

Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation

Sven Burgdorf¹, Christian Schölz², Andreas Kautz¹, Robert Tampé² & Christian Kurts¹

Antiviral or antitumor immunity requires activation of cytotoxic CD8⁺ T cells by dendritic cells, which present viral or tumor antigens on major histocompatibility complex (MHC) class I molecules. The intracellular mechanisms facilitating MHC class I-restricted presentation of extracellular antigens ('cross-presentation') are unclear. Here we demonstrate that cross-presentation of soluble antigen occurred in an early endosomal compartment distinct from the endoplasmic reticulum where endogenous antigen is loaded onto MHC class I. Efficient cross-presentation required endotoxin-induced, Toll-like receptor 4- and signaling molecule MyD88-dependent relocation of the transporter associated with antigen processing, essential for loading of MHC class I, to early endosomes. Transport of cross-presented antigen from endosomes to the cell surface was inhibited by primaquine, which blocks endosomal trafficking. Thus, cross-presentation is spatially and mechanistically separated from endogenous MHC class I-restricted antigen presentation and is biased toward antigens containing microbial molecular patterns.

The mechanism of cross-presentation allows 'professional' antigen-presenting cells (APCs), in particular dendritic cells (DCs)^{1,2}, to induce protective immunity against intracellular microbes that do not infect DCs or against tumors that do not originate from DCs^{3,4}. For this, DCs take up extracellular antigens, process them intracellularly into antigenic peptides and load them onto major histocompatibility (MHC) class I molecules. These complexes are transported to the APC surface to be presented to antigen-specific CD8⁺ T lymphocytes (CTLs), which causes T cell activation, proliferation and acquisition of cytotoxic function against infected or malignant target cells^{3,4}. Although cross-presentation is biased toward 'high-dose' antigens, cell-associated antigens and antigens released during cellular destruction, autoantigens and soluble antigens are also constitutively cross-presented, albeit with lower efficiency⁵⁻⁷. Cross-presentation is enhanced by microbial molecular patterns, in particular by Toll-like receptor (TLR) ligands⁸⁻¹⁰, whose effects include induction of DC maturation, upregulation of costimulatory molecule expression and augmented antigen uptake and processing¹¹⁻¹⁶. The cell-biological mechanisms underlying cross-presentation and the stimulatory effect of TLR ligands on it are not fully resolved.

Once activated, CTLs survey the cells of the body for the presence of tumor or microbial antigens in complex with MHC class I molecules to destroy malignant or infected target cells. Such MHC class I-restricted presentation of antigens synthesized in a cell (endogenous antigens) occurs constitutively in all nucleated cells of the body, in contrast to cross-presentation, which is mostly restricted to 'professional' APCs. The intracellular mechanisms of the endogenous MHC

class I presentation pathway are better understood than are those of cross-presentation. Endogenous antigens are degraded in the cytoplasm by the proteasome, and the resulting peptides are transported by the transporter associated with antigen processing (TAP), a heterodimer of TAP1 (A002608) and TAP2 (A002609) subunits, into the endoplasmic reticulum, where they are loaded onto MHC class I molecules¹⁷. MHC class I-peptide complexes are then transported through the Golgi apparatus via the secretory pathway to the cell surface for presentation to CTLs.

It has been proposed that APCs might also use mechanisms of the endogenous MHC class I-restricted presentation pathway for cross-presentation¹⁸. In support of that idea, cross-presentation of soluble antigens¹⁹⁻²⁶ and particulate antigens^{21,27-29} has required the cytosolic proteasome and TAP in most studies. Phagosomes contain endoplasmic reticulum-associated molecules and components of the MHC class I loading 'machinery' such as TAP, which suggests that antigenic peptides generated by the proteasome might be reimported by TAP directly into the phagosomes for MHC class I loading there^{21,28,29}. The mechanisms by which components of the endoplasmic reticulum reach the phagosome remain controversial³⁰. Furthermore, such studies have been unable to distinguish the functional requirements of TAP in the endoplasmic reticulum versus phagosomal TAP and thus cannot formally distinguish between cross-presentation in the endoplasmic reticulum and that in phagosomes. Also, for cross-presentation of soluble antigen, direct evidence is lacking for peptide loading in the endoplasmic reticulum. Thus, the elusive compartment for cross-presentation remains to be identified.

¹Institute of Molecular Medicine and Experimental Immunology, Friedrich-Wilhelms University, 53105 Bonn, Germany. ²Institute of Biochemistry, Biocenter, Johann Wolfgang Goethe University, 60438 Frankfurt am Main, Germany. Correspondence should be addressed to C.K. (ckurts@web.de) or S.B. (sven.burgdorf@ukb.uni-bonn.de).

Received 15 January; accepted 29 February; published online 30 March 2008; doi:10.1038/ni.1601

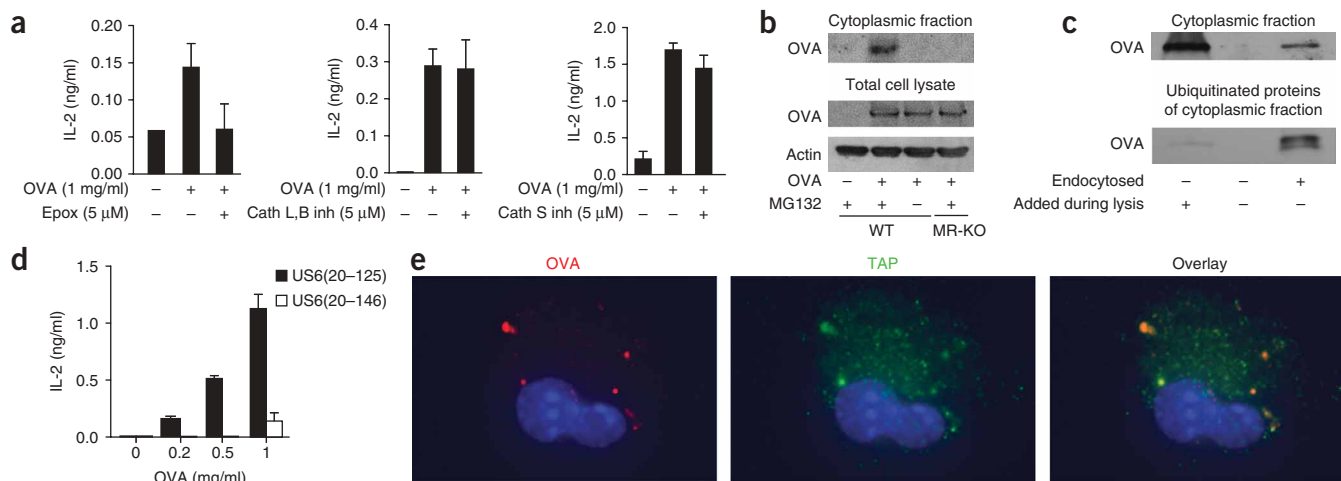


Figure 1 TAP is essential for cross-presentation and is present in OVA-containing endosomes. **(a)** ELISA of IL-2 in supernatants of OT-I cells cultured with DCs treated with OVA and the proteasome inhibitor epoxomicin (Epox; left) or inhibitors of cathepsins L and B (Cath L,B inh; middle) or cathepsin S (Cath S inh; right). **(b)** Immunoblot analysis of the OVA content of total cell lysates or the cytoplasmic fraction of wild-type (WT) and mannose receptor-deficient (MR-KO) DCs incubated with OVA and the proteasome inhibitor MG132. Actin, loading control. **(c)** Immunoblot analysis of the ubiquitination status of cytoplasmic OVA in DCs transfected with mRNA encoding polyhistidine-tagged ubiquitin and then cultured with OVA; ubiquitinated proteins in the cytosolic fraction were enriched with nickel-nitrilotriacetic acid agarose. Added during lysis (bottom), OVA externally added during DC lysis, not endocytosed (negative control). **(d)** ELISA of IL-2 in supernatants of OT-I cells incubated with DCs treated with OVA and US6(20–146). Inactive US6(20–125) serves as control for TAP-independent effects. **(e)** Immunofluorescence microscopy of DCs after endocytosis of fluorochrome-labeled OVA (red), stained for TAP (green) and with DAPI (blue). Original magnification, $\times 600$. Data are representative of three independent experiments (error bars, mean + s.e.m. of triplicates).

