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Access of soluble antigens to the endoplasmic reticulum can explain cross-presentation by dendritic cells

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In dendritic cells (DCs), peptides derived from internalized particulate substrates are efficiently cross-presented by major histocompatibility complex (MHC) class I molecules. Exogenous soluble antigens are also presented by DCs but with substantially lower efficiency. Here we show that particulate and soluble antigens use different transport pathways. Particulate antigens have been shown to access peripheral endoplasmic reticulum (ER)–like phagosomes that are competent for cross-presentation, whereas we show here that soluble proteins that escape proteolysis enter the lumen of the ER. From there, they may be translocated into the cytosol by the pathway established for ER-associated degradation and their derived peptides may be transported back into the ER for binding by MHC class I molecules. MHC class I presentation involving the constitutive retrograde transport of soluble proteins to the ER by DCs may facilitate DC tolerance to components of their extracellular environment.

Cytotoxic $CD8^+$ T lymphocytes (CTLs) are crucial for immunological control of viral and intracellular pathogen infections. These T cells target infected cells through the recognition of surface major histocompatibility complex (MHC) class I molecules bearing peptide antigens derived from endogenously expressed pathogen proteins. Before becoming competent effector cells, however, CTLs first must be primed against these antigens by dendritic cells (DCs). For pathogens that do not infect DCs[1] or that inhibit DC function[2], DCs acquire exogenous antigens from infected cells and present peptides derived from them in association with MHC class I molecules to initiate adaptive immune responses. This process, called 'cross-presentation', provides internalized proteins access to cytosolic proteasomes and their derived peptides access to the MHC class I processing machinery, an endoplasmic reticulum (ER)–based oligomeric complex containing dimers of MHC class I heavy chain and β_2-microglobulin (β_2M), the transporter associated with antigen processing (TAP), calreticulin, the thiol oxidoreductase ERp57 and tapasin[3].

The description of ER-mediated phagocytosis has suggested a pathway that explains both the intersection of exogenous molecules with the ER and the high efficiency of cross-presentation observed for phagocytosed antigens[4]. In DCs and macrophages, the ER functions as a membrane donor during phagocytosis, creating peripheral phagosomes by contributing membrane[5]. Thus, internalized antigens initially enter a compartment that typically contains ER-based proteins. These include the TAP-associated loading complex and the retrotranslocation machinery, putatively involving the Sec61 translocon[6], which is responsible for moving misfolded proteins from the ER into the cytosol. These early phagosomes are competent to load peptides derived from exogenous proteins onto MHC class I molecules, creating a unique peripheral organelle optimized for cross-presentation[6,7].

The ability to prime CTL responses in mice is restricted to $CD8^+$ DCs[8,9]. Although the human correlate of mouse $CD8^+$ DCs remains unknown, it is likely that DCs are the crucial cross-priming cells in humans[10]. The potency of DCs as antigen-presenting cells, however, means that to prevent the induction of autoimmune responses DCs must distinguish between self and non-self at the level of antigen presentation. This discrimination may derive in part from the ability of DCs to generate better immune responses against exogenous particulate antigens than against soluble antigens[11,12]. Neither internalization of increased antigen amounts nor differential antigen stability during phagocytosis explains this differential response to particular and soluble antigens, as the inclusion of latex beads with soluble antigens increases their cross-presentation efficiency by DCs[13].

Whereas particulate antigens access ER-like phagosomes, soluble exogenous antigens in human DCs and the human dendritic-like cell (DLC) line KG-1 access a macropinocytotic compartment with apparent ER-like characteristics[14]. By immunofluorescence microscopy, soluble antigens appear early after internalization in pinocytotic vesicles containing ER-resident chaperones. Both latex bead–conjugated and soluble monoclonal antibodies (mAbs) to tapasin, an ER-resident component, interact with tapasin and tapasin-associated MHC class I molecules after internalization.

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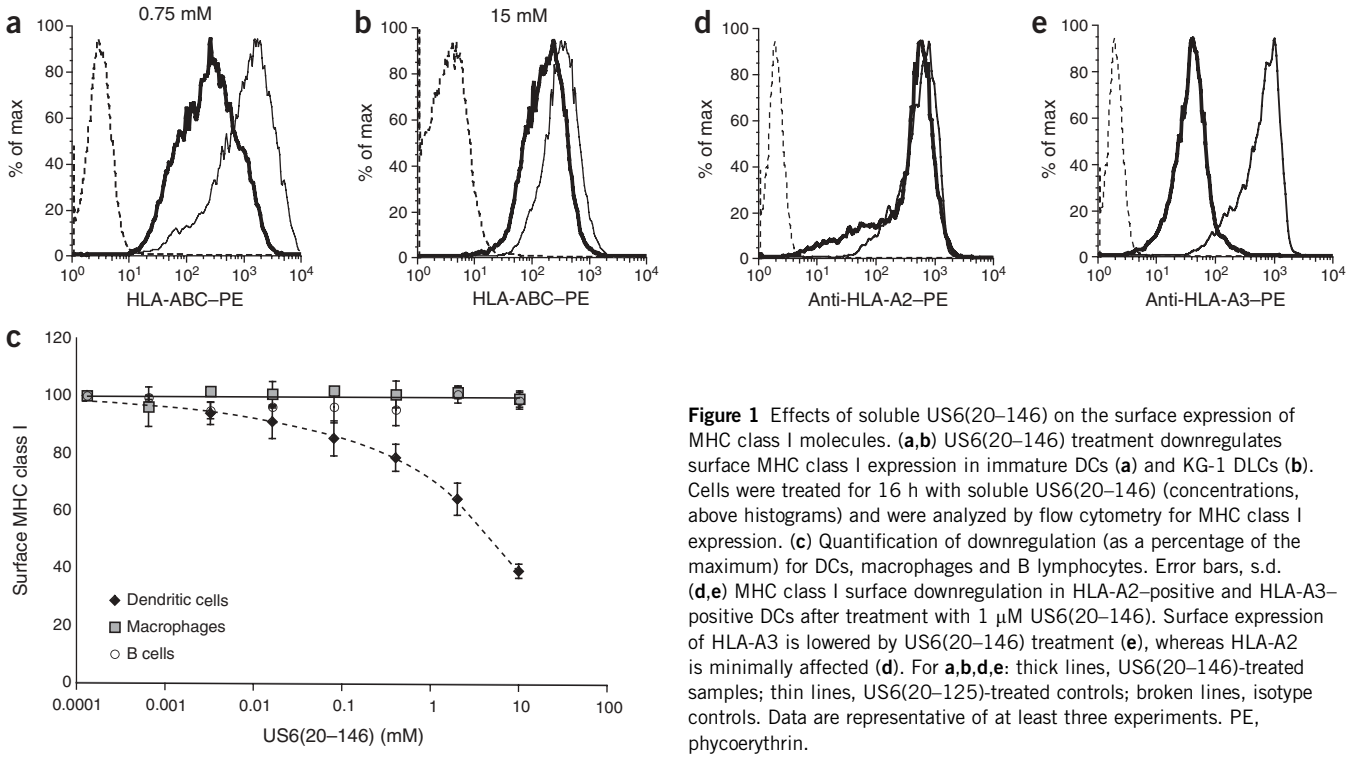

