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Enhanced ER entry of tumor antigen is crucial for cross-presentation induced by dendritic cell-targeted vaccination

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Abstract

Efficient cross-presentation of protein antigens to cytotoxic T lymphocytes (CTLs) by dendritic cells (DCs) is essential for the success of prophylactic and therapeutic vaccines. Here, we report a previously underappreciated pathway involving antigen entry into the endoplasmic reticulum (ER) critically needed for T-cell cross-priming induced by a DC-targeted vaccine. Directing the clinically relevant, melanoma antigen Gp100 to mouse-derived DCs by molecular adjuvant and chaperone Grp170 substantially facilitates antigen access to the ER. Grp170 also strengthens the interaction of internalized protein antigen with molecular components involved in ER-associated protein dislocation and/or degradation, which culminates in cytosolic translocation for proteasome-dependent degradation and processing. Targeted disruption of protein retrotranslocation causes exclusive ER retention of tumor antigen in mouse bone marrow-derived DCs and splenic $CD8^+$ DCs. This results in the blockade of antigen ubiquitination and processing, which abrogates the priming of antigen-specific $CD8^+$ T cells *in vitro* and *in vivo*. Therefore, the improved ER entry of tumor antigen serves as a molecular basis for the superior cross-presenting capacity of Grp170-based vaccine platform. The ER access and retrotranslocation represents a distinct pathway that operates within DCs for cross-presentation, and is required for the activation of antigen-specific CTLs by certain vaccines. These results also reinforce the importance of the ER-associated protein quality-control machinery and the mode of the antigen delivery in regulating DC-elicited immune outcomes.

Keywords

dendritic cell; cross-presentation; endoplasmic reticulum; vaccine; melanoma antigen; molecular chaperone

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that are highly efficient in processing and presenting antigen to $CD8^+$ cytotoxic T lymphocytes (CTLs),

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which constitute a critical component of host defense against pathogens or cancers (1). In addition to direct presentation of cytosolic antigen, DCs also acquire antigen exogenously and display it on major histocompatibility complex (MHC) class I molecules through a process termed cross-presentation (2). Cross-presentation of extracellular soluble antigens by DCs plays a pivotal role in the induction of antiviral and antitumor immune responses (3, 4). Since DCs represent a major target for many experimental or clinical cancer vaccination strategies, antigen cross-presentation is also crucial for the development of prophylactic and therapeutic vaccines to induce or activate $CD8^+$ T-cell immunity (5, 6).

The cross-presentation efficiency of DCs is not only determined by their ability to capture antigen, but also dictated by several other critical factors, such as the route of antigen uptake and trafficking, antigen stability and retention, or entry into the MHC-I pathway (7–10). The complexity of this process is highlighted by the fact that various pathways involved in cross-presentation have been proposed (11, 12). A major mechanism underlying this process appears to involve the translocation of endocytosed antigen into the cytoplasm for proteasomal degradation, followed by peptide transportation *via* transporter associated with antigen processing (TAP) and peptide loading onto MHC class I molecules in the endoplasmic reticulum (ER) (13, 14). Alternatively, antigenic peptides can be generated in the endocytic pathway and bind to recycling MHC class I molecules (15–18). Although the broad pathways leading to cross-presentation have been elucidated, it remains less understood how internalized protein antigens, when delivered in a therapeutic vaccine targeting DCs, gain access to MHC class I processing machinery.

Endoplasmic reticulum–associated degradation (ERAD) is an essential protein quality-control process that retrotranslocates misfolded or unfolded proteins in the ER to the cytosol for proteasome degradation (19, 20). Several lines of evidence imply that the ERAD machinery is either present on or recruited to the phagosomes/endosomes in antigen-presenting cells (APCs) and that it may be involved in antigen translocation to the cytosol for degradation (21–24). The possible relevance of ERAD in processing exogenous antigens is now apparent. Intriguingly, a few exogenous soluble proteins, such as the model antigen ovalbumin and US6 (a transmembrane protein from human cytomegalovirus), can be transported into the ER prior to ERAD (25, 26), though the mechanistic details of this retrograde trafficking pathway have remained elusive. Its potential involvement in cross-presenting soluble tumor antigens and prospective role in priming $CD8^+$ CTLs in the setting of therapeutic vaccination is largely unknown.

Soluble protein antigens are typically poorly cross-presented by DCs. Therefore, the choice of adjuvant to enhance this process will play a critical role in the success of cancer vaccines. Many evolutionarily-conserved stress/heat shock proteins (HSPs) function as molecular chaperones and are key players in the maintenance of protein homeostasis, e.g., folding/refolding, translocation and degradation (27). Extensive studies also demonstrate that HSPs are highly effective in directing associated antigen for cross-presentation by DCs and eliciting antigen-specific CTL responses, which has been attributed to their natural polypeptide-chaperoning capability and the presence of specific HSP-binding receptors (e.g., scavenger receptors) on the surface of DCs (28). The large stress proteins Hsp110 and glucose-regulated protein 170 (Grp170) are distant “Hsp70 superfamily” members that exhibit distinct structural and functional features compared to conventional chaperone molecules, such as Hsp70 (29). The exceptional antigen-holding capacity of these large stress proteins enables them to be exploited for the development of chaperoning-based cancer vaccines that are created by complexing Hsp110 or Grp170 with a clinically relevant, full-length protein antigen, e.g., Gp100 (30) or HER2/Neu (31). Full-length tumor proteins are suitable for vaccine development because they contain multiple epitopes recognized by both $CD4^+$ and $CD8^+$ T lymphocytes. Preclinical studies have shown that these reconstituted

chaperone-protein-complex vaccines generate a robust CTL response to associated tumor protein antigens (30–33). Indeed, a phase I clinical trial is currently underway to test a recombinant chaperone complex vaccine for treatment of metastatic melanoma. Although large stress proteins are superior holders of protein antigens, a feature that is essential for resultant vaccine efficacy (32, 33), the molecular pathways in DCs that are responsible for large chaperone-promoted cross-presentation and T-cell priming have not been elucidated.

In this study, we have investigated how Grp170-based chaperoning directs the intracellular compartmentalization, processing, and subsequent cross-presentation of internalized full-length tumor protein antigen by DCs. We show that the ER is a major organelle accessed by the Grp170-gp100 protein-chaperone complex vaccine following internalization by DCs. Functional ERAD machinery is required for the retrotranslocation of vaccine target antigen from the ER lumen to the cytosol for ubiquitination and integration into the MHC class I antigen-processing pathway. Our data reveal that the adjuvant activity of large chaperones (e.g., Grp170) in promoting cross-presentation relies on their ability to facilitate associated protein antigen into the distinct ER compartment within DCs, further highlighting the importance of vaccine composition and the nature of antigen delivery platform in influencing antigen trafficking routes and processing mechanisms. These findings extend the previous reports by the Cresswell group on the role of ERAD in cross-presentation (24, 25) and identify a previously underappreciated pathway involving antigen entry into the ER and ERAD critically needed for a therapeutic vaccine-induced cross-presentation and antitumor T-cell response.

Material and methods

Mice

C57BL/6 mice were purchased from the National Institutes of Health (Bethesda, MD). Tap1 knockout mice ($Tap1^{-/-}$) and pmel transgenic mice carrying the TCR transgene specific for the mouse homolog (pmel-17) of human gp100 (34) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were bred and maintained under pathogen-free conditions. All experimental procedures were conducted according to the protocols approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Reagents and antibodies

“KNDEL”-truncated mouse Grp170 and human Gp100 proteins were expressed using a BacPAK baculovirous expression system (BD Biosciences Clotech, Palo Alto, CA) and prepared using a nickel nitriloacetic acid (Ni-NTA)-agarose (Qiagen, Valencia, CA) column, as previously described (30, 35, 36). All glassware was depyrogenated at 250 °C for 4 h to reduce potential endotoxin contamination. Endotoxin levels in recombinant proteins (<30 EU/mg protein) were measured using a Limulus amebocyte lysate kit (Biowhittaker, Walkersville, MD). Protein labeling was carried out using the FITC-conjugation kit from Sigma-Aldrich (St. Louis, MO) as described previously (37). Briefly, 20-fold excess of FITC was incubated with the proteins in 0.1-M sodium bicarbonate/carbonate buffer (pH9.5) for 2 h at room temperature with slow rocking. The excess of free-FITC was then removed with a Sephadex G-25 column (Pharmacia, Piscataway, NJ). Proteins were concentrated with Centriplus (Millipore, Bedford, MA) and quantified using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The FITC and protein molar ratio was typically 2:3.