RESULTS

TAP is present in ovalbumin-containing endosomes

Distinct antigen uptake mechanisms have been shown to dictate whether endocytosed antigen is cross-presented to CTLs or is presented on MHC class II molecules to CD4⁺ T helper cells^{26,31}. DCs exclusively use the mannose receptor (A003686) to take up the widely used model antigen soluble ovalbumin (OVA) into distinct early endosomal compartments specialized for cross-presentation^{26,31}. We found that the resulting activation of OVA-specific CTLs (OT-I cells) could be blocked by the proteasome inhibitors MG132 (ref. 31) and epoxomicin (**Fig. 1a**), consistent with studies reporting a requirement for the proteasome for cross-presentation^{22,25,26,28,29}. In contrast to studies describing endosomal antigen degradation by cathepsins for cross-presentation³², OT-I cell activation in our system was unaltered by inhibitors of those enzymes (**Fig. 1a**), which suggested that soluble antigen must leave early endosomes to reach the proteasome, as proposed before^{22,33,34}. This indeed was the case, as we detected the presence of OVA endocytosed by the mannose receptor (called 'mannose receptor-endocytosed OVA' here) in the cytosol when the proteasome was prevented from instantly degrading it after export from early endosomes (**Fig. 1b**). Furthermore, such OVA was ubiquitinated, which demonstrated its susceptibility to proteasomal degradation (**Fig. 1c**).

Cross-presentation of OVA has been reported to be TAP dependent^{19,20,24,25,27}. Also in our system, cross-presentation of mannose receptor-endocytosed OVA required TAP, as DCs from TAP-deficient mice were unable to activate OT-I cells (**Supplementary Fig. 1** online). However, it has been shown that TAP-deficient cells express small amounts of surface MHC class I (ref. 35), which also might explain the inability of such cells to activate CTLs. Hence, we used the recombinant protein US6, a low-molecular-weight luminal TAP inhibitor of the cytomegalovirus³⁶. Consistent with published studies^{21,23}, we noted that a fragment of recombinant US6 (amino acids 20–146; US6(20–146)) comprising the TAP-inhibiting domain

completely abolished cross-presentation of mannose receptor-endocytosed OVA by TAP-competent DCs, in contrast to results obtained with a further truncated, inactive fragment of US6 (amino acids 20–125 (US6(20–125))); **Fig. 1d**). This inhibition of cross-presentation was not due to small amounts of MHC class I, because DCs in our system were fixed after 2 h of culture with OVA and expression of MHC class I was not visibly altered by US6 in this short time frame (**Supplementary Fig. 2** online).

As it has been shown that OVA-transporting endosomes contain some endoplasmic reticulum components such as calnexin^{21,24}, we investigated whether endosomes containing mannose receptor-endocytosed OVA also contained TAP, as has been reported for phagosomes carrying cell-associated antigen for cross-presentation^{21,28,29}. Indeed, immunofluorescence staining for TAP showed distinct localization of TAP together with endocytosed OVA (**Fig. 1e**; staining control with TAP-deficient DCs, **Supplementary Fig. 3** online). In addition, we noted perinuclear TAP staining that localized together with calnexin, an endoplasmic reticulum marker often used (data not shown). These findings suggested that not only the endoplasmic reticulum but also early endosomes containing antigen destined for cross-presentation contained TAP, which raised the possibility that proteasome-generated peptides might be imported into early endosomes and loaded there on MHC class I molecules.

TAP reimports antigenic peptides into early endosomes

To investigate whether endosomal TAP was involved in the cross-presentation of OVA, we needed an approach to selectively deliver the inhibitor US6 into early endosomes. We achieved this by using transferrin as a 'Trojan horse'. When this molecule has bound iron, it is internalized into cells by binding to the cell surface-expressed transferrin receptor, after which transferrin plus the receptor enter recycling endosomes; in endosomes, receptor and cargo separate and then the receptor recycles back to the cell surface³⁷. Notably, in DCs,

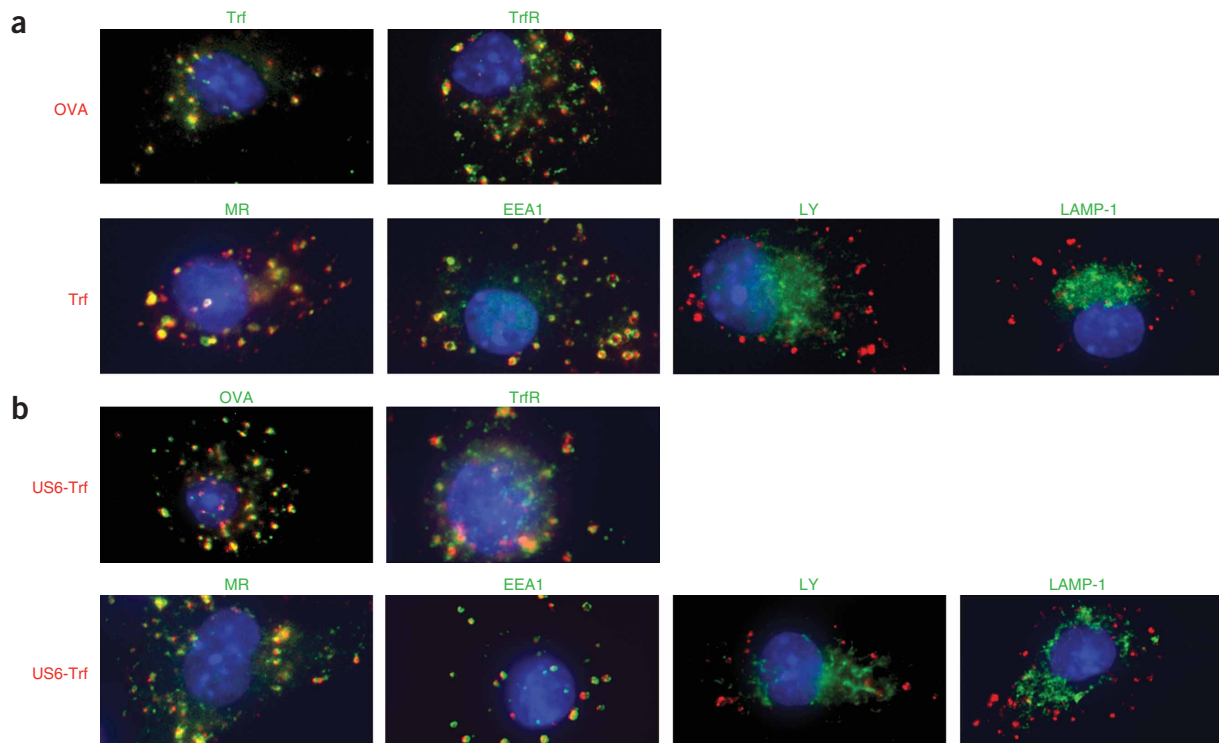


Figure 2 US6 linked to Trf is specifically targeted into early endosomes. Immunofluorescence microscopy of DCs cultured with fluorochrome-labeled OVA and Trf (red; **a**) or US6-Trf (red; **b**) and counterstained with antibody to transferrin receptor (TrfR), mannose receptor (MR), EEA1, LAMP-1 or pinocytosed Lucifer yellow (LY; all green); nuclei are stained with DAPI (blue). Original magnification, $\times 600$. Data are representative of two independent experiments.

transferrin and its receptor localized together strictly with mannose receptor–endocytosed OVA and the early endosomal marker EEA1 (Fig. 2a; separate channel images, **Supplementary Fig. 4** online), which indicated that transferrin plus the receptor trafficked through the early endosomes dedicated to cross-presentation. Transferrin plus the receptor localized together with neither Lucifer yellow, a substance taken up by fluid-phase pinocytosis and rapidly targeted to lysosomal compartments²⁶, nor the lysosomal marker LAMP-1, which demonstrated its exclusion from the late endosomal–lysosomal MHC class II loading compartment. We reasoned that this intracellular routing

pattern of transferrin plus receptor might allow the selective introduction of US6 coupled to transferrin into early endosomes.