Figure 1 Effects of soluble US6(20–146) on the surface expression of MHC class I molecules. (**a**,**b**) US6(20–146) treatment downregulates surface MHC class I expression in immature DCs (**a**) and KG-1 DLCs (**b**). Cells were treated for 16 h with soluble US6(20–146) (concentrations, above histograms) and were analyzed by flow cytometry for MHC class I expression. (**c**) Quantification of downregulation (as a percentage of the maximum) for DCs, macrophages and B lymphocytes. Error bars, s.d. (**d**,**e**) MHC class I surface downregulation in HLA-A2–positive and HLA-A3–positive DCs after treatment with 1 μM US6(20–146). Surface expression of HLA-A3 is lowered by US6(20–146) treatment (**e**), whereas HLA-A2 is minimally affected (**d**). For **a**,**b**,**d**,**e**: thick lines, US6(20–146)-treated samples; thin lines, US6(20–125)-treated controls; broken lines, isotype controls. Data are representative of at least three experiments. PE, phycoerythrin.

Thus, both macropinosomes and phagosomes may acquire ER membrane during their formation. The published data suggest that, regardless of the method of internalization, exogenous antigens can reach a compartment that has characteristics of the ER.

US6, a transmembrane protein from human cytomegalovirus, blocks TAP-mediated peptide transport by interacting with the lumenal region of the TAP heterodimer[15,16]. Endogenous expression of US6 leads to a reduction in the surface expression of MHC class I molecules because empty class I molecules are retained in the ER[15–17]. A truncated soluble form of US6 containing amino acids 20–146, US6(20–146), retains the ability to inhibit TAP transport, but further truncation of US6 to US6(20–125) produces an inactive protein that also binds TAP[18,19]. Internalization by DCs of soluble exogenous US6(20–146) allows its association with intracellular TAP molecules, downregulates surface MHC class I expression and inhibits cross-presentation[14]. This study, however, did not identify the compartment accessed by soluble US6.

Exogenous US6 might exert its effects in macropinosomes or it might have direct access to the ER, either by transient continuities between the ER and macropinosomes or by a separate retrograde vesicular transport mechanism. If this is generally true for soluble proteins, then cross-presentation of soluble antigens could occur after their delivery to the perinuclear ER, which contains the retrotranslocation machinery, associated proteasomes and the MHC class I peptide-loading complex. To distinguish between these possibilities, here we have investigated the potential access of exogenous molecules to the ER lumen in B cells, macrophages and DCs.

RESULTS

Inhibition of MHC class I peptide loading by US6

We incubated primary human peripheral B cells, monocyte-derived macrophages, immature DCs and KG-1 DLCs for 16 h with soluble active US6(20–146) or inactive US6(20–125). Only exogenous soluble US6(20–146) induced a substantial dose-dependent downregulation of surface MHC class I expression when added to both immature DCs (**Fig. 1a**) and KG-1 DLCs (**Fig. 1b**), as determined by flow cytometry. The magnitude of the downregulation was donor specific, reaching a level of 90% in cells from one donor (**Fig. 1a**) and exceeding 60% at concentrations of US6 higher than 10 μM in another (**Fig. 1c**). This observation may reflect allele-specific differences in TAP dependence. Surface expression of MHC class I molecules on primary macrophages and B cells (**Fig. 1c**) and PeCr2 B lymphoblastoid cells (data not shown) was unaffected, suggesting that only in DCs can exogenous US6(20–146) disrupt surface MHC class I expression.

To show that the effect of exogenous US6(20–146) did not result from altered internalization or degradation of pre-existing surface MHC class I complexes, we biotinylated surface proteins before treating the cells with US6(20–125) or US6(20–146) and incubating them at 37 °C. Immunoblot analysis for surface-biotinylated MHC class I heavy chains isolated from both macrophages and DCs (data not shown) indicated that there was no difference between the degradation rate of surface MHC class I in cells treated with US6(20–146) and that in cells treated with the inactive form. Thus, an increase in endocytosis of surface MHC class I molecules did not explain the observed effects.

