals absent in THP-treated cells (Supplemental Fig. 2E, 2F), including HMGB1 (58) and elevated levels of ATP (59), may be required to confer stronger immunogenicity to tumor cells. Consequently, although THP has some potentially desirable attributes...
for a chemotherapeutic, including the abilities to induce stronger phagocytic uptake of treated cells by DCs (Fig. 7) and elicit stronger cytokine production by DCs (Figs. 5, 6), these seem to be insufficient signals to confer antimicrobial immune protection. These observations are highly relevant to the ongoing clinical trials evaluating THP prodrugs for the treatment of advanced solid tumors [ClinicalTrials.gov Identifier: NCT01056029 (60)]. Because phagocytic uptake and cytokine levels can influence T cell priming steps, our results highlight the importance of further research investigating the impact of different forms of ER stress on the priming of CD8 and CD4 T cell responses.

Acknowledgments

We thank the transgenic mouse core for providing mouse bone marrow for DC preparations; Joanne Sonstein, Natasha Del Cid, and Dr. Jeff Curtis for assistance with the development of phagocytosis assays; Elise Jeffery for assistance with the project; and Drs. Malathi Kandarpa and Andzej Jakubowiak for the indicated cell lines.

Disclosures

The authors have no financial conflicts of interest.

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