Thus, we chemically linked US6 to transferrin, producing complexes (Trf-US6) with a molecular size above 250 kilodaltons (**Supplementary Fig. 5** online). Trf-US6 was targeted as intended, as shown by its localization together with the transferrin receptor, the mannose receptor, mannose receptor–endocytosed OVA and EEA1 but not with Lucifer yellow or LAMP-1 (Fig. 2b; separate channel images, **Supplementary Fig. 4**; kinetic analysis of intracellular Trf-US6 routing, **Supplementary Fig. 6** online). We confirmed the absence of routing

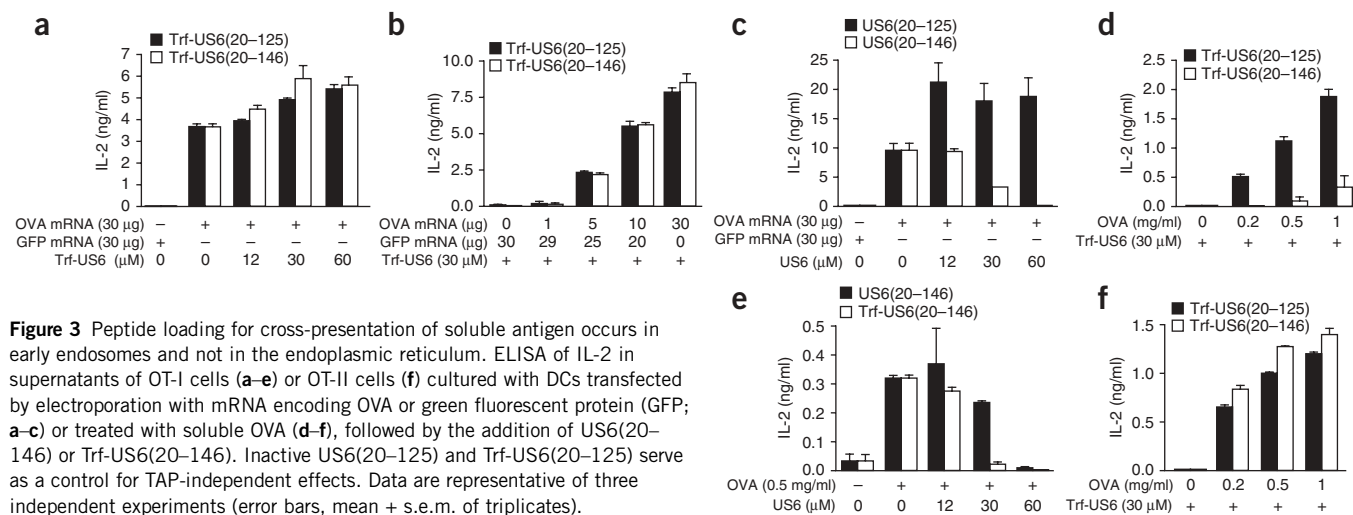


Figure 3 Peptide loading for cross-presentation of soluble antigen occurs in early endosomes and not in the endoplasmic reticulum. ELISA of IL-2 in supernatants of OT-I cells (**a–c**) or OT-II cells (**d–f**) cultured with DCs transfected by electroporation with mRNA encoding OVA or green fluorescent protein (GFP; **a–c**) or treated with soluble OVA (**d–f**), followed by the addition of US6(20–146) or Trf-US6(20–146). Inactive US6(20–125) and Trf-US6(20–125) serve as a control for TAP-independent effects. Data are representative of three independent experiments (error bars, mean + s.e.m. of triplicates).

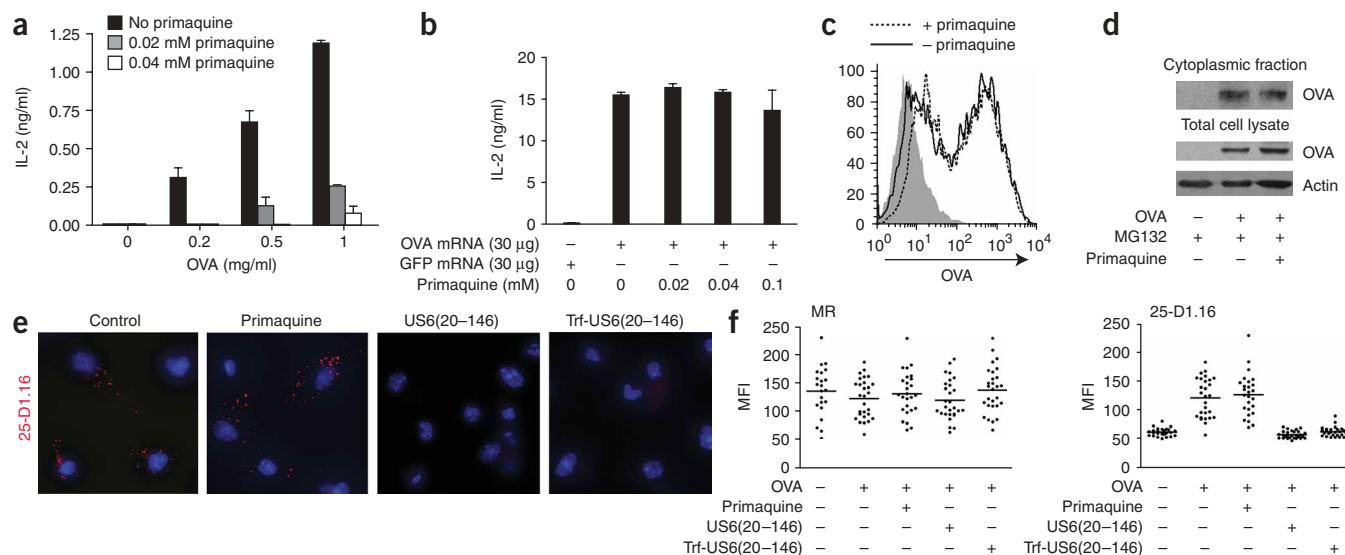


Figure 4 Cross-presented OVA is transported to the DC surface from endosomes, not from the endoplasmic reticulum. **(a)** ELISA of IL-2 in supernatants of OT-I cells cultured with DCs previously exposed to primaquine and OVA. **(b)** ELISA of IL-2 in supernatants of OT-I cells cultured with DCs previously transfected by electroporation with mRNA encoding OVA or GFP, then treated with primaquine. **(c)** Flow cytometry of OVA uptake by DCs in the presence or absence of primaquine shaded histogram, DCs not treated with OVA. **(d)** Immunoblot analysis of OVA in total cell lysates or the cytoplasmic fraction of DCs incubated with OVA, primaquine and MG132. Actin, loading control. **(e)** Immunofluorescence microscopy of DCs incubated with OVA, primaquine, US6(20–146) or Trf-US6(20–146). Cross-presented OVA is stained with 25-D1.16 (red); nuclei are counterstained with DAPI (blue). Original magnification, $\times 600$. **(f)** Quantitative analysis of mannose receptor and 25-D1.16 staining in mannose receptor–positive endosomes in DCs treated as described in **e**. Each symbol represents an individual endosome; small horizontal lines indicate the mean. MFI, mean fluorescence intensity. Data are representative of three independent experiments (error bars, mean + s.e.m. of triplicates).

of Trf-US6 into the endoplasmic reticulum using DCs transfected with mRNA encoding OVA, which resulted in endogenous expression of this antigen. In these DCs, Trf-US6(20–146) did not inhibit OT-I cell activation over a wide range of antigen concentrations (**Fig. 3a,b**), which demonstrated that it did not inhibit TAP in the endoplasmic reticulum. In contrast, activation of OT-I cells by endogenous OVA was abrogated by US6(20–146) (**Fig. 3c**), a protein known to block endogenous presentation²³ as well as cross-presentation of OVA²¹. Notably, Trf-US6(20–146) indeed inhibited cross-presentation, whereas the inactive construct, Trf-US6(20–125), did not (**Fig. 3d**). These findings functionally confirmed the presence of endosomal TAP and showed that it was needed to mediate access of OVA-derived peptides into early endosomes for cross-presentation. Trf-US6 was more efficient in inhibiting cross-presentation on the molar level than was free US6 (**Fig. 3e**), which demonstrated the high efficiency of Trf-mediated delivery of US6 to endosomes. Activation of OVA-specific CD4⁺ T cells (OT-II cells) was unaffected by Trf-US6, which excluded the possibility of toxic effects and demonstrated the specificity of inhibition for MHC class I–restricted presentation of exogenous antigens (**Fig. 3f**).