Peptide loading of HLA-A2, which has a hydrophobic peptide-binding motif, is relatively independent of TAP-mediated transport because it can bind signal sequence peptides cleaved in the ER from nascent soluble or transmembrane proteins[20]. By contrast, HLA-A3 binds mainly TAP-transported peptides, making its surface expression dependent on TAP activity[20]. We examined the surface expression of HLA-A2 and HLA-A3 in immature DCs derived from a donor bearing both alleles. Whereas HLA-A2 surface expression was minimally affected by treatment with exogenous US6(20–146) (**Fig. 1d**), HLA-A3 was substantially downregulated (**Fig. 1e**). This is consistent with the possibility that HLA-A2 molecules encounter signal sequence

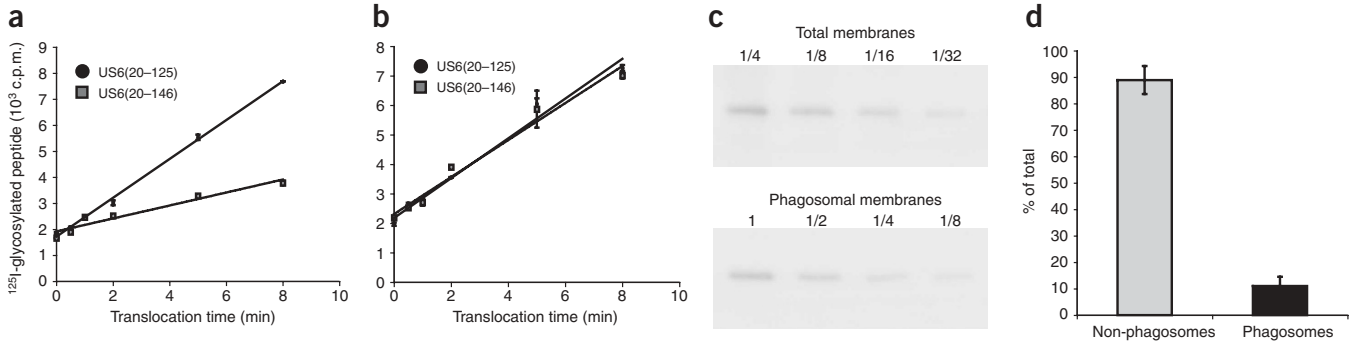

Figure 2 Exogenous soluble US6(20–146) inhibits ER-based translocation by TAP. (**a**,**b**) Translocation is substantially inhibited in human DCs (**a**) but not macrophages (**b**). After 2 h in 100 μM US6(20–125) or US6(20–146), human DCs and macrophages were permeabilized with streptolysin O, and TAP-dependent translocation of a ^{125}I-labeled glycosylation acceptor peptide into the ER was measured. (**c**,**d**) Phagosomes contain roughly 10% of total cellular TAP. (**c**) Solubilized phagosomal (bottom) or total cellular membranes (top) at various relative concentrations (above lanes) were analyzed by immunoblot for TAP. (**d**) Quantification of the band intensities in **c** using a FluorImager and normalization of these values to the dilution factors indicates that phagosomes contain about 10% of the total cellular TAP. Error bars, s.d.

peptides. DC and macrophage phagosomes, although they contain ER membrane and lumenal components, lack membrane-associated ribosomes[5]. If macropinosomes have similar characteristics, then their supply of signal sequence peptides should be minimal. The lack of an effect of exogenous US6(20–146) on HLA-A2 expression therefore suggests that US6(20–146) affects MHC class I surface expression by interfering with ER-based processing.

To confirm that treatment with exogenous US6(20–146) inhibits TAP function after its internalization by DCs, we measured TAP-dependent peptide translocation in human monocyte–derived macrophages and immature DCs after their preincubation for 2 h with US6(20–146) or US6(20–125). We permeabilized the cells with streptolysin O and incubated them with an ^{125}I-labeled glycosylation acceptor peptide and an ATP regenerating system[21]. Subsequent recovery of peptides glycosylated by ER-resident enzymes using concanavalin A–Sepharose provided a measure of TAP-mediated transport into the ER.

Internalization of active but not inactive US6 inhibited TAP-dependent peptide transport in DCs (**Fig. 2a**). Comparison of the rates of translocation indicated that US6(20–146) inhibited total peptide transport by more than 70%. We noted this trend in several experiments. Because the initial rates of peptide transport are proportional to the number of TAP molecules available[22], this suggests that most TAP in DCs treated with US6(20–146) is unable to transport peptides. Abrogation of peptide glycosylation by inclusion of apyrase or the TAP inhibitor ICP47(1–35), a synthetic peptide corresponding to the N-terminal 35 residues of the herpes simplex ICP47 immune evasion protein, in the reaction showed that transport was dependent on both ATP and TAP (data not shown). Treatment of macrophages with active US6(20–146), however, did not inhibit TAP-mediated peptide translocation (**Fig. 2b**). In addition, although we could reproduce the inhibition of TAP-mediated peptide translocation in KG-1 cells, we did not observe inhibition in a B lymphoblastoid cell line (data not shown).

To estimate the relative amounts of TAP in peripheral ER-like compartments, we examined its distribution throughout the cell after saturation of phagocytosis. After incubating immature DCs with more than 200 latex beads per cell, we isolated phagosomal membranes from the total cellular membrane pool. Quantitative immunoblot analysis of these samples indicated that, even in these presumably optimal circumstances, only 10% of cellular TAP was present in peripheral phagosomes (**Fig. 2c**,**d**). Thus, the magnitude of the inhibition of cellular TAP-mediated peptide translocation is consistent with the hypothesis that soluble US6 accesses the ER lumen.