MG132, lactacystin, exotoxin A (Exo A), primaquine, and chloroquine were obtained from Calbiochem (San Diego, CA) in addition to Eeyarestatin I, which was obtained from TOCRIS Bioscience (San Francisco, CA). Antibodies against CHIP (C-0), VCP (G-20),

Gp100 (H-300), TFR1 (YTA 74.4), Giantin (N-18), and ubiquitin (P4D1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against KDEL (10C3) and Bip/Grp78 (SPA-826) (Enzo Life Sciences, Farmingdale, NY), Sec61α (ab15575), calnexin (ab22595) and EEA1 (polyclonal antibodies, ab2900; monoclonal antibodies, 1D4, Abcam, Cambridge, MA), Rab5 (C8B1) and Lamp1 (C54H11) (Cell Signaling Biotechnology, Beverly, MA), luciferase (G745A, Promega, Madison, WI), Hrd1 (ABGent, San Diego, CA), and histidine (Qiagen, Valencia, CA) were acquired from commercial resources as indicated. Alexa Fluor 488, Alexa Fluor 594, and ER-Tracker Red (BODIPY® TR Glibenclamide) were obtained from Invitrogen (Carlsbad, CA), as well as luciferase protein and pepstatin A from Sigma Aldrich.

Cell preparation

Mouse bone marrow-derived DCs (BMDCs) were prepared in the presence of GM-CSF as described previously (38, 39). Briefly, BM cells were flushed from the femurs and tibias of mice using phosphate-buffered saline (PBS) and then cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), 20-ng/mL recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ), 50-μM 2-Mercaptoethanol, and 1 % penicillin-streptomycin antibiotics at 37°C in an atmosphere of humidified air and 5% CO2. The culture media was replenished at days 3 and 6. Nonadherent cells were harvested on day 8 and cultured for an additional 24 h in fresh medium containing GM-CSF. The purity of $CD11C^+MHCII^+$ was consistently >90% at day 8–9, as assessed by flow cytometry. To prepare splenic $CD8^+$ DCs, spleens from naïve mice were minced and digested in RPMI media containing collagenase D (2 mg/mL) and DNase I (100 ng/mL) for 90 min at 37°C. $CD8^+CD11c^+$ DCs were isolated from single-cell suspensions using flow cytometry-based cell sorting. DC1.2 cells, a murine dendritic cell line (40), were kindly provided by Dr. Kenneth Rock (University of Massachusetts Medical Center, Worcester, MA). $CD8^+$ T cells were purified from pmel mice using a $CD8^+$ T-cell isolation kit (CL121, Cedarlane Labs, Burlington, NC).

Chaperone complex preparation and DC treatment

Chaperone complex vaccine was prepared as previously described (30, 33). Briefly, recombinant Grp170 protein was incubated with luciferase or Gp100 (1:1 molar ratio) for 30 min at 43 °C, followed by incubation at 37 °C for an additional 30 min. BMDCs were seeded in 12-well plates with glass coverslips (1×10^5 cells/well) at 37°C for 24 h. The cells were pulsed with FITC-labeled chaperone complexes, e.g., Grp170 (30 μg/mL)-gp100 (20 μg/mL), for 15 min and washed extensively with PBS. Cells were chased for different periods of time before collection for analyses.

Antigen cross-presentation

Cross-presentation of Gp100 protein chaperoned by Grp170 was carried out as previously described (41). BMDCs were pulsed with Grp170-gp100 protein complexes for 3 h. DCs (1×10^4) were washed with PBS and co-cultured with 1×10^5 naïve T cells derived from pmel mice in a round-bottom 96-well microtiter plate. Cells were cultured for 60 h and pulsed with ^{3}H-thymidine (^{3}H-TdR, 0.5 μCi/well) during the last 16 h of culture. T cell proliferation was assessed using ^{3}H-TdR incorporation assays. Levels of IL-2 in the culture supernatant were determined using ELISA kits from eBiosciences (San Diego, CA). In some cases, cells were pre-treated with or without various inhibitors for 2 h and washed extensively prior to co-culture with antigen-specific T cells.

Gene silencing

Lentiviruses (LVs) encoding mouse Sec61α short hairpin RNA (shRNA) or scrambled shRNA were packaged using Phoenix cells co-transfected with pLKO.1, pMD.G, and

pCMVΔR8.91 constructs as previously described (42). Sec61α shRNA sequences were 5′-GTCCAACCTGTATGTCATC-3′ and 5′-GAGTGATTCTGGCCTCTAA-3′. Day-3 BMDCs were infected with lentiviruses at room temperature for 2 h in the presence of 4 μg/mL polybrene (Sigma-Aldrich) and 20-ng/mL GM-CSF. Cells were collected on day 8 for studies.

Immunoblotting and immunoprecipitation

To prepare cell lysates, cells were washed twice with ice-cold PBS and solubilized in a lysis buffer containing 50-mM Tris-HCl (PH 7.4), 1% NP-40, 150-mM NaCl, 1-mM EDTA, 1-mM PMSF, 1-μg/mL each of aprotinin and leupeptin, and 1 -mM Na_3VO_4). For immunoprecipitation, 1 mg of cell extract was incubated with 2 μg of antibodies for 2 h at 4°C, followed by incubation with 40-3L Protein A/G-plus sepharose beads (Santa Cruz Biotechnology) at 4°C for 3 h. The beads were washed extensively and immune complexes were eluted by boiling them in SDS loading buffer for 5 min. Cell lysates or precipitated protein complexes were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were immunoblotted with primary antibodies, followed by HRP-conjugated secondary antibodies. Specific bands were visualized using enhanced chemiluminescence detection reagents from Thermo Scientific (Rockford, IL). The relative intensity values were normalized against β-actin.

Immunofluorescence and confocal microscopy

Cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After permeabilization with 0.1% Triton X-100 for 5 min and blocking with 10 % goat serum for 1 h, cells were stained with primary antibodies in 1% goat serum at room temperature for 2 h. After washing, cells were incubated with fluorescent-conjugated secondary antibodies for 1 h. Nuclei were visualized with 1 μg/mL of 4′-6-diamidino-2-phenylindole (DAPI). Images were acquired with a 63x (1.4 N.A.) oil-immersion objective on a Leica TCS SP2 spectral confocal microscope and processed using Photoshop software (Adobe Systems Inc.). Adjustments to brightness and contrast were applied to the whole image. Colocalization was quantified using ImageJ software (http://rsb.info.nih.gov/ij//index.html). Images were processed using the Colocalization Threshold plugin of ImageJ with the channel ratio set at 90% to obtain Pearson's correlation coefficient (Rtotal). A minimum 90 cells were analyzed per staining.

Live-imaging analysis

BMDCs were incubated with ER-Tracker Red (1 μM) in staining solution for 15 min at 37 °C and replaced with fresh complete medium. Cells were pulsed with FITC-labeled chaperone complexes for 15 min and washed extensively with PBS. Live cell images were collected using a Zeiss Cell Observer SD spinning disc confocal microscope equipped with a Yokogawa CSU-X1A spinning disc, 2 Photometrics Evolve 512 cameras (16-bit), a high-resolution piezo-driven Z-stage, an acousto-optic tunable filter, 4 lasers (405 nm, multi-line Argon 458/488/514 nm, 561 nm, 635 nm), a PeCon A-stage incubation system, and a 63x/1.2 numerical aperture water-corrected C-Aprochromat objective lens. Stage and chamber temperatures were set at 37 ºC and the CO_2 level was set at 5%. Images were collected sequentially in green (488 nm excitation, 525/50 nm emission) and red (561 nm excitation, 629/62 nm emission) channels. For 4-dimensional imaging (3-dimensional imaging over time), 30 images were collected through-focus (with a step size of 0.31 μm) at 5-minute intervals for 1 h. The resultant voxel dimensions were 0.21 μm × 0.21 μm × 0.31 μm. For finer temporal resolution, single-plane multi-channel images were collected at 1-minute intervals for 2 h.

Ubiquitination assays

DC1.2 cells were pulsed with Grp170-luciferase complexes for 30 min and washed extensively with PBS. Cells were treated with the proteasome inhibitors lactacystin (20 μM) or MG132 (20 μM) for 8 h, and cell lysates were immunoprecipitated with anti-luciferase antibodies. The immune complexes were subjected to immunoblotting with antibodies against luciferase or ubiquitin.