We noted that the addition of inactive US6(20–125) increased the activation of OT-I cells by endogenous OVA (**Fig. 3c**). This may have been due to endotoxins resulting from its production in recombinant bacteria, as LPS treatment has been shown to stimulate proteasomal and TAP activity in DCs¹⁴. In support of that interpretation, we noted upregulation of costimulatory molecules in DCs treated with US6 (**Supplementary Fig. 7** online).

Cross-presented OVA travels from endosomes to DC surfaces

The inhibition of cross-presentation after specific blockade of endosomal TAP demonstrated that antigenic peptides had to enter early

endosomes to be loaded onto MHC class I molecules. Next we investigated whether those complexes were directly transported from early endosomes to the cell surface. We used the drug primaquine, which prevents trafficking of endosomes to the cell membrane^{38,39}. Primaquine indeed prevented activation of OT-I cells by cross-presentation in a dose-dependent way (**Fig. 4a**), which showed that cross-presented OVA on the cell surface originated from endosomes. In contrast, primaquine did not affect the activation of OT-I cells by DCs transfected with mRNA encoding OVA (**Fig. 4b**), which confirmed that the classical endogenous MHC class I presentation pathway through the endoplasmic reticulum and the Golgi apparatus was not compromised by this drug. Primaquine affected neither OVA uptake (**Fig. 4c**) nor export of OVA from endosomes to the cytoplasm (**Fig. 4d**), which ruled out the possibility that it inhibited other steps of cross-presentation.

The findings presented above indicated that Trf-US6 and primaquine are specific inhibitors of cross-presentation that act at different points in the cross-presentation pathway. To confirm that interpretation, we used the antibody 25-D1.16, which specifically recognizes the OVA peptide 'SIINFEKL' bound to the MHC class I molecule H-2K^b (ref. 40). This has been used to show the presence of cross-presented OVA in early endosomes, where it localizes together with mannose receptor–endocytosed, unprocessed OVA²⁶. Indeed, despite primaquine treatment, cross-presented OVA remained detectable in early endosomes (**Fig. 4e,f** and **Supplementary Fig. 8** online), which confirmed that this drug did not abrogate the generation of but instead prevented the transport of cross-presented OVA to the cell surface. In contrast, DCs treated with US6 or Trf-US6 showed no 25-D1.16 staining (**Fig. 4e,f** and **Supplementary Fig. 8**), which once more demonstrated that endosomal TAP activity was required for the generation of MHC class I–peptide complexes for cross-presentation.

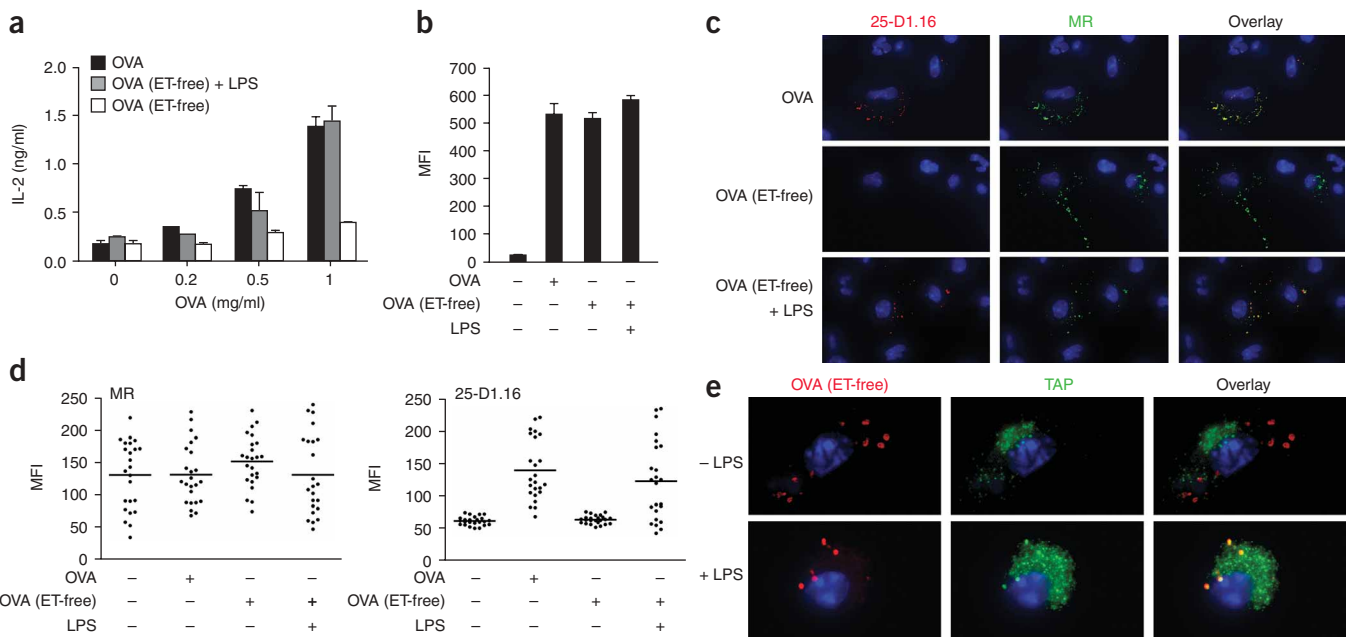


Figure 5 Recruitment of TAP to early endosomes depends on endotoxin. **(a)** ELISA of IL-2 in supernatants of OT-I cells cultured with DCs previously exposed to 'ordinary' OVA or endotoxin-free OVA (OVA(ET-free)) with or without LPS. **(b)** Flow cytometry of the uptake of 'ordinary' or endotoxin-free OVA by DCs in the presence or absence of LPS. **(c)** Immunofluorescence microscopy of DCs incubated with 'ordinary' OVA or endotoxin-free OVA with or without LPS. Cross-presented OVA is stained with 25-D1.16 (red), mannose receptor is stained with a specific antibody (green) and nuclei are counterstained with DAPI (blue). **(d)** Quantitative analysis of mannose receptor and 25-D1.16 staining in individual mannose receptor-positive endosomes in DCs treated as described in **c**. Each symbol represents an individual endosome; small horizontal lines indicate the mean. **(e)** Immunofluorescence microscopy of DCs after endocytosis of fluorochrome-labeled endotoxin-free OVA (red) in the presence or absence of LPS. DCs are counterstained for TAP (green) and with DAPI (blue). Original magnification, $\times 600$ (**c,e**). Data are representative of three independent experiments (error bars, mean + s.e.m. of triplicates).

In summary, these findings demonstrated spatial and mechanistic separation of cross-presentation and endogenous antigen presentation (**Supplementary Fig. 9** online).

Endotoxin causes recruitment of TAP to early endosomes

Immunity requires DC maturation induced, for example, by microbial endotoxins such as LPS, which increase expression of costimulatory molecules on the DC surface^{9,10,13,41}. Studies of MHC class II-restricted antigen presentation have demonstrated that endotoxins also increase lysosomal antigen processing by several mechanisms^{42–45}. There is also evidence of enhancement of the intracellular mechanisms of cross-presentation^{11,14,15}. As pathogen-derived antigens as well as commonly used model antigens such as 'ordinary' OVA often contain traces of endotoxins^{9,46}, we investigated the influence of endotoxin on the cross-presentation of mannose receptor-endocytosed OVA. We noted that endotoxin-free OVA was cross-presented far less efficiently than was 'ordinary' OVA (**Fig. 5a**) and that this was restored by the addition of LPS (**Fig. 5a**). The impaired cross-presentation was not due to less antigen uptake, as both endotoxin-free and 'ordinary' OVA were internalized by DCs to a similar extent (**Fig. 5b**). It was not due to a requirement of endotoxins for intracellular antigen routing, as

'ordinary' OVA and endotoxin-free OVA trafficked through Trf⁺EAA1⁺ mannose receptor-positive early endosomes but not through LAMP-1⁺ late endosomes-lysosomes, as did pinocytosed Lucifer yellow (**Supplementary Fig. 10** online). Finally, the diminished cross-presentation was also not due to lack of expression