A likely explanation for the lack of sensitivity of macrophages to US6(20–146) is that they are substantially more proteolytic[23]. We therefore examined the degradation of US6 after its internalization by macrophages and immature DCs. We allowed cells to internalize US6(20–146) for 30 min. After extensive washing, we isolated equal cell numbers at intervals throughout a subsequent 6-hour incubation at 37 °C. Immunoblotting indicated that in macrophages US6 became almost undetectable within 1 h of internalization (**Fig. 3**, bottom). Although the initial rate of US6 degradation in DCs was similar to that in macrophages, the fraction remaining after 1 h was stable throughout the 6-hour incubation period (**Fig. 3**, top). Thus, internalized US6 is rapidly degraded in macrophages, whereas a fraction of US6 is protected from proteolysis in DCs.

Exogenous β_2M can access the ER in DCs

The above data suggested that soluble US6 must access the ER lumen to alter MHC class I expression. Attempts to show this directly, by immunofluorescence microscopy for example, were unsuccessful, as were attempts to demonstrate the accumulation of other

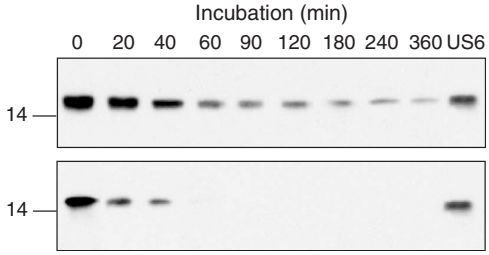

Figure 3 DCs but not macrophages accumulate US6 after its internalization. DCs (top) and macrophages (bottom) were allowed to internalize US6(20–46), then washed and incubated for various durations (above lanes). Cell-associated US6(20–46) was measured by SDS-PAGE of cell extracts followed by immunoblotting. Far right lane, purified US6(20–46), used as a positive control. Left margin, mass (in kDa) of one of the standard proteins used. Data are representative of three independent experiments.

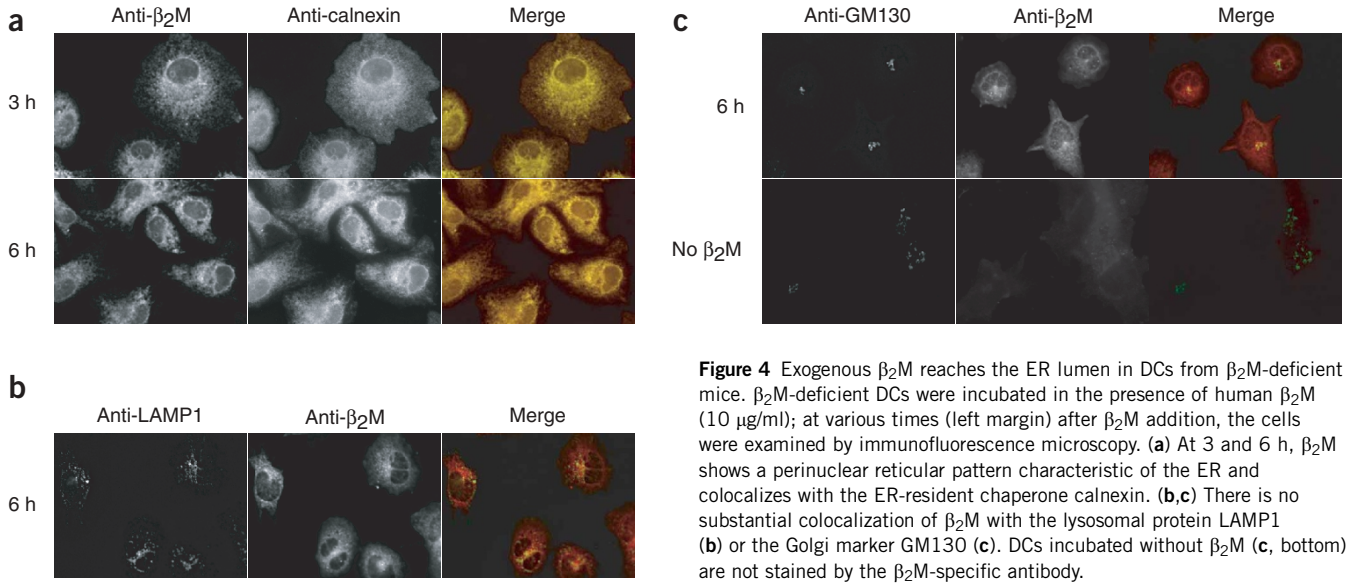

Figure 4 Exogenous β_2M reaches the ER lumen in DCs from β_2M-deficient mice. β_2M-deficient DCs were incubated in the presence of human β_2M (10 μg/ml); at various times (left margin) after β_2M addition, the cells were examined by immunofluorescence microscopy. (**a**) At 3 and 6 h, β_2M shows a perinuclear reticular pattern characteristic of the ER and colocalizes with the ER-resident chaperone calnexin. (**b**,**c**) There is no substantial colocalization of β_2M with the lysosomal protein LAMP1 (**b**) or the Golgi marker GM130 (**c**). DCs incubated without β_2M (**c**, bottom) are not stained by the β_2M-specific antibody.

fluid-phase markers in the ER. A possible explanation is that exogenous proteins do not accumulate in sufficient concentrations, either because of degradation, secretion or retrotranslocation into the cytosol or simply because dilution in the ER makes detection difficult. We therefore chose to examine β_2M, a protease-resistant protein that might accumulate, at least transiently, in the ER. To enhance the likelihood of such an accumulation, we used bone marrow–derived DCs from β_2M-deficient mice and incubated them with human β_2M for 3 or 6 h before examining them by immunofluorescence microscopy. Internalized β_2M showed an ER-like reticular pattern with strong perinuclear staining and colocalized with calnexin, an ER-resident chaperone (**Fig. 4a**). It did not significantly colocalize with either LAMP-1 or GM130 (**Fig. 4b,c**), which identify lysosomal or Golgi structures, respectively. These results indicate that in DCs, exogenous β_2M is transported back to the ER lumen.