Adoptive cell transfer and T-cell cross-priming *in vivo*

2×10^6 T cells from pmel mice were transferred into recipient C57BL/6 mice by tail-vein injection one day prior to immunization. BMDCs were pre-treated with MG132 (20 μM) or Exo A (1 μg/mL) for 2 h and washed with PBS. Cells were then pulsed with Grp170-gp100 complexes for 4 h, washed, and injected subcutaneously into mice. Five-days later, cells from the spleen or lymph nodes were analyzed for T-cell expansion by first staining with anti-CD90.1-CY5 and anti-CD8-FITC antibodies and followed by FACS analysis. For intracellular cytokine assays, cells were stimulated with MHC class I- restricted $Gp100_{25–33}$ peptide (1 μg/mL) and then treated with PMA (10 nM) and ionomycin (1 μM) in the presence of brefeldin A (5 μg/mL) for 5 h. Cells were stained with anti-CD8-FITC antibodies and permeabilized using a Cytofix/Cytoperm kit from BD Biosciences (San Jose, CA) according to the manufacturer's instructions, prior to staining with anti-IFN-γ-PE antibodies (XMG1.2) from Biolegend (San Diego, CA). Cells were then analyzed using FACS by gating on $CD8^+CD90.1^+$ T cells.

Statistical analysis

Data are presented as means ± S.E. Statistical significance between groups within experiments was determined using unpaired two-tailed Student's *t*-tests, with a value of $p<0.05$ considered to be statistically significant.

Results

Internalized Grp170 interacts with the ERAD machinery involving Sec61α

During the study of Grp170 enhanced antigen cross-presentation *in vitro*, we observed that the stimulation of antigen-specific T cells by a Grp170-gp100 protein-complex vaccine was highly sensitive to treatment of DCs with either exotoxin A (Exo A) or the proteasome inhibitor lactacystin (Fig. 1A). Since Exo A, a *Pseudomonas aeruginosa*-derived toxin, blocks protein retrotranslocation *via* the Sec61 channel, a major component of ERAD (24, 43, 44), we sought todetermine the potential interaction between internalized Grp170 and molecules associated with ERAD or protein retrotransportation from the ER. Immunoprecipitation analyses were performed using DC1.2 cells after incubation with His-tagged Grp170 protein. The internalized Grp170 that was pulled-down by anti-penta-His antibodies was associated with Sec61α, the carboxyl terminus of Hsc70-interacting protein (CHIP, (45)), the AAA ATPase VCP/p97 (46), and ER-resident chaperone Bip/Grp78 (Fig. 1B). Reciprocal immunoprecipitations confirmed thatexogenous Grp170 bound to ERAD components, including Sec61α (Fig. 1C), CHIP (Fig. 1D), VCP/p97 (Fig. 1E) and Bip/Grp78 (Fig. 1F). CHIP and VCP/p97 were also present in the Sec61α immunocomplex (Fig. 1C). However, we were unable to detect the association between Grp170 and ERAD E3 ligase Hrd1 (47) (Fig. 1B). These data suggest that Grp170, a chaperonewith highly efficient cross-priming activity, interacts with molecules involved in ERAD upon internalization. We also showed that, analogous to intracellular Bip/Grp78, the ER-resident endogenous Grp170 also interacts with Sec61α (Supplementary Fig. S1).

Grp170-based chaperoning facilitates antigen access to the ER and its interaction with Sec61α

We first used luciferase as a model antigen to test the hypothesis that Grp170 chaperoning influences antigen transportation and processing. To mimic physiological conditions more closely, we incubated bone marrow-derived DCs (BMDCs) with luciferase or Grp170-luciferase complexes at 37°C. The immunoprecipitated Sec61α from DCs was associated with increased luciferase when delivered in a chaperone complex with Grp170 (Fig. 2A), implicating chaperone-facilitated antigen interactions with the ERAD machinery. In support of this observation, confocal microscopy analysis showed that Grp170 chaperoning enhanced the colocalization of luciferase with Sec61α in comparison to luciferase alone (Fig. 2B).

We subsequently used the Gp100 protein, a melanoma antigen, to further examine the effects of chaperoning on the subcellular compartmentalization of internalized vaccine complex. Soluble Gp100 itself was not associated with Sec61α after DC uptake; however, the presence of Grp170 substantially enhanced Gp100 binding with intracellular Sec61α (Fig. 2C). Similarly, colocalization of Gp100 and Sec61α significantly increased following internalization of Grp170-gp100 complex compared to Gp100 alone (Fig. 2D). Since we removed the ER retention signal (i.e., “KNDEL” sequence) from the recombinant His-tagged Grp170 protein in our studies (Supplementary Fig. S2A), we used antibodies for KDEL, a commonly used ER marker, to examine the subcellular localization of the vaccine complex. Gp100, chaperoned by Grp170, showed an ER-like perinuclear reticular staining pattern colocalized with KDEL (Fig. 2E), as well as calnexin, an ER-resident chaperone (Supplementary Fig. S2B). However, Gp100 in the chaperone complex did not significantly colocalize with LAMP-1 or Giantin, which identify lysosomal/late endosomal or Golgi compartments, respectively (Supplementary Fig. S2B). Biochemical fractionation analysis showed that both Grp170 and Gp100 were present in the purified ER fractions (Supplementary Fig. S2C). In addition, we used ER tracker red, which binds to the sulphonylurea receptors of ATP-sensitive K^+ channels that are prominent on the ER, in conjunction with live-cell imaging to track the internalized Grp170-gp100 complexes in BMDCs. We showed that FITC-labeled Gp100 in the chaperone complex was progressively and efficiently transported to and entered the lumen of the ER (Fig. 2F and Supplementary video).

The uptake of chaperone-antigen complexes was previously reported to depend on receptor-mediated endocytosis (48–50). To confirm this, we incubated BMDCs with Grp170-gp100 complexes on ice for 30 min prior to washing and transferring to 37°C. It was observed that the vaccines were initially associated with the surface of BMDCs, presumably *via* receptor binding (37), and subsequently gained access to the ER (Supplementary Fig. S2D). Another “Hsp70 superfamily” member, known as Hsp110 and considered to be the cytosolic paralogue of Grp170, also enhanced Gp100 entry into the ER. However, no significant colocalization of Gp100 and KDEL was detected when Hsp70 was used to deliver Gp100 protein (unpublished observations).

Grp170-gp100 complexes are transported to the ER from endosomal compartments

A pulse-chase strategy was exploited to define the dynamic trafficking of the Grp170-gp100 complex after internalization. BMDCs were incubated for 15 min with FITC-labeled Gp100 complexed with Grp170 and extensively washed with PBS (set as 0 min), followed by chasing for different times. At early time points, cell staining of Sec61α indicated low levels of colocalization with Gp100; however, colocalization progressively increased until it peaked at 120 min, followed by a decrease to baseline at 3 h (Fig. 3A). A similar trend was observed for KDEL staining (Fig. 3B), although its colocalization with Gp100 occurred

earlier (i.e., 15 and 30 min) when compared to Sec61α. Since other chaperone molecules were reported to access endosome structures in professional APCs (51, 52), we examined the subcellular localization of Gp100 relative to EEA1 (for early endosomes) or TFR1 (for recycling endosomes). Surprisingly, the Grp170-gp100 protein complexes were only present in the $EEA1^+$ (Fig. 3C) or TFR^+ (Fig. 3D) endosomal compartments shortly after uptake and graduated disappeared from these vesicles while they were entering the $Sec61\alpha^+$ ER.

Internalization of the Grp170-gp100 complex is associated with colocalization of ER and endosomal components

The observation of Gp100 antigen colocalization with KDEL and EEA1 or TFR1 at early time points after internalization prompted us to further assess the potential 'fusion' of ER and endosomal structures in response to vaccine uptake. We examined BMDCs by co-staining KDEL and EEA1 (Fig. 4A) or TFR1 (Fig. 4B) in the setting of the pulse-chase study. KDEL did not colocalize with either EEA1 or TFR1 in untreated BMDCs. Immediately after internalization of the Grp170-gp100 complex, the presence of $KDEL^+EEA1^+$ (Fig. 4A) or $KDEL^+TFR1^+$ (Fig. 4B) structures substantially increased (i.e., 15 to 30 min). These double-positive vesicles began to significantly decrease after 1 h and were observed to have completely diminished in 2 h. Interestingly, there was little colocalization of Sec61α with either EEA1 or TFR1 throughout the uptake process of the Grp170-gp100 complex (unpublished observations). The formation of $KDEL^+EEA1^+$ vesicles was not seen when Gp100 was delivered to DCs in the absence of Grp170 (Fig. 4C). It is likely that internalization of the vaccine complex triggers the formation of an intermediate structure that contains "KDEL" sequence-containing ER proteins (e.g., Bip/Grp78, Gp96/Grp94) and $EEA1^+$ or $TFR1^+$, but not Sec61α.