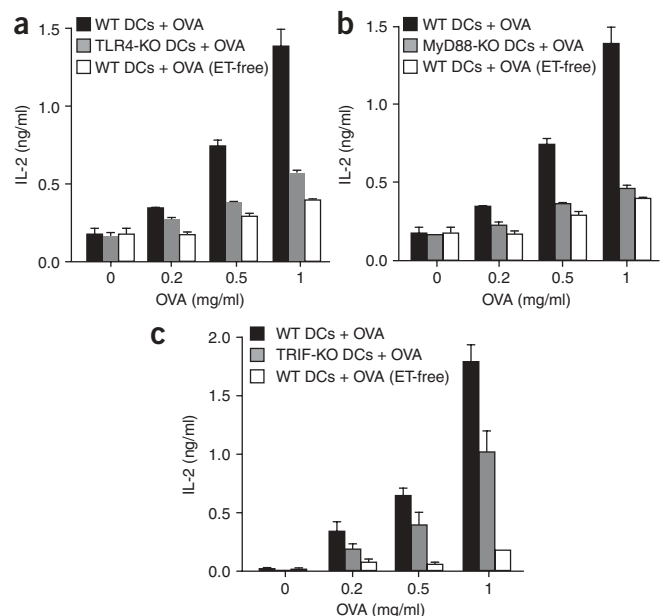


Figure 6 The endotoxin-mediated increase in cross-presentation depends on TLR4 and MyD88 and partially on TRIF. ELISA of IL-2 in supernatants of OT-I cells cultured with TLR4-deficient DCs (TLR4-KO; **a**), MyD88-deficient DCs (MyD88-KO; **b**), TRIF-deficient DCs (TRIF-KO; **c**) or wild-type DCs exposed to 'ordinary' OVA or endotoxin-free OVA. Data are representative of two independent experiments (error bars, mean + s.e.m. of triplicates).

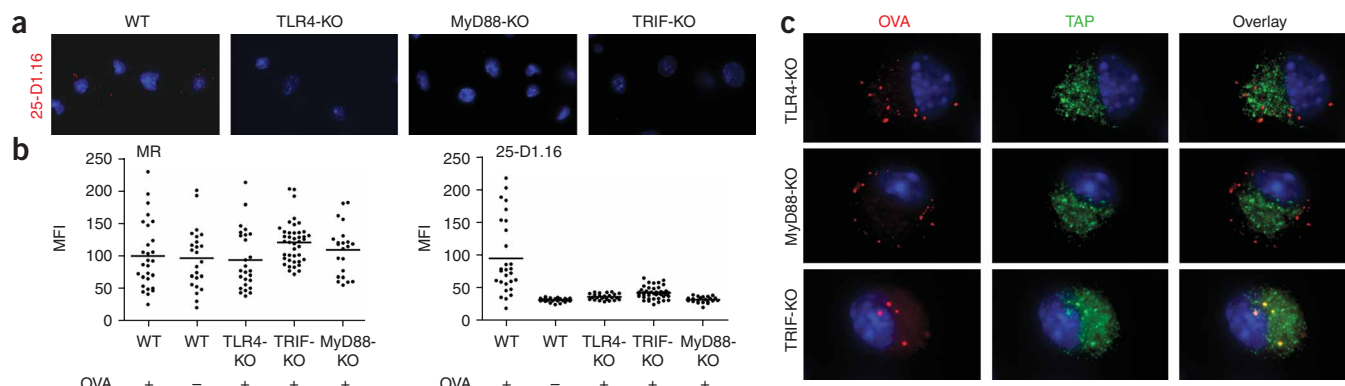


Figure 7 Recruitment of TAP to endosomes depends on TLR4 and MyD88 but not on TRIF. (a) Immunofluorescence microscopy of wild-type DCs and DCs deficient in TLR4, MyD88 or TRIF, incubated with OVA. Cross-presented OVA is stained with 25-D1.16 (red); nuclei are counterstained with DAPI (blue). (b) Quantitative analysis of mannose receptor and 25-D1.16 staining in mannose receptor-positive endosomes in DCs treated as described in a. Each symbol represents an individual endosome; small horizontal lines indicate the mean. (c) Immunofluorescence microscopy of DCs deficient in TLR4, MyD88 or TRIF, after endocytosis of fluorochrome-labeled OVA (red). DCs are counterstained for TAP (green) and with DAPI (blue). Original magnification, $\times 600$ (a,c). Data are representative of three independent experiments.

of costimulatory molecules; we fixed DCs 2 h after culture with OVA and found no altered expression of these molecules in this time frame (Supplementary Fig. 11 online). Therefore, we speculated that the stimulatory effect of endotoxin might instead have resulted from increased activity of the cross-presentation ‘machinery’.

To investigate that hypothesis, we first assessed the influence of endotoxin on the generation of cross-presented OVA by immunofluorescence microscopy with the 25-D1.16 antibody. When we used endotoxin-free OVA, which was cross-presented much less efficiently (Fig. 5a), no 25-D1.16 signal was detectable in early endosomes (Fig. 5c,d). The addition of LPS quantitatively restored the generation of cross-presented OVA in early endosomes (Fig. 5c,d), consistent with the greater activation of OT-I cells in these conditions (Fig. 5a). Because endosomal TAP was crucial for cross-presentation of OVA (Fig. 3d), we speculated that the stimulatory effect of endotoxins resulted from the recruitment of TAP to OVA-containing endosomes. Indeed, TAP localized together with endocytosed ‘ordinary’ OVA in 53 of 100 DCs examined (Fig. 1e), but after internalization of endotoxin-free OVA, only 13% of the DCs showed such colocalization (Fig. 5e). Again this deficiency was overcome by the addition of exogenous LPS, after which 43% of the DCs showed localization of endotoxin-free OVA together with TAP (Fig. 5e). These findings collectively demonstrated that endotoxin was required for recruitment of TAP to early endosomal compartments and for the efficient generation of cross-presented OVA therein.

Endosomal recruitment of TAP is TLR4-MyD88 dependent

The requirement of endotoxin for the recruitment of TAP to early endosomes suggested involvement of TLR4 and its signaling molecules MyD88 and TRIF^{9,10,13} in cross-presentation. To investigate that hypothesis, we used DCs from mice deficient in TLR4, MyD88 or TRIF. Indeed, in the absence of TLR4 or MyD88, OT-I cell activation by OVA was as low as that obtained with endotoxin-free OVA (Fig. 6a,b). These findings excluded the possibility that residual traces of endotoxin in the endotoxin-free OVA were responsible for its cross-presentation. TRIF deficiency also resulted in diminished OT-I cell activation, albeit by only 35–40% (Fig. 6c).

Consistent with the lower OT-I cell responses, cross-presented OVA was undetectable by 25-D1.16 staining in DCs deficient in TLR4 or

MyD88 (Fig. 7a,b; additional staining for localization together with the mannose receptor, Supplementary Fig. 12 online). In TRIF-deficient DCs, cross-presented OVA was also not detected with this technique (Fig. 7a,b and Supplementary Fig. 12), despite some residual OT-I cell activation by these DCs (Fig. 6c), perhaps because of sensitivity limitations of the 25-D1.16 antibody.

Finally, we studied whether TLR4 and its signaling molecules were needed to recruit TAP to early endosomes. TAP was localized together with endocytosed OVA in 53% of wild-type DCs but in only 16% or 17% in DCs deficient in TLR4 or MyD88, respectively. In contrast, we noted this colocalization in 56% of TRIF-deficient DCs, similar to the results obtained with wild-type DCs (Fig. 7c). These findings indicated that TLR4 and MyD88 signaling were required for endotoxin-induced recruitment of TAP to early endosomes, which was essential for cross-presentation in these organelles (Figs. 1 and 3). The absence of TLR4 signaling through TRIF partially impaired cross-presentation by a distinct molecular mechanism, which must have compromised antigen processing, as cross-presented antigen was absent from endosomes in TRIF-deficient DCs (Fig. 7a,b) despite intact recruitment of TAP to these organelles (Fig. 7c). This distinct effect on antigen processing might result, for example, from increased antigen export into the cytoplasm or from augmented antigen processing by the cytosolic proteasome.