MHC class I molecules do not fold correctly in the absence of β_2M and are retained in the ER for destruction by ER-associated degradation. As a result, MHC class I molecules are absent from the surface of β_2M-deficient cells. If internalized β_2M could access the ER lumen, however, it might 'rescue' the transport of MHC class I in β_2M-deficient DCs. We examined the surface expression of H-2K^b on bone

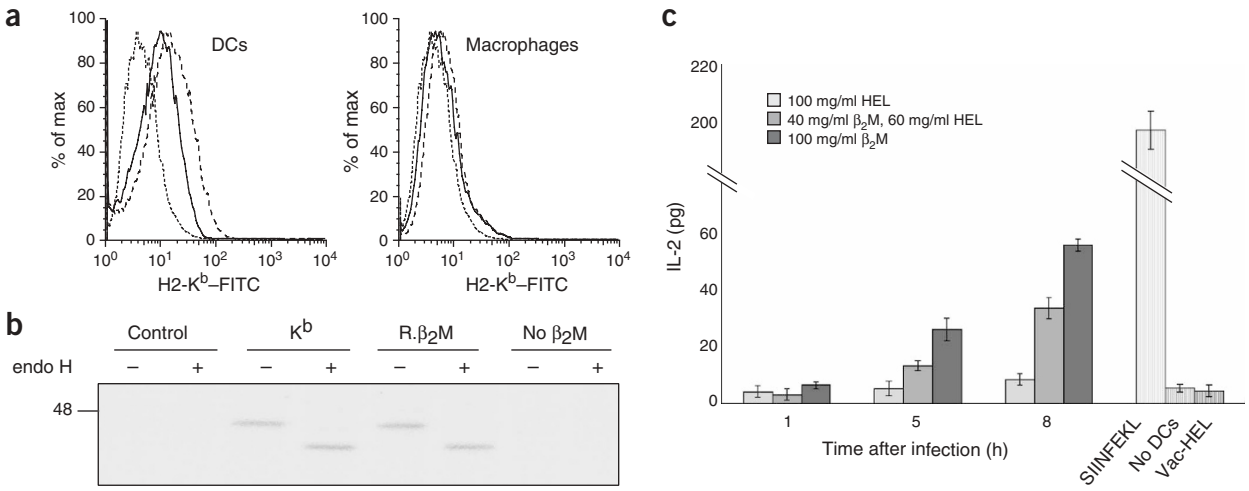

Figure 5 Exogenous human β_2M interacts functionally with MHC class I heavy chains in the ER of mouse DCs. (**a**) β_2M-deficient DCs (left) and macrophages (right) were incubated overnight with human β_2M (dashed lines, 25 μg/ml; solid lines, 1.5 μg/ml) or in its absence (dotted lines). Surface H-2K^b expression was assessed by flow cytometry. FITC, fluorescein isothiocyanate.(**b**) β_2M-deficient DCs were incubated for 1 h with human β_2M (25 μg/ml), labeled for 30 min with [^{35}S]methionine and lysed in detergent. Immunoprecipitation with human β_2M antiserum (lanes 5 and 6) or a conformation-dependent H-2K^b mAb (lanes 3 and 4) identified ER-resident heavy chains sensitive to endoglycosidase H (endo H). There is no signal after incubation without β_2M (lanes 7 and 8) or after immunoprecipitation with a nonspecific antiserum (Control; lanes 1 and 2). Left margin, mass (in kDa) of one of the standard proteins used. (**c**) Incubation of β_2M-deficient DCs with exogenous human β_2M restores their ability to present endogenous OVA to an H-2K^b-restricted T cell hybridoma. DCs were incubated with β_2M, HEL or both, and then were infected with Vac-OVA, a control Vac-HEL virus or the specific OVA peptide SIINFEKL for various times (horizontal axis) before fixation. Release of IL-2 by the B3Z hybridoma was measured by enzyme-linked immunosorbent assay. Data are representative of three independent experiments. Error bars, s.d.