Processing of Grp170-chaperoned protein antigen requires ER retrotranslocation for ubiquitination and degradation

Since the Grp170-gp100 vaccine gains access to the ER and interacts with the Sec61 complex, we next examined whether Gp100 processing depends on proteasome activity and protein retrotranslocation from the ER lumen to the cytosol. Inhibition of ERAD-mediated transportation with Exo A or inhibition of proteasome activity with lactacystin (53) blocked the degradation of luciferase (Fig. 5A). However, treatment of DCs with chloroquine, a lysosome inhibitor, failed to rescue the degradation (Fig. 5A), suggesting that chaperone complex formation permits antigen to escape lysosome-mediated protein degradation, which is often seen for many internalized soluble proteins. The involvement of the ERAD pathway in the processing of Grp170-chaperoned antigen is also indicated by immunoprecipitation analysis, which showed that inhibition of proteasome activity in BMDCs by lactacystin increased luciferase association with Sec61α (Fig. 5B).

ERAD-mediated retrotranslocation is associated with protein ubiquitination prior to degradation. Indeed, chaperoning by Grp170 markedly increased the ubiquitination of luciferase (Fig. 5C), suggesting that chaperoning by Grp170 may also facilitate the processing of associated antigen. We used luciferase as a model protein antigen instead of Gp100 in this study because the anti-Gp100 antibodies available are not suitable for Gp100 immunoprecipitation. Strikingly, inhibition of retrotranslocation with Exo A completely blocked the ubiquitination of luciferase (Fig. 5D), which is consistent with the observation that pretreatment of BMDCs with Exo A resulted in the internalized Grp170-gp100 complex being retained exclusively in the ER (Fig. 5E–F). However, disruption of the ERAD pathway did not affect the access of the Grp170-gp100 vaccine to $EEA1^+$ structures after uptake (Fig. 5G). These results suggest that the chaperone complex gains access to the ER before retrotranslocation into the cytosol for ubiquitination and degradation. Use of

Eeyarestatin 1, another inhibitor for ERAD or ER translocation (54), caused the similar retention of Grp170-gp100 complex in the $KDEL^+$ compartment (Supplementary Fig. S3A).

Since $CD8^+$DCs have been shown to be major contributors in antigen cross-presentation (55), we purified these cells from the spleens of mice using flow cytometry-based cell sorting (Fig. 6A) and then examined the subcellular localization of internalized Grp170-gp100 chaperone complex. Compared to BMDCs, primary $CD8^+$ DCs displayed a rounded morphology with a relatively large nuclear volume (Fig. 6B). As expected, Gp100 carried by Grp170 primarily colocalized with Sec61α or KDEL but not EEA1 (Fig. 6B). Inhibition of ERAD-mediated retrotranslocation also resulted in Gp100 accumulation in the $KDEL^+$ ER compartment (Fig. 6C).

Considering previously reported ER recruitment to phagosomes (23), we examined the potential involvement of phagocytosis in directing the internalized chaperone-protein complex to the ER. Treatment of BMDCs with cytochalasin D had no effect on the colocalization of Gp100 with KDEL (Supplementary Fig. S3B). Blocking phagocytosis with cytochalasin D in addition did not affect the ubiquitination of antigen (i.e., luciferase) carried by Grp170 (Supplementary Fig. S3C), suggesting that uptake of the Grp170-protein antigen complex by DCs and subsequent ER entry does not depend on the phagocytic processes.

Interference of the ERAD pathway inhibits Grp170-enhanced cross-presentation

We used *in vitro* T-cell-stimulation assays to examine the contribution of the ERAD pathway in cross-presentation of tumor protein antigen Gp100 elicited by the chaperone-protein complex vaccine. Blocking the ER retrotranslocation or proteasome activity in BMDCs with Exo A (Fig. 7A) or MG132 (Fig. 7B), respectively, profoundly suppressed the activation of Gp100-specific naïve $CD8^+$ T cells, as indicated by T cell proliferation and IL-2 production. This inhibitory effect was not due to cellular toxicity because direct presentation of the $Gp100_{25–33}$ peptide by BMDCs was not affected by the presence of these inhibitors (Supplementary Fig. S4A–B). We also showed that silencing Sec61α in BMDCs completely eliminated Grp170-promoted cross-presentation (Fig. 7C). The absence of Tap1, a peptide transporter for MHC class I-restricted antigen presentation (56), in DCs significantly reduced T-cell stimulation by theGrp170-gp100 chaperone complex (Fig, 7D).

In contrast to the dramatic effect of Exo A or MG132, pretreatment of BMDCs with pepstatin A and NH_4Cl that inhibit endosomal/lysosomal proteolysis did not interfere with Grp170-enhanced cross-presentation. Blocking endosomal recycling to the plasma membrane with primaquine, however, showed a modest inhibitory effect (Supplementary Fig. S4C).

The ERAD pathway is crucial for the cross-priming of $CD8^+$ T-cells *in vivo*

An adoptive T-cell transfer model was used to further examine the impact of ER retrotranslocation and proteasome activity on Grp170-gp100-complex vaccine–elicited CTL response *in vivo*. BMDCs were pretreated with Exo A or MG132 and pulsed with the Grp170-gp100 protein complexes, followed by injection into mice that had received naïve $Gp100_{25–33}$-speific $CD90.1^+CD8^+$ T cells. Consistent with the T-cell stimulation *in vitro* data, DC treatment with Exo A or MG132 led to reduced expansion and IFN-γ production of $CD90.1^+CD8^+$ T cells in the spleens (Fig. 8A) and draining lymph nodes (Supplementary Fig. S4D). Similarly, Sec61α downregulation in DCs by a lentivirus-encoding shRNA also blocked the Grp170-gp100 vaccine induced activation of Gp100-specific CTLs (Fig. 8B and Supplementary Fig. S4E), suggesting that Grp170-promoted cross-priming of $CD8^+$T cells relies on proteasome activity and protein retrotranslocation from the ER to the cytosol.

Discussion

Although large chaperones (e.g., Grp170) promote the cross-presentation of associated cancer protein antigens (30–33), the mechanisms underlying DC interaction with the chaperone-complex vaccine and resultant T-cell cross-priming are enigmas. Here, we have investigated the antigen cross-presentation pathways operating in DCs upon capture of the Grp170-based vaccine. We demonstrate that the formation of chaperone complexes directs soluble protein antigens efficiently to the ER lumen after their internalization. In particular, Grp170-enhanced antigen processing and presentation is dependent on retrotranslocation machinery involving Sec61 in the ER, which targets antigens for ubiquitination and proteasome-mediated cytosolic degradation and integration into the conventional MHC class I antigen-processing pathways. We also provide the experimental evidence that large chaperone molecules are extraordinarily competent in preserving internalized protein antigen target from being degraded by lysosomes and routing exogenous tumor antigen from endosomes into the ER compartment, which therefore contributes to the improved cross-priming capacity of DCs. Thus, ER access and ERAD-mediated protein retrotranslocation within DCs are crucial events required for large chaperones, molecular adjuvants, enhanced cross-presentation, and activation of tumor antigen-specific $CD8^+$ CTLs.

Using a combination of molecular, biochemical, immunofluorescence microscopy, and live-cell imaging techniques, we have provided compelling evidence that Grp170-based chaperoning enhances antigen access to ER following the capture of the chaperone-complex vaccine by DCs; including $CD8^+$ DCs that are believed to play a prominent role in antigen cross-presentation *in vivo*. Evidently, chaperoning by Grp170 protects associated tumor antigens from destruction by lysosomal proteases, which may be one of the key factors responsible for the highly efficient cross-presentation of soluble antigens when targeted to DCs in a chaperone complex. This is also supported by the observation of Chatterjee et al, who found that antigens destined for more degradative late endosomes or lysosomes are often poorly cross-presented (10). Although the Grp170-gp100 complex is transiently present in $EEA1^+$ or $TFR1^+$ endocytic structures after internalization, the complex is transported into the ER, as indicated by co-immunoprecipitation with ER-resident molecules (e.g., Sec61, Bip/Grp78), progressively increased colocalization with Sec61α, KDEL, calnexin, or ER tracker in the pulse-chase study, and fractionation analysis.