DISCUSSION

In this study we have provided formal experimental evidence that cross-presentation is spatially separated from endogenous MHC class I-restricted antigen presentation in DCs. Our findings support the following mechanism for cross-presentation of soluble antigen: OVA is taken up by the mannose receptor into stable early endosomes²⁶, is exported into the cytoplasm, is processed by the proteasome, and is reimported by means of TAP into the same endosomal compartment for loading onto MHC class I molecules. This model explains the observation that mannose receptor–endocytosed OVA and cross-presented OVA localize together only in early endosomes²⁶. From these endosomes, cross-presented OVA is transported directly to the cell membrane for presentation to CTLs. TAP-mediated import into the endoplasmic reticulum is required only for endogenous presentation, not for cross-presentation.

That scenario is reminiscent of a model for cross-presentation of phagocytosed particulate antigens that requires antigen export from phagosomes to the cytoplasmic proteasome, followed by TAP-mediated transport of antigen-derived peptides^{21,28,29}. Although TAP was detected in the phagosomal membrane, formal experimental evidence for involvement of phagosomal TAP in cross-presentation has not been reported. Such evidence may be attained by specific targeting of the TAP inhibitor US6 into phagosomes by an approach similar to that described here.

A study examining cross-presentation of soluble antigens has reported that externally added β_2 -microglobulin, a small component of MHC class I, is targeted to the endoplasmic reticulum by unknown pathways²³. On the basis of that observation, it has been proposed that a mechanism that can introduce extracellular proteins into the endoplasmic reticulum may facilitate cross-presentation by using the presentation mechanisms for endogenous antigen²³. However, in those studies, extracellular OVA was found to be targeted to “ER-like structures”; a term used to describe vesicles spatially distinct from the perinuclear endoplasmic reticulum yet containing certain endoplasmic reticulum components such as calnexin²¹. Because extracellular proteins such as OVA are usually internalized into endosomes, direct access of antigen to the endoplasmic reticulum is probably restricted to a subset of antigens.

Here we have provided several lines of evidence that endosomes carrying soluble antigens for cross-presentation contained TAP and that its function was essential for cross-presentation. The endosomal content of TAP depended on the presence of endotoxin during antigen uptake and on TLR4-MyD88 signaling. Such recruitment of TAP to early endosomes may represent a mechanism by which efficient cross-presentation can be restricted to microbial antigens carrying endotoxin or other pathogen-associated molecular patterns. Future studies should elucidate whether further stimuli exist that can induce such recruitment and whether it occurs by vesicular fusion or, as discussed for particulate antigens, by endoplasmic reticulum-mediated endocytosis^{11,21,28–30,47}.

Consistent with TLR4-MyD88-dependent recruitment of TAP to early endosomes, DCs deficient in the endotoxin sensor TLR4 and its signaling adaptor molecule MyD88 (refs. 8–10) produced comparably weak cross-presentation responses. Because in our system endosomal TAP was essential for cross-presentation, some of this transporter may have been present in early endosomes that permitted low-level cross-presentation in the absence of TLR4-MyD88 signaling or endotoxin and that we could not detect by immunofluorescence. That interpretation may explain why we found weak cross-presentation responses to endotoxin-free OVA and why published studies have reported constitutive *in vivo* cross-presentation of OVA expressed as a transgenic autoantigen^{2,5}, although autoantigens are usually devoid of endotoxins. In those studies, only high-dose autoantigen was found to be cross-presented⁶, and in the absence of endotoxins, only high doses may be cross-presented with sufficient efficiency for activation of CD8⁺ T cells. However, those previous studies examined cross-presentation of OVA expressed by islet β -cells, and cross-presentation of cell-associated antigen is mechanistically distinct from that of soluble antigen studied here^{7,31}.

The recruitment of TAP to the organelles of cross-presentation may represent a further mechanism by which TLR ligands such as endotoxins can stimulate CTL-mediated immunity. For MHC class II-restricted antigen presentation, endotoxins have been shown to increase CD4⁺ T cells activation by a variety of mechanisms beyond mere upregulation of costimulatory molecules, including stimulatory effects on phagocytosis and phagosome maturation⁴⁸, lysosomal

antigen processing⁴⁴, loading of processed antigens onto MHC class II molecules⁴⁵, and routing and stabilization of MHC class II-peptide complexes on the DC surface^{42,43}. The effect of endotoxin on cross-presentation is less clear. It has been shown that endotoxin-treated DCs internalize antigen-antibody complexes through Fc receptors more efficiently and show higher proteasomal activity in their cytosolic fraction and more TAP-mediated transport of peptide into the endoplasmic reticulum¹⁴, but the contribution of these effects to cross-presentation has not been clarified. Our finding that endotoxin caused TLR4-MyD88-mediated relocation of TAP to early endosomes and thereby permitted the entry of antigenic peptides for subsequent cross-presentation has identified a mechanism by which an essential component of the cross-presentation ‘machinery’ can be recruited and regulated. This mechanism differs from that of MHC class II-restricted presentation of extracellular particulate antigen in phagosomes, in which peptides are generated in these organelles and require TLR4-MyD88 signaling to be loaded onto MHC class II molecules⁴⁵. In addition to TAP recruitment, TLR4-MyD88 may mediate further stimulatory effects on cross-presentation, such as increasing proteasomal activity, as is reported to occur after treatment of DCs with LPS¹⁴, or (theoretically) by increasing translocation of antigen to the cytosolic proteasome. Those two mechanisms may have been responsible for the observed impairment of cross-presentation in TRIF-deficient DCs, as these cells also lacked cross-presented OVA in their endosomes despite having intact TAP content. The exact molecular effects of TRIF and MyD88 signaling on antigen processing and cross-presentation remain to be elucidated.

The spatial separation of MHC class I-restricted antigen processing for endogenous and extracellular antigens may ensure that peptides from extracellular antigens do not have to compete with the large pool of endogenous peptides for MHC class I molecules in the endoplasmic reticulum. Such a separation may hinder viruses that inhibit endogenous presentation in infected APCs from compromising cross-presentation. Furthermore, distinct loading sites theoretically may allow distinct proteasomal subsets to selectively process endogenous and extracellular antigens and may permit the development of drugs that selectively inhibit one of these processes, such as primaquine did. Finally, our findings pertain to the development of therapeutic vaccines against viruses or tumors by identifying the intracellular compartments in which a vaccine is processed when pathways such as mannose receptor-mediated endocytosis are used and by showing the necessity of TLR ligands able to recruit TAP for efficient cross-presentation.

METHODS

Mice. Mannose receptor-deficient mice were provided by M.C. Nussenzweig, and OT-II and OT-I recombination-activating gene 1-deficient mice were provided by W.R. Heath. For all experiments, mice between 8 and 16 weeks of age bred in specific pathogen-free conditions were used in accordance with local animal experimentation guidelines.

Antibodies and reagents. Rat antibody to mannose receptor (anti-mannose receptor; MR5D3) was from Serotec; rabbit anti-Lucifer yellow (A-5751) and all secondary antibodies were from Invitrogen; rat anti-LAMP-1 (1D4B) was from Pharmingen-BD Biosciences; rabbit anti-actin (20-33) was from Sigma; rabbit anti-EEA1 (PA1-063A) was from Dianova; and goat anti-TAP (M-18) was from Santa Cruz Biotechnologies. Rat anti-transferrin receptor (TIB-219) was a gift from A. Haas. Alexa Fluor 647-labeled transferrin was from Invitrogen. US6(20-146) and US6(20-125) were prepared as described³⁶. Endotoxin-free OVA (EndoGrade) was from Profos. All other reagents, if not specified otherwise, were from Sigma, including LPS from *Escherichia coli*.

Generation of bone marrow-derived DCs. DCs were generated with granulocyte-macrophage colony-stimulating factor as described^{26,31}. At day 7, CD11c⁺ cells were isolated by magnetic separation with the autoMACS system (Miltenyi). The purity of DCs was typically higher than 98%.