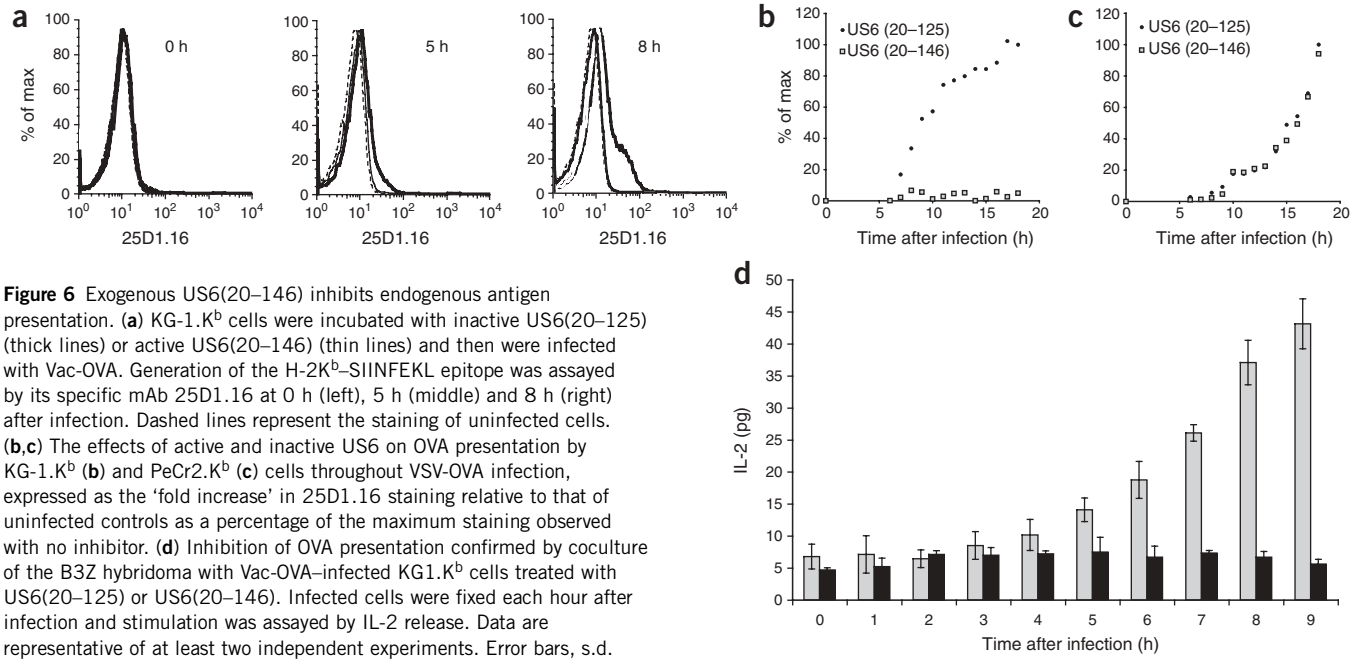

Figure 6 Exogenous US6(20–146) inhibits endogenous antigen presentation. (**a**) KG-1.K^b cells were incubated with inactive US6(20–125) (thick lines) or active US6(20–146) (thin lines) and then were infected with Vac-OVA. Generation of the H-2K^b–SIINFEKL epitope was assayed by its specific mAb 25D1.16 at 0 h (left), 5 h (middle) and 8 h (right) after infection. Dashed lines represent the staining of uninfected cells. (**b**,**c**) The effects of active and inactive US6 on OVA presentation by KG-1.K^b (**b**) and PeCr2.K^b (**c**) cells throughout VSV-OVA infection, expressed as the 'fold increase' in 25D1.16 staining relative to that of uninfected controls as a percentage of the maximum staining observed with no inhibitor. (**d**) Inhibition of OVA presentation confirmed by coculture of the B3Z hybridoma with Vac-OVA–infected KG1.K^b cells treated with US6(20–125) or US6(20–146). Infected cells were fixed each hour after infection and stimulation was assayed by IL-2 release. Data are representative of at least two independent experiments. Error bars, s.d.

marrow–derived DCs and macrophages from β_2M-deficient mice by flow cytometry after incubating the cells for 16 h with recombinant human β_2M. We noted a slight increase in surface MHC class I in macrophages, but the amount of H-2K^b on the surface of β_2M-deficient DCs increased substantially (**Fig. 5a**). The identity of the cell populations was confirmed by the differential expression of I-A^b, CD11b and CD11c[24]. These results suggest that exogenous β_2M may reverse the ER retention and degradation of MHC class I heavy chains in β_2M-deficient DCs by allowing proper assembly of MHC class I–β_2M dimers in the ER lumen.

In β_2M-deficient cells, the asparagine-linked glycans of ER-retained MHC class I heavy chains remain sensitive to digestion by endoglycosidase H. To confirm that exogenous β_2M interacts with MHC class I heavy chains in the ER, we incubated DCs and macrophages with β_2M for 1 h in the absence of methionine and cysteine, and then labeled the cells for 30 min with [^{35}S]methionine. We isolated β_2M-associated H-2K^b molecules by immunoprecipitation, digested them with endoglycosidase H, and separated them by SDS-PAGE (**Fig. 5b**). The glycans of the isolated MHC class I molecules were sensitive to endoglycosidase H, indicating that the complexes had yet to move out of the ER. These results confirm that exogenous β_2M restores MHC class I surface expression in β_2M-deficient DCs by pairing with newly synthesized MHC class I heavy chains in the ER and facilitating their transport to the cell surface.

If intact exogenous β_2M can form MHC class I heterodimers in the ER lumen, then it should restore the ability of β_2M-deficient DCs to present MHC class I–restricted cytosolic antigens to T cells. To test this prediction, we infected β_2M-deficient DCs with vaccinia virus expressing ovalbumin (Vac-OVA) after a 1-hour preincubation with exogenous human β_2M. We used a vaccinia virus expressing hen egg lysozyme (Vac-HEL) as a control virus and HEL as a control protein. We incubated fixed infected cells with the H-2K^b-restricted T cell hybridoma B3Z, which is specific for the ovalbumin peptide SIINFEKL (OVA(257–264)). Assessment of presentation by B3Z stimulation (**Fig. 5c**) showed that exogenous β_2M restored the ability of β_2M-deficient DCs to present endogenous OVA in a dose-dependent way. These data indicate that intact exogenous β_2M and, by analogy, US6, can enter the lumen of the ER.

Inhibition of endogenous presentation by US6

Conventional MHC class I presentation depends on the translocation of peptides into the ER by TAP. If soluble active US6 readily accesses the ER lumen, in addition to blocking the cross-presentation of a soluble antigen[14] it should also block the presentation of a conventional cytoplasmic antigen. Because US6 does not affect the translocation of peptides by mouse TAP, we examined the effect of soluble US6 on antigen presentation in the human DLC KG-1 and the human B lymphoblastoid cell line PeCr2, each expressing transfected H-2K^b. We infected cells with either vesicular stomatitis virus encoding recombinant OVA (VSV-OVA) or Vac-OVA after a 1-hour preincubation with recombinant US6.