Immunofluorescence staining shows substantially enhanced, but not complete colocalization of the chaperone complex with Sec61α. This may be explained by the observation that the chaperone complex also interacts with other molecules in the ER lumen, e.g., VCP, CHIP, and BiP. Nonetheless, when antigen is delivered using the Grp170-based platform, the ER compartment within DCs likely serves as an antigen portal for subsequent cytosolic entry, followed by processing and cross-presentation. Our results are, in general, consistent with the previous finding of Ackerman et al that the exogenous model- or foreign-antigens (e.g., ovalbumin) are able to enter the ER (25). However, our data provide additional insight into this phenomenon by identifying and elucidating the mechanism of the peculiar efficiency of a vaccine adjuvant in promoting the cross-presentation of clinically-relevant tumor antigen (Fig. 8C). We therefore propose that Grp170-based chaperoning in a vaccine regimen offers an optimal setting for priming of $CD8^+$ T-cells by promoting the efficient access of protein antigen, such as Gp100, to the ER compartment.

The function of ERAD, a protein quality-control mechanism in the ER, is to retrotranslocate misfolded or unfolded proteins to the cytosol for ubiquitination and proteasomal degradation (19). Using two different ERAD inhibitors, we demonstrate that disruption of ERAD-mediated protein export leads to retention of internalized Gp100 antigen in the ER, not in the endocytic structures or cytoplasm, which further supports the notion of direct ER access

by Grp170-chaperoned protein antigen. Additionally, interfering with retrotranslocation substantially reduces the ubiquitination and proteasomal degradation of Gp100, subsequently eliminating cross-presentation *in vitro* and *in vivo*. The involvement of ERAD in Grp170-promoted T-cell cross-priming is also indicated by the suppression of cross-priming of Gp100-specific naïve T cells when Sec61α, a major component of the ERAD machinery (44), is downregulated in DCs. These results are consistent with and further reinforce the previously reported role of the ER retrotranslocation machinery involving Sec61 in the cross-presentation of soluble antigen (24, 57). Although our data show that internalized vaccine carrier Grp170 physically binds to several molecular components associated with the ERAD pathway (e.g., Sec61, CHIP, VCP/p97, and Bip/Grp78), and that molecular chaperoning enhances antigen association with Sec61, the exact ERAD channel responsible for dislocation of tumor antigen remain unclear. It has also been suggested that Sec61 mediates ER retrotranslocation (43, 44), though Sec61-independent polypeptide dislocation, however, also exists (58).

Molecular chaperones are known to play significant roles in the ER and cytosol during ERAD (59). In our study, chaperoning by Grp170 substantially enhances the interaction between internalized antigen and the molecular components (e.g., Sec61α, VCP/p97, CHIP, Bip/Grp78) associated with the ERAD pathway, which may explain the increased ubiquitination of antigen carried by Grp170. Since ERAD only recognizes non-native substrate conformation, tumor protein antigen (i.e., Gp100) that is already partially unfolded by heat shock and chaperoned by Grp170 in the vaccine may serve as an ERAD target. Intriguingly, like Bip/Grp78, ER-resident Grp170 can also be co-immunoprecipitated with Sec61α. Since the cytosolic chaperone Hsp90 was recently reported to promote antigen access to the cytosol (60, 61), it is reasonable to speculate that internalized Grp170 together with ER-resident chaperone molecules may collaboratively guide Gp100 to the ERAD machinery and support protein retrotranslocation for further processing.

The intracellular pathways involved in transporting the Grp170-gp100 complex from endocytic compartments to the ER have not been addressed in our study. The transient presence of ER markers, such as KDEL, in early endosomes or recycling endosomes, followed by their progressive disappearance from these endocytic compartments, support the possibility that these “intermediate structures” with ER components may represent the mechanism by which exogenous Grp170-gp100 protein complexes reach the ER. Indeed, recruitment of ER-resident proteins to phagosomes or ER-phagosome fusion compartment during engulfment of particulate antigens has been documented (9, 23, 62). Thus, the concept of an “ER–endosome fusion” remains an attractive model for explaining how the chaperone complex vaccine is directed into the ER from the extracellular environment. Despite the fact that internalized Gp100 carried by Grp170 does not localize within the Golgi apparatus, involvement of the retrograde trafficking route previously reported for pathogens and bacterial toxins cannot be excluded (63–65).

It has been proposed that trafficking routes of internalized antigens can be dictated by the size and structure of the antigen (14), as particulate antigens are often more efficiently targeted for cross-presentation compared to their soluble counterparts (66). However, phagocytosis is not involved in the uptake and transportation of the Grp170-gp100 complex to the ER in our system because treatment of DCs with cytochalasin D has no effect on Gp100 access to the ER, as well as its ubiquitination when chaperoned by Grp170. This is also supported by the general view that MHC class I-restricted cross-presentation of chaperone-associated antigen depends on receptor-mediated endocytosis (48–50).

In contrast to the high sensitivity of Grp170-gp100 vaccine to inhibition of protein retrotranslocation and proteasome activity, we observed that pharmacological blocking of

endosomal recycling also modestly reduces the cross-presentation of Gp100. It appears that no single pathway is likely to account for all the observed instances of cross-presentation. It remains possible that the dominant, proteasome-dependent cytosolic pathway may co-exist with the vacuolar pathway in Grp170-amplified T cell cross-priming. The ER entry of the Grp170-gp100 vaccine revealed in the current study also highlights a difference in the intracellular behavior of large chaperone-protein complexes upon internalization compared to other chaperone-peptide complexes, which appear to be primarily targeted toward $EEA1^+$ static early endosomes for cross-presentation (51, 52, 67). In the context of shuttling full-length protein tumor antigen (e.g., gp100), the superior chaperoning property of high molecular weight stress proteins appears to be a crucial factor in determining enhanced ER access. This inconsistency may also be attributed to the structure and nature of antigens shuttled by chaperone molecules. It should be noted that model antigens, such as ovalbumin, are often used in these previous studies, whereas we employed a clinically relevant tumor antigen (i.e., Gp100) in the setting of cancer vaccine therapy.

In conclusion, we demonstrate that direct ER access and protein dislocation *via* the ERAD pathway in DCs is required for the generation of a CTL response to tumor protein antigen carried by large chaperones. Our findings provide molecular insights into the mechanism by which the recombinant large chaperone-complex vaccine provokes antitumor cellular immunity, and support the notion that the protein quality-control machinery in the ER can actively participate in immune surveillance and cross-presentation of soluble tumor protein antigens captured by large chaperone molecules. The implication of our results is that the choice of vaccine adjuvant and the mode of antigen formulation or delivery can define the cross-presenting capacity of DCs. A better understanding of these details should assist in the design and optimization of vaccination strategies aimed at critical tumor targets in the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CTL	cytotoxic T lymphocyte
DC	dendritic cell
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
Grp	Glucose-regulated protein

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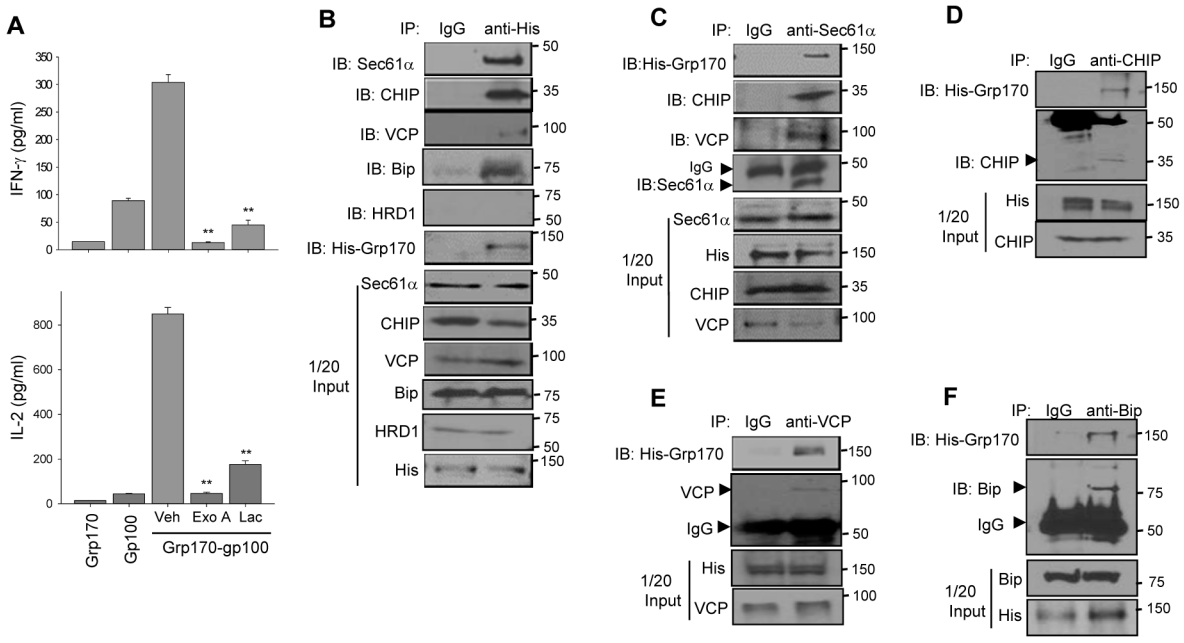