Isolation and analysis of the cytoplasmic fraction of cells lysates. For analysis of the OVA content of the cytoplasmic fraction of cells lysates, OVA was biotinylated with the Sulfo-NHS-LC Biotinylation kit (Perbio). DCs were incubated for 15 min with biotinylated OVA (0.5 mg/ml) in the presence or absence of 0.04 mM primaquine or 5 μM MG132. Then, medium was removed and replaced with medium containing the inhibitors but not OVA. After another 45 min, cytoplasmic fractions were isolated with a sub-cellular proteome extraction kit (Calbiochem). Neither endosomal proteins (such as the mannose receptor) nor lysosomal proteins (such as LAMP-1) were detected in this fraction by immunoblot (data not shown). For measurement of OVA concentration, cytoplasmic fractions were incubated for 1 h with streptavidin-agarose and washed extensively and bound proteins were eluted for analysis by immunoblot with horseradish peroxidase-conjugated streptavidin (Biomedica). Total cell lysates were analyzed after being incubated for 10 min on ice in lysis buffer (20 mM NaPO₄, 140 mM NaCl, 3 mM MgCl₂, 0.5% (vol/vol) nonidet-P40 and 50 μM leupeptin, pH 8.0).

Analysis of the ubiquitination state of cytoplasmic OVA. For analysis of whether cytoplasmic OVA was ubiquitinated, mRNA encoding six-histidine-tagged ubiquitin was synthesized with the mMessage mMachine T3 and the Poly(A) Tailing kit (Ambion). DCs were transfected with mRNA by electroporation as described^{26,49}. After electroporation, cells were treated with biotinylated OVA in the presence of MG132 as described above before the cytoplasmic fractions were collected. Total OVA in this fraction was isolated by incubation of the lysate with streptavidin-agarose, whereas ubiquitinated proteins were isolated by incubation with nickel-nitrilotriacetic acid agarose (Ni-NTA Agarose; Qiagen) as described⁵⁰. Ubiquitinated OVA was detected by immunoblot with a streptavidin-horseradish peroxidase conjugate. For exclusion of ubiquitination after cell disruption, OVA was added to a separate control sample during cell lysis.

OT-I and OT-II activation assays. OT-I cells from OT-I recombination-activating gene 1-deficient mice and OT-II cells were isolated as described^{5,26,31} and were further purified with CD8⁺ and CD4⁺ T cell isolation kits, respectively (Miltenyi). For cross-presentation assays, 2.5 × 10⁵ DCs were incubated with OVA in the presence or absence of the cathepsin S inhibitor Z-FL-COCHO (5 μM; Calbiochem), the cathepsin L-cathepsin B inhibitor Z-FF-FMK (5 μM; Calbiochem), US6 (1 mg/ml), US6-Trf (1 mg/ml) or the proteasome inhibitor epoxomicin (1 μM). After 2 h, cells were washed, were fixed for 3 min with 0.008% (wt/vol) glutaraldehyde and were incubated together with 7 × 10⁵ OT-I or OT-II cells. The concentration of interleukin 2 (IL-2) in the supernatant was determined after 18 h by enzyme-linked immunosorbent assay (ELISA). For analysis of the effect of primaquine on cross-presentation, cells were incubated for 10 min with OVA and primaquine. Then, medium was removed and replaced for an additional 110 min with medium containing primaquine but no OVA. Afterward, cells were fixed and cultured together with OT-I cells as described above. For the MHC class I-restricted presentation of endogenous antigens, mRNA encoding OVA was synthesized with the T7 mMessage mMachine Ultra Kit (Ambion). Cells were transfected with mRNA by electroporation as described^{26,49}; this typically yielded efficiencies of about 80%. After electroporation, cells were cultured for 2 h in the presence or absence of various concentrations of primaquine, US6 or Trf-US6, then were fixed and cultured together with OT-I cells as described above.

Preparation of fluorescent OVA and uptake experiments. Soluble OVA was rendered fluorescent by chemical conjugation to Alexa Fluor 647 (Invitrogen). The labeling procedure involved gel-filtration for the removal of low-molecular-weight molecules such as peptide fragments or unbound fluorochrome. Uptake experiments were done as described²⁶.

Crosslinking of Trf and US6. 'Heterobifunctional' crosslinker reagents from Perbio were used for coupling experiments according to the manufacturer's

instructions. Maleimide groups were coupled to 1 mg US6 by incubation for 30 min at 25 °C with 19 μl of a solution of sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate. Sulfhydryl groups were coupled to 1 mg transferrin by incubation for 30 min with 0.2 mg N-succinimidyl S-acetylthioacetate, followed by reduction achieved by the addition for 2 h of 10% (vol/vol) hydroxylamine solution (0.62 M hydroxylamine hydrochloride and 25 mM EDTA, pH 7.4). Afterward, modified US6 and transferrin were coupled by being incubated together for 30 min at a molar ratio of 5:1 (US6/transferrin). After coupling, free US6 was removed by extensive washing with a Vivaspin column with a molecular cutoff of 30 kilodaltons (Sartorius). For coupling of Alexa Fluor 647-labeled US6 to transferrin, 1 mg US6 was coupled to Alexa Fluor 647 (Invitrogen) in the presence of 0.336 mg of the 'heterobifunctional' crosslinking reagent succinimidyl 4-formylbenzoate. After 30 min, free Alexa Fluor 647 and succinimidyl 4-formylbenzoate were removed by gel filtration. In parallel, 1 mg transferrin was conjugated to 75 μg succinimidyl 4-hydrazinonicotinate acetone hydrazone. Afterward, Alexa Fluor 647-labeled US6 was coupled to transferrin and free US6 was removed as described above.

Immunofluorescence. Cells were pulsed for 15 min with fluorochrome-labeled OVA (10 μg/ml), Lucifer yellow (0.3 mg/ml), US6 (10 μg/ml), Trf (10 μg/ml) or US6-Trf (10 μg/ml) and were chased for another 15 min with medium. Staining experiments, including 25-D1.16 staining, were done as described²⁶. Nuclei were visualized with the DNA-intercalating dye DAPI (4,6-diamidino-2-phenylindole; 1 μg/ml). Cells were analyzed with a IX71 microscope with analySIS software (Olympus). For quantitative analysis of 25-D1.16 staining, the fluorescence intensity of individual mannose receptor-positive endosomes was calculated with CellF software (Olympus).

Flow cytometry. An LSR (BD Biosciences) was used for flow cytometry; data were analyzed with FlowJo software (TreeStar).

Accession codes. UCSD-Nature Signaling Gateway (<http://www.signaling-gateway.org>): A003686, A002608 and A002609.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank M.C. Nussenzweig (Rockefeller University) for mannose receptor-deficient mice; W.R. Heath (The Walter and Eliza Hall Institute of Medical Research) for OT-I and OT-II mice; R. Germain (National Institutes of Health) for 25-D1.16; A. Haas for the transferrin receptor antibody; and P. Knolle for critically reading the manuscript. We acknowledge the technical support of the flow cytometry core facility of the Institute of Molecular Medicine and Experimental Immunology and the House for Experimental Therapy of the Medical Faculty of Bonn University. Supported by the BONFOR program of Friedrich-Wilhelms University (O-173.0009 to S.B.) and by the Deutsche Forschungsgemeinschaft (SFB704 to C.K. and Ta157/7-1 to R.T.).

AUTHOR CONTRIBUTIONS

S.B. designed experiments, did research, analyzed and interpreted data, and wrote the paper; C.S. and A.K. did experiments; R.T. contributed critical analytical tools and analyzed and interpreted data; C.K. designed experiments, analyzed and interpreted data, and wrote the paper.