As measured by binding of the mAb 25D1.16, which recognizes complexes of SIINFEKL and H-2K^b, addition of US6(20–146) inhibited the presentation of endogenous OVA by KG-1.K^b cells, whereas inactive US6(20–125) had no effect (**Fig. 6a**,**b**). Presentation by PeCr2.K^b cells infected with VSV-OVA was unaffected by the addition of either active or inactive US6 (**Fig. 6c**). Soluble US6 had no effect on cell viability, presentation of exogenous SIINFEKL peptide, or the surface abundance of H-2K^b, HLA-DR or the transferrin receptor (data not shown). We confirmed these results with Vac-OVA and the B3Z hybridoma, which recognizes the same peptide–MHC class I complex (**Fig. 6d**). OVA expressed by viral infection requires cytoplasmic processing and the subsequent transport of antigenic peptides into the ER lumen by TAP for loading onto MHC class I molecules. Thus, inhibition of endogenous OVA presentation in KG-1.K^b DLCs confirms that exogenous US6(20–146) reaches the lumen of the ER and interferes with TAP-mediated peptide translocation and MHC class I peptide loading in this compartment.

DISCUSSION

The discovery of the ER as a membrane donor in phagosome formation identified a functional link between the ER and the

phagocytic pathway and suggested a mechanism that enables exogenous antigens to access the ER-based antigen-processing machinery directly[6,7]. Here we have provided substantial evidence to show that soluble proteins can gain access to the lumen of the perinuclear ER after their internalization by DCs. Exogenous β_2M can 'rescue' both MHC class I surface expression and endogenous presentation in β_2M-deficient cells by interacting with free heavy chains in the ER. The soluble TAP inhibitor US6(20–146) can substantially reduce MHC class I surface expression, ER-based TAP peptide translocation and the ability of cells to present endogenously expressed antigens. We infer from these data that soluble protein antigens can access the retrotranslocation machinery of DCs in the ER and that peptides derived from these proteins can access the MHC class I antigen-processing machinery directly in the ER, facilitating their cross-presentation to $CD8^+$ T cells.

Macrophages are similar to DCs, being derived from a common hematopoietic precursor. They express ample MHC class I and II molecules, have a high endocytic capacity and form ER-like phagosomes that are competent for cross-presentation[5,6]. None of the approaches used in the study reported here, however, that exogenous proteins can access the ER of macrophages. The most likely explanation for these cellular differences is the disparate proteolytic capacity of macrophages and DCs[23]. Whereas internalized US6(20–146) is rapidly degraded in macrophages, a fraction is protected from proteolysis in DCs and probably escapes this process by sequestration in the ER. Lymphocytes and other cells that are not exceptionally active in endocytosis may not allow soluble proteins to access the ER, precluding efficient cross-presentation. The high efficiency of cross-presentation in DCs compared with that in macrophages, however, is likely to result from the ability of DCs to protect exogenous antigens from destruction by lysosomal proteases.

The cellular transport pathway that facilitates the trafficking of protein back to the ER remains unclear. Visualization of macrophages by electron microscopy after treatment with bafilomycin, an inhibitor of the vacuolar H^+ ATPase, identified physical continuities between the lumen of the ER and nascent phagosomes[5]. If these intermediate structures represent the mechanism by which exogenous proteins reach the ER, then the ability of soluble proteins to access the ER lumen is consistent with our previous suggestion that macropinocytosis may also use the ER as a membrane donor[14].

An alternative mechanism is retrograde vesicular trafficking, which has been observed for both soluble proteins and whole pathogens. SV40 viral particles can be transported to the ER via caveolin-containing structures called 'caveosomes'[25]. A subset of proteins, mainly plant and bacterial toxins such as ricin, cholera and shiga toxins, gain access to the ER by retrograde transport through the Golgi. Each of these proteins, however, uses a different mechanism to achieve this transport[26]. The mechanism of transport back to the ER may depend on the nature of the substrate, but it seems equally likely that DCs have a specialized pathway that provides retrograde transport for all soluble exogenous proteins or fragments derived from them. Although further studies will be necessary to elucidate the mechanism, the availability of this retrograde transport pathway clearly correlates with the capacity of DCs to cross-present exogenous soluble antigens.

It is essential for an organism to maintain tolerance to self proteins present in the extracellular environment to which immature DCs are exposed in the periphery. An ER-based cross-presentation pathway available to soluble exogenous proteins may be unable to generate sufficiently high numbers of specific peptide–MHC class I complexes to prime naive CTLs in the absence of inflammation. Although newly synthesized proteins are the main source of peptides presented by MHC class I molecules[27,28], ER-based cross-presentation may allow low constitutive presentation, leading to peripheral tolerance. During infection, the crucial antigens for which cross-presentation is required to generate a $CD8^+$ T cell response are likely to be contained in phagocytic substrates; that is, either whole pathogens or fragments of infected cells. In these situations, phagosomes may efficiently generate peptide–MHC class I complexes while stimulation via Toll-like receptors induces DC maturation[29] and subsequent $CD8^+$ T cell activation. Internalization into the ER and low-efficiency constitutive presentation of exogenous self antigens may thus provide a safeguard against the induction of autoimmunity. The separation by DCs of internalized antigens into two different pathways, based on the mechanism of internalization, may facilitate the proper balance of immune tolerance and activation.