Figure 1. Exogenous Grp170 associates with ERAD machinery following internalization by DCs

A. Grp170-enhanced cross-presentation is highly sensitive to inhibition of ER retrotranslocation and proteasome activity. BMDCs were pre-treated with vehicle (Veh), Exo A (100 ng/mL) or Lactacystin (Lac, 20 μM) for 2 h and then washed extensively. Cells were pulsed with the Grp170-gp100 complex and cocultured with $Gp100_{25–33}$-specific, naïve $CD8^+$ T cells for 72 h. Cells treated with Grp170 or Gp100 proteins serve as controls. IFN-γ (upper) or IL-2 (lower) in the supernatant was determined using ELISA. **, $p<0.001$ *vs* vehicle treatment. **B.** Internalized His-Grp170 interacts with molecular components associated with the ERAD pathways. DC1.2 cells were incubated with His-Grp170 at 37 °C for 30 min and washed extensively. Cell lysates were immunoprecipitated with anti-Penta-His antibodies to pull down His-Grp170. The immune complexes were probed with antibodies against Sec61α, CHIP, VCP, Bip, or Hrd1. Total cell lysates, as the input, were also examined. **C–F.** Reciprocal immunoprecipitation analysis of His-Grp170-association with Sec61α (**C**), CHIP (**D**), VCP (**E**), or Bip/Grp78 (**F**) using respective antibodies. CHIP and VCP were also seen in the immune complex of Sec61α (**C**). The experiments were repeated three times with similar results.

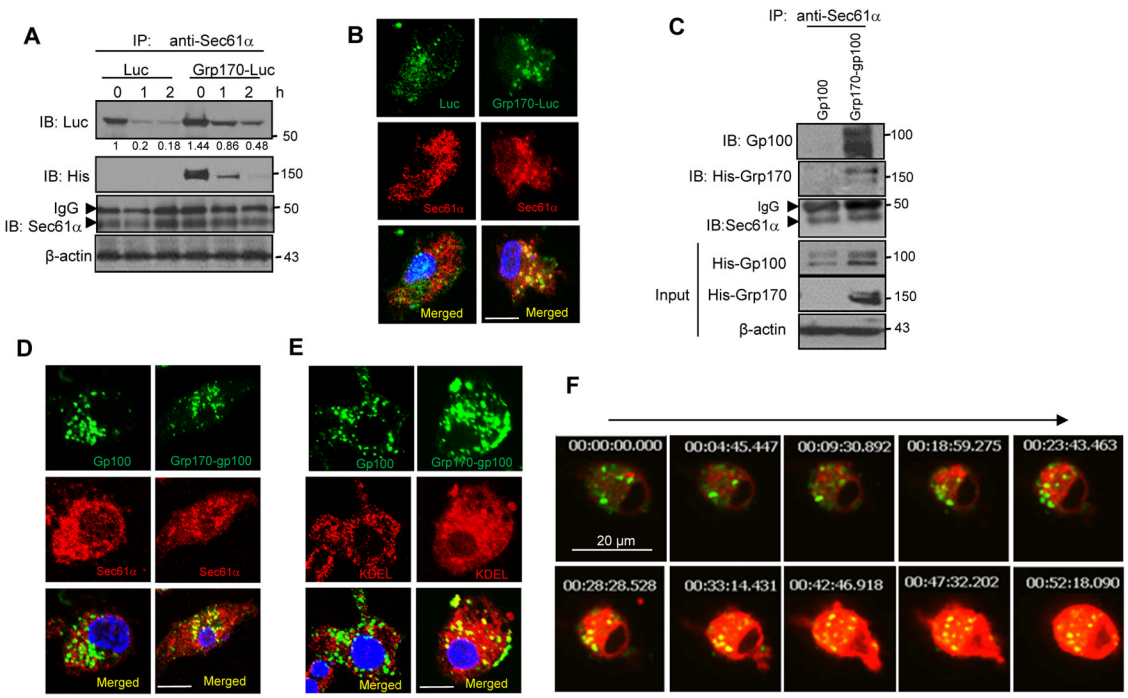

Figure 2. Soluble protein antigen chaperoned by Grp170 gains access to the ER upon internalization by DCs

A. Grp170 enhances association of internalized luciferase (Luc) with Sec61α. BMDCs were cocultured with Luc or His-Grp170-Luc complex at 37 °C for 30 min. Sec61α was immunoprecipitated at the indicated times after wash and examined for the presence of Luc or His-Grp170 in the immune complexes. **B.** Grp170 increases colocalization of Luc and Sec61α. After coculture with Luc or His-Grp170-Luc for 30 min, BMDCs were subjected to immunofluorescence staining for Luc and Sec61α. **C.** Grp170 chaperoning enhances melanoma antigen Gp100 interaction with Sec61α. BMDCs were cocultured with His-Gp100 or reconstituted His-Grp170-gp100 complex, followed by immunoprecipitation assays for Sec61α association with His-Gp100 or His-Grp170. **D–E.** Grp170 carries Gp100 into the ERcompartment. After coculture with FITC-labeled Gp100 or Grp170-gp100 complex, BMDCs were stained with antibodies against Sec61α (**D**) or KDEL (**E**). DAPI was used to counterstain nuclei. **F.** Time-lapse live-cell imaging of the complex vaccine access to the ER. BMDCs were treated with the ER-Tracker Red (1 μM) for 15 min. Cells were pulsed with the Grp170-gp100 complex for 15 min and washed (time = 0). Time-lapse images were captured using a Zeiss Cell Observer SD spinning disc confocal microscope. See Supplementary Video 1 for the full time course. Data shown are representative of at least two independent experiments with similar results. Scale bar, 5 μM

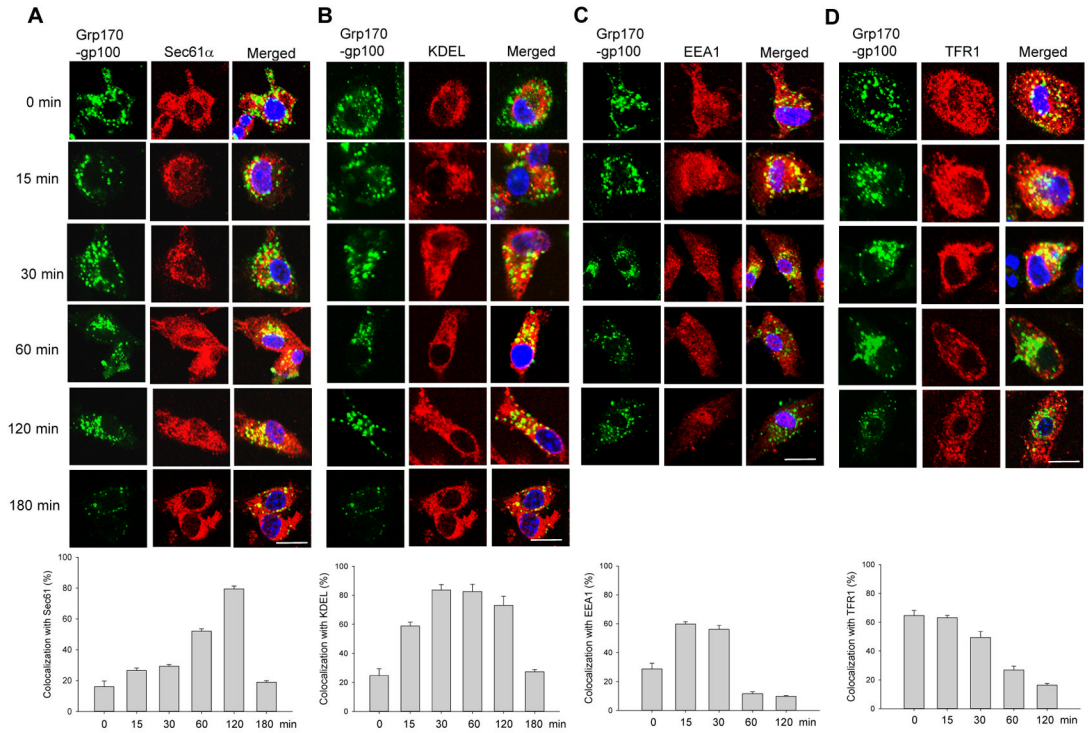

Figure 3. Intracellular trafficking of the Grp170-gp100 chaperone-complex from endocytic structures to the Sec61α^{+} ER