Published online at <http://www.nature.com/natureimmunology>
Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions>

- den Haan, J.M., Lehar, S.M. & Bevan, M.J. CD8⁺ but not CD8⁻ dendritic cells cross-prime cytotoxic T cells in vivo. *J. Exp. Med.* **192**, 1685–1696 (2000).
- Kurts, C., Cannarile, M., Klebba, I. & Brocker, T. Dendritic cells are sufficient to cross-present self-antigens to CD8 T cells in vivo. *J. Immunol.* **166**, 1439–1442 (2001).
- Bevan, M.J. Cross-priming. *Nat. Immunol.* **7**, 363–365 (2006).
- Villadangos, J.A., Heath, W.R. & Carbone, F.R. Outside looking in: the inner workings of the cross-presentation pathway within dendritic cells. *Trends Immunol.* **28**, 45–47 (2007).
- Kurts, C. *et al.* Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J. Exp. Med.* **184**, 923–930 (1996).
- Kurts, C., Miller, J.F., Subramaniam, R.M., Carbone, F.R. & Heath, W.R. Major histocompatibility complex class I-restricted cross-presentation is biased towards

- high dose antigens and those released during cellular destruction. *J. Exp. Med.* **188**, 409–414 (1998).
7. Li, M. *et al.* Cell-associated ovalbumin is cross-presented much more efficiently than soluble ovalbumin in vivo. *J. Immunol.* **166**, 6099–6103 (2001).
 8. Beutler, B., Hoebe, K., Du, X. & Ulevitch, R.J. How we detect microbes and respond to them: the Toll-like receptors and their transducers. *J. Leukoc. Biol.* **74**, 479–485 (2003).
 9. Kopp, E. & Medzhitov, R. Recognition of microbial infection by Toll-like receptors. *Curr. Opin. Immunol.* **15**, 396–401 (2003).
 10. Hemmi, H. & Akira, S. TLR signalling and the function of dendritic cells. *Chem. Immunol. Allergy* **86**, 120–135 (2005).
 11. Maurer, T. *et al.* CpG-DNA aided cross-presentation of soluble antigens by dendritic cells. *Eur. J. Immunol.* **32**, 2356–2364 (2002).
 12. Datta, S.K. *et al.* A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. *J. Immunol.* **170**, 4102–4110 (2003).
 13. Hoebe, K. *et al.* Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways. *Nat. Immunol.* **4**, 1223–1229 (2003).
 14. Gil-Torregrosa, B.C. *et al.* Control of cross-presentation during dendritic cell maturation. *Eur. J. Immunol.* **34**, 398–407 (2004).
 15. Schulz, O. *et al.* Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* **433**, 887–892 (2005).
 16. Weck, M.M. *et al.* TLR ligands differentially affect uptake and presentation of cellular antigens. *Blood* **109**, 3890–3894 (2007).
 17. Abele, R. & Tampé, R. The ABCs of immunology: structure and function of TAP, the transporter associated with antigen processing. *Physiology (Bethesda)* **19**, 216–224 (2004).
 18. Ackerman, A.L. & Cresswell, P. Cellular mechanisms governing cross-presentation of exogenous antigens. *Nat. Immunol.* **5**, 678–684 (2004).
 19. Brossart, P. & Bevan, M.J. Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. *Blood* **90**, 1594–1599 (1997).
 20. Norbury, C.C., Chambers, B.J., Prescott, A.R., Ljunggren, H.G. & Watts, C. Constitutive macropinocytosis allows TAP-dependent major histocompatibility complex class I presentation of exogenous soluble antigen by bone marrow-derived dendritic cells. *Eur. J. Immunol.* **27**, 280–288 (1997).
 21. Ackerman, A.L., Kyritsis, C., Tampé, R. & Cresswell, P. Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. *Proc. Natl. Acad. Sci. USA* **100**, 12889–12894 (2003).
 22. Norbury, C.C. *et al.* CD8⁺ T cell cross-priming via transfer of proteasome substrates. *Science* **304**, 1318–1321 (2004).
 23. Ackerman, A.L., Kyritsis, C., Tampé, R. & Cresswell, P. Access of soluble antigens to the endoplasmic reticulum can explain cross-presentation by dendritic cells. *Nat. Immunol.* **6**, 107–113 (2005).
 24. Imai, J., Hasegawa, H., Maruya, M., Koyasu, S. & Yahara, I. Exogenous antigens are processed through the endoplasmic reticulum-associated degradation (ERAD) in cross-presentation by dendritic cells. *Int. Immunol.* **17**, 45–53 (2005).
 25. Ackerman, A.L., Giodini, A. & Cresswell, P. A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells. *Immunity* **25**, 607–617 (2006).
 26. Burgdorf, S., Kautz, A., Bohnert, V., Knolle, P.A. & Kurts, C. Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science* **316**, 612–616 (2007).
 27. Song, R. & Harding, C.V. Roles of proteasomes, transporter for antigen presentation (TAP), and beta 2-microglobulin in the processing of bacterial or particulate antigens via an alternate class I MHC processing pathway. *J. Immunol.* **156**, 4182–4190 (1996).
 28. Houde, M. *et al.* Phagosomes are competent organelles for antigen cross-presentation. *Nature* **425**, 402–406 (2003).
 29. Guernonprez, P. *et al.* ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature* **425**, 397–402 (2003).
 30. Touret, N. *et al.* Quantitative and dynamic assessment of the contribution of the ER to phagosome formation. *Cell* **123**, 157–170 (2005).
 31. Burgdorf, S., Lukacs-Kornek, V. & Kurts, C. The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation. *J. Immunol.* **176**, 6770–6776 (2006).
 32. Riese, R.J. & Chapman, H.A. Cathepsins and compartmentalization in antigen presentation. *Curr. Opin. Immunol.* **12**, 107–113 (2000).
 33. Rodriguez, A., Regnault, A., Kleijmeer, M., Ricciardi-Castagnoli, P. & Amigorena, S. Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat. Cell Biol.* **1**, 362–368 (1999).
 34. Hotta, C., Fujimaki, H., Yoshinari, M., Nakazawa, M. & Minami, M. The delivery of an antigen from the endocytic compartment into the cytosol for cross-presentation is restricted to early immature dendritic cells. *Immunology* **117**, 97–107 (2006).
 35. Song, R., Porgador, A. & Harding, C.V. Peptide-receptive class I major histocompatibility complex molecules on TAP-deficient and wild-type cells and their roles in the processing of exogenous antigens. *Immunology* **97**, 316–324 (1999).
 36. Kyritsis, C. *et al.* Molecular mechanism and structural aspects of transporter associated with antigen processing inhibition by the cytomegalovirus protein US6. *J. Biol. Chem.* **276**, 48031–48039 (2001).
 37. Ciechanover, A., Schwartz, A.L. & Lodish, H.F. Sorting and recycling of cell surface receptors and endocytosed ligands: the asialoglycoprotein and transferrin receptors. *J. Cell. Biochem.* **23**, 107–130 (1983).
 38. Reid, P.A. & Watts, C. Cycling of cell-surface MHC glycoproteins through primaquine-sensitive intracellular compartments. *Nature* **346**, 655–657 (1990).
 39. van Weert, A.W., Geuze, H.J., Groothuis, B. & Stoorvogel, W. Primaquine interferes with membrane recycling from endosomes to the plasma membrane through a direct interaction with endosomes which does not involve neutralisation of endosomal pH nor osmotic swelling of endosomes. *Eur. J. Cell Biol.* **79**, 394–399 (2000).
 40. Porgador, A., Yewdell, J.W., Deng, Y., Bennink, J.R. & Germain, R.N. Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity* **6**, 715–726 (1997).
 41. Bancherau, J. & Steinman, R.M. Dendritic cells and the control of immunity. *Nature* **392**, 245–252 (1998).
 42. Cella, M., Engering, A., Pinet, V., Pieters, J. & Lanzavecchia, A. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* **388**, 782–787 (1997).
 43. Turley, S.J. *et al.* Transport of peptide-MHC class II complexes in developing dendritic cells. *Science* **288**, 522–527 (2000).
 44. Trombetta, E.S., Ebersold, M., Garrett, W., Pypaert, M. & Mellman, I. Activation of lysosomal function during dendritic cell maturation. *Science* **299**, 1400–1403 (2003).
 45. Blander, J.M. & Medzhitov, R. Toll-dependent selection of microbial antigens for presentation by dendritic cells. *Nature* **440**, 808–812 (2006).
 46. Watanabe, J., Miyazaki, Y., Zimmerman, G.A., Albertine, K.H. & McIntyre, T.M. Endotoxin contamination of ovalbumin suppresses murine immunologic responses and development of airway hyper-reactivity. *J. Biol. Chem.* **278**, 42361–42368 (2003).
 47. Gagnon, E. *et al.* Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell* **110**, 119–131 (2002).
 48. Blander, J.M. & Medzhitov, R. Regulation of phagosome maturation by signals from toll-like receptors. *Science* **304**, 1014–1018 (2004).
 49. Van Meirvenne, S. *et al.* Efficient genetic modification of murine dendritic cells by electroporation with mRNA. *Cancer Gene Ther.* **9**, 787–797 (2002).
 50. Burgdorf, S., Leister, P. & Scheidtmann, K.H. TSG101 interacts with apoptosis-antagonizing transcription factor and enhances androgen receptor-mediated transcription by promoting its monoubiquitination. *J. Biol. Chem.* **279**, 17524–17534 (2004).