METHODS

Cells, viruses and peptides. Immature mouse DCs and macrophages were cultured from the bone marrow of β_2M-deficient male mice 6–8 weeks of age[30] (a gift from L. van Kaer, Vanderbilt University, Nashville, Tennessee) as described[31]. Experiments involving mice were approved by the Yale Institutional Animal Care and Use Committee (New Haven, Connecticut). The KG-1, KG-1.K^b, PeCr2 and PeCr2.K^b cells have been described[14,32]. Recombinant Vac-OVA, Vac-HEL and VSV-OVA were gifts from J. Yewdell (National Institutes of Health, Bethesda, Maryland) and L. Lefrancois (University of Connecticut Health Center, Farmington, Connecticut). We prepared soluble recombinant US6(20–146) and US6(20–125) as described[18].

Antibodies. Phycoerythrin-conjugated anti–human CD14, anti–human B220, anti–human CD83, anti-HLA-DR and anti–I-A^b, fluorescein isothiocyanate–conjugated anti–H-$2K^b$, anti-GM130, anti-LAMP1 and anti–HLA-ABC, biotinylated anti–mouse CD11b, CyChrome-conjugated streptavidin and allophycocyanin-conjugated anti–mouse CD11c were obtained from PharMingen. R.US6.9218 was affinity purified from a rabbit antiserum[18]. We obtained a rabbit antiserum to β_2M (R.β_2M) from Boehringer Ingleheim. The rabbit antiserum specific for calnexin (R.CNX)[33] and the mAbs specific for human β_2M (BBM.1)[34], TAP1 (148.3)[35], HLA-A3 (GAP.A3)[36] and HLA-A2 (BB7.2)[37] have been described. Alexa Fluor 647–conjugated 25D1.16 (ref. 38) was a gift from J. Yewdell. Y3 is a mouse mAb specific for β_2M-associated H-$2K^b$ molecules[39]. Rat mAb 3B10.7 has been described[40].

US6-mediated MHC class I downregulation. We incubated primary DCs, macrophages and B cells, and the KG-1 and PeCr2 cells lines for 16 h with the indicated concentrations (**Fig. 1**) of US6(20–146) or US6(20–125) in RPMI 1640 medium supplemented with 20% FCS. After the cells were washed, MHC class I surface downregulation was assessed by flow cytometry as described[14].

Radiolabeling and immunoprecipitation. For examination of the association of exogenous human β_2M (PharMingen) with H-$2K^b$ and H-$2D^b$, bone marrow–derived β_2M-deficient DCs were deprived of methionine and cysteine in the presence of 25 μg/ml of human β_2M for 1 h. Cells were labeled for 30 min with [^{35}S]methionine/cysteine (0.25 mCi per 1×10^6 cells; ICN) in the presence of exogenous human β_2M (25 μg/ml). Cells were extracted in Tris-buffered saline, pH 7.4, containing 1% Triton X-100 (Sigma), as described[41]. Postnuclear supernatants were subjected to immunoprecipitation with anti-H-$2K^b$ (Y3) or rabbit anti-β_2M serum, followed by digestion with endoglycosidase H[41] and SDS-PAGE.

Analysis of US6 stability in DCs and macrophages. Primary human immature DCs and macrophages were incubated for 30 min with 10 μM US6(20–146). After extensive washing, cells were cultured for an additional 6 h. At various times, equal cell numbers were removed and were extracted in 1% Triton X-100. Postnuclear supernatants were then subjected to SDS-PAGE and immunoblotting for US6 with R.US6.9218. Gel band intensities were quantified on a FluorImager (Molecular Dynamics).

Infection with recombinant OVA-expressing viruses. Bone marrow–derived immature DCs were preincubated for 1 h at 37 °C with human β_2M (10 μg/ml)

for DCs from β_2M-deficient mice or in 100 μM US6(20–146) or US6(20–125) for KG1.K^b cells. Cells were then infected with VSV-OVA, Vac-OVA or Vac-HEL as described[42,43] at a multiplicity of infection of 10 for 1 h in the continued presence of β_2M (DCs) or US6 (KG1.K^b cells). KG-1.K^b cells were cultured for an additional 7 h after both the virus and inhibitor were removed. For the DCs, β_2M was present throughout the 8-hour time course. We isolated and fixed cells for analysis both before infection and at the indicated time points (**Figs. 5c** and **6**) throughout infection. The formation of H-2K^b–SIINFEKL complexes on the surface of infected cells from was detected by flow cytometry with Alexa Fluor 647–conjugated 25D1.16 or the B3Z T cell hybridoma as described[44].

Upregulation of surface MHC class I in β_2M-deficient cells. Bone marrow–derived immature DCs and macrophages from β_2M-deficient mice were incubated overnight at 37 °C with varying concentrations of exogenous recombinant human β_2M. Cells were then stained as described[44] for H-2K^b by Y3 and were examined by flow cytometry. Costaining for I-A^b, CD11b, and CD11c confirmed macrophage and DC populations.

Immunofluorescence microscopy. Bone marrow–derived immature DCs derived from β_2M-deficient mice were incubated for up to 6 h in recombinant human β_2M (10 μg/ml). Cells were fixed in 3.7% formaldehyde at various times throughout this process and then were stained with a combination of fluorescein isothiocyanate–conjugated anti–human β_2M and R.CNX or fluorescein isothiocyanate–conjugated anti-GM130 or anti-LAMP1 and R.β_2M. Rabbit antibodies were visualized with Alexa Fluor 594–conjugated goat anti-rabbit IgG (Molecular Probes). Cells were visualized by an Axiophot 2 fluorescence microscope (Zeiss). Digital images were acquired with a CCD camera (Princeton Instruments).

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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