BMDCs were pulsed for 15 min with FITC-labeled Gp100 in complex with Grp170 and washed extensively with cold PBS (time = 0). Cells were chased for the indicated times and stained with antibodies against Sec61α (**A**), KDEL (**B**), EEA1 (**C**) or TFR1 (**D**). Colocalization of Gp100 with the various organelle markers was quantified using ImageJ software (lower panels). Data representative of three independent experiments with similar results are shown. Scale bar, 5 μM

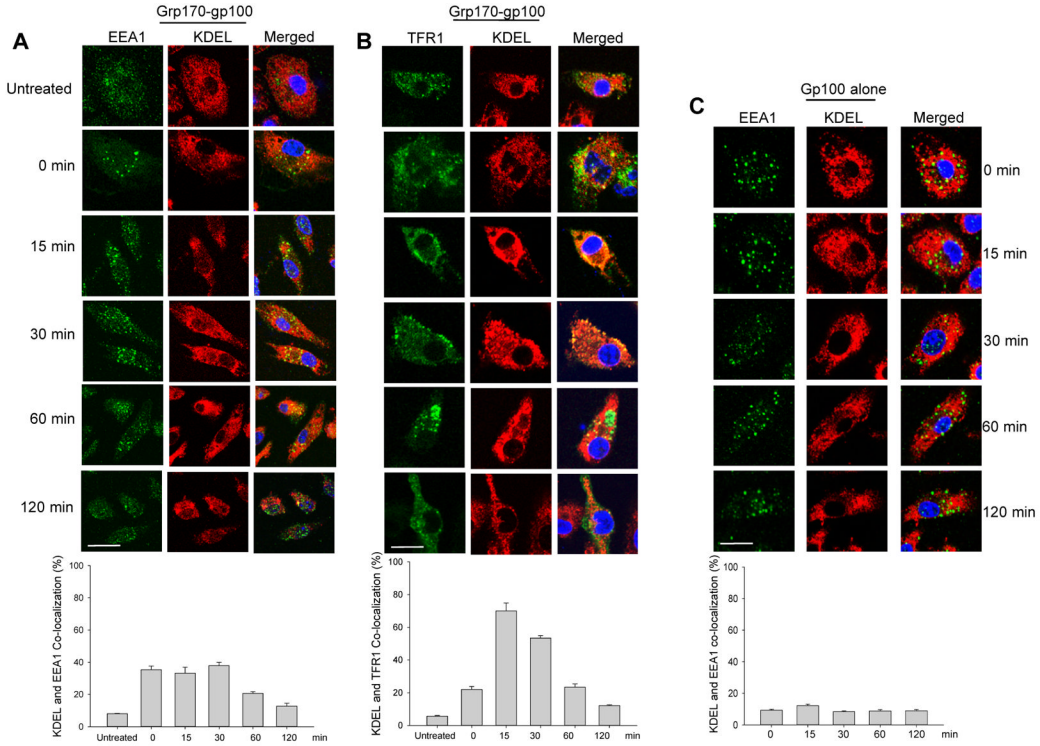

Figure 4. Recruitment of the ER components to the endocytic vesicles in response to uptake of the complex vaccine

BMDCs were pulsed with the Grp170-gp100 complex (**A–B**), washed, and chased for the indicated times. Colocalization of EEA1 and KDEL (**A**), or TFR1 and KDEL (**B**) was analyzed with an immunofluorescence microscopy. **C**. Costaining of EEA1 and KDEL after DCs were pulsed with Gp100 alone. Colocalization is quantified using ImageJ software. Data represent three independent experiments with similar results. Scale bar, 5 μM

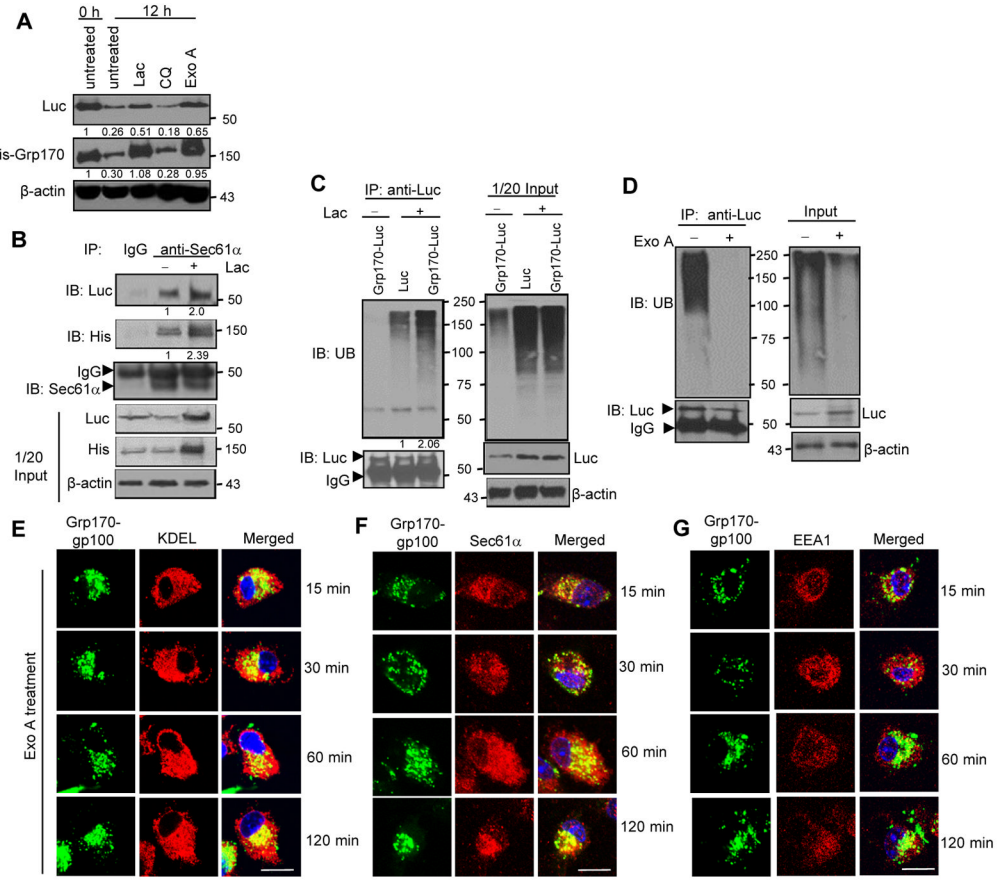

Figure 5. The ERAD pathway is responsible for processing of Grp170-chaperoned protein antigen

A. Proteasome and protein retrotranslocation-dependent degradation of the Grp170-antigen complex. DC1.2 cells were pulsed with the Grp170-Luc complex for 30 min, washed, and cultured in the presence of lactacystin (Lac, 20 μM), chloroquine (CQ, 50 μM), or Exo A (100 ng/mL) for 12 h. Intracellular levels of Luc or Grp170 were examined by immunoblotting. **B.** Increased Luc association with Sec61α after inhibition of proteasome activity. DC1.2 cells were treated with the Grp170-Luc complex in the presence of Lac. Sec61α was immunoprecipitated and analyzed for the presence of Luc or His-Grp170. **C.** Increased ubiquitination of Luc in the chaperone complex. DC1.2 cells were pulsed with Luc or Grp170-Luc complex, washed, and treated with Lac for 8 h. Luc was immunoprecipitated and examined for ubiquitination with anti-ubiquitin (UB) antibodies. **D**. Inhibition of Luc ubiquitination by disrupting ER retrotranslocation. DC1.2 cells were pulsed with Grp170-Luc complex, washed, and treated with Exo A. Ubiquitination was examined after pull-down of Luc using anti-Luc antibodies. **E–F.** Inhibition of ER retrotranlocation causes the accumulation of the Grp170-gp100 vaccine in the ER. BMDCs were pretreated with Exo A for 2 h, pulsed with FITC-labeled Gp100 in complexes with Grp170 and chased for the indicated times. Cells were stained with antibodies for KDEL (**E**), Sec61α (**F**), or EEA1 (**G**). Data shown are representative of three independent experiments with similar results. Scale bar, 5 μM

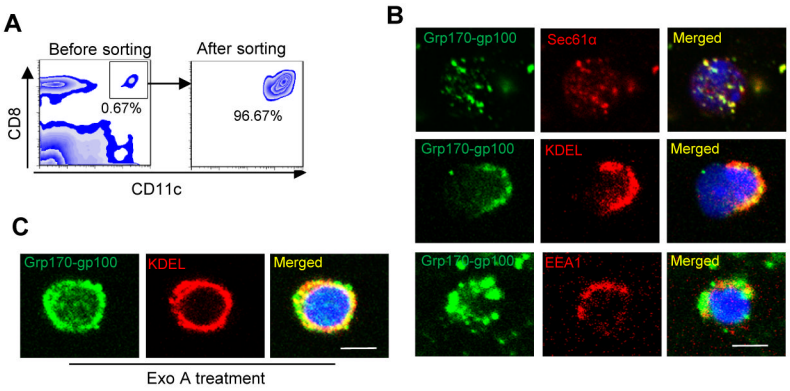

Figure 6. Grp170-gp100 protein complexes access the ER of CD8$^+$ DCs

CD8$^+$ DCs were sorted from mouse spleens after staining with anti-CD11c and -CD8 antibodies (**A**). Cells were pulsed with FITC-labeled Gp100 in complex with Grp170, washed, and cultured for an additional 30 min. Cells were stained with antibodies against Sec61α, KDEL, or EEA1 (**B**). Isolated CD8$^+$ DCs were pretreated with Exo A, pulsed with the chaperone complex for 15 min, and washed. Cells were cultured for an additional 30 min and stained with anti-KDEL antibodies (**C**). Data shown are representative of two independent experiments. Scale bar, 5 μM

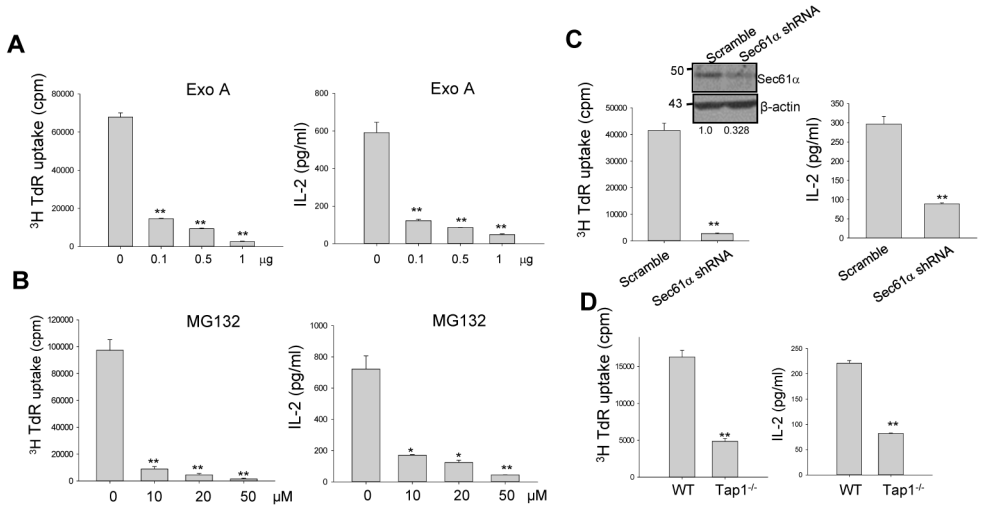

Figure 7. Inhibition of the ERAD pathway disrupts Grp170-enhanced cross-presentation of Gp100

A–B. BMDCs were pretreated with indicated concentrations of Exo A (**A**) or MG132 (**B**) and washed with PBS. Cells were pulsed with Grp170-gp100 complexes and incubated with Gp100-specific, naïve CD8$^+$ pmel cells at a ratio of 1:10. T-cell proliferation was assessed using ^{3}H-TdR incorporation assays. IL-2 in the supernatants was determined using ELISAs. **C.** Reduced Gp100 cross-presentation by Sec61α-silencing in DCs. BMDCs infected with lentiviruses encoding scrambled shRNA or Sec61α shRNA were pulsed with the Grp170-gp100 complex. T-cell proliferation and activation were examined after coculture with CD8$^+$ pmel cells. **D.** Requirement of TAP for Grp170-promoted cross-presentation. BMDCs from WT or Tap1$^{-/-}$ mice were pulsed with the Grp170-gp100 complex and used to stimulate CD8$^+$ pmel cells. *, p <0.05; **, p <0.01, Data represent at least three independent experiments with similar results.

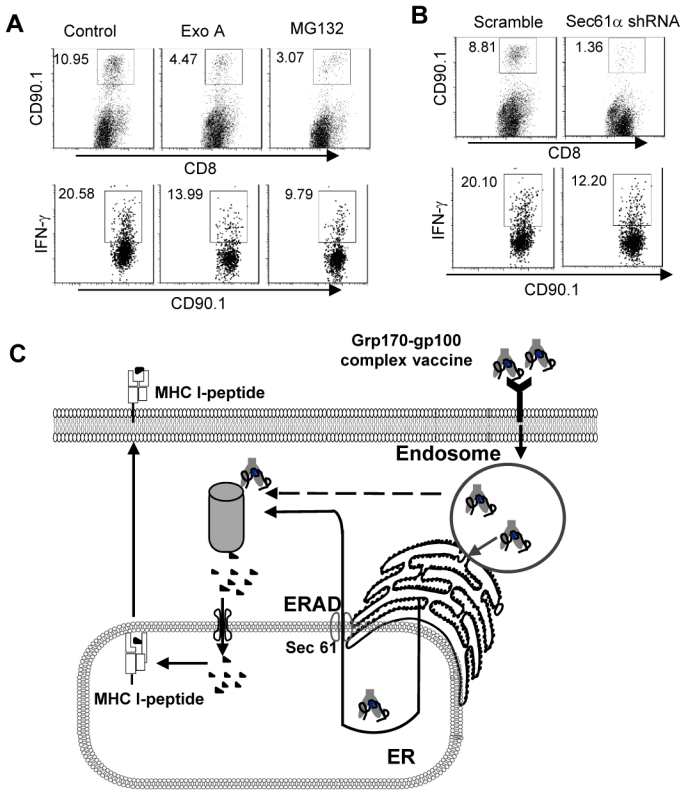

Figure 8. The ERAD pathway is crucial for Grp170-gp100-complex vaccine-induced T-cell cross-priming

A. BMDCs were pretreated with Exo A or MG132, and pulsed with the Grp170-gp100 complex. After washes, the cells were used to immunize C57BL/6 mice that had been adoptively transferred with Gp100-speific, $CD90.1^+CD8^+$ naïve T cells (day 0). Five-days later the expansion and IFN-γ production of $CD90.1^+CD8^+$ T cells in the spleens were examined using FACS analysis (upper) or intracellular cytokine assays (lower), respectively. **B.** Expansion and activation of $CD90.1^+CD8^+$ T cells was analyzed in mice immunized with BMDCs that had been infected with lentiviruses encoding scrambled or Sec61α shRNA and pulsed with the Grp170-gp100 complex. Representative results from three independent experiments with similar results are shown. **C.** The proposed model for the trafficking and processing of recombinant large chaperone-complex vaccines. Grp170-gp100 protein complexes are efficiently captured and internalized by the surface receptors (e.g., scavenger receptors) on specialized APCs (e.g., $CD8^+$ DCs). The chaperone complexes are directed to the ER possibly through dynamic interactions between the ER and endosome organelles (e.g., membrane fusion, recruitment of ER-resident chaperone molecules to endocytic structures). The ERAD machinery involving Sec61 is required to export the complex from the ER lumen to the cytosol for ubiquitination and proteasome-mediated processing. The generated antigenic peptides are transported by TAP and loaded on the MHC class I molecules in the ER.