

Antigen Loading of MHC Class I Molecules in the Endocytic Tract

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Major histocompatibility complex (MHC) class I molecules bind antigenic peptides that are translocated from the cytosol into the endoplasmic reticulum by the transporter associated with antigen processing. MHC class I loading independent of this transporter also exists and involves peptides derived from exogenously acquired antigens. Thus far, a detailed characterization of the intracellular compartments involved in this pathway is lacking. In the present study, we have used the model system in which peptides derived from measles virus protein F are presented to cytotoxic T cells by B-lymphoblastoid cells that lack the peptide transporter. Inhibition of T cell activation by the lysosomotropic drug ammoniumchloride indicated that endocytic compartments were involved in the class I presentation of this antigen. Using immunoelectron microscopy, we demonstrate that class I molecules and virus protein F co-localized in multivesicular endosomes and lysosomes. Surprisingly, these compartments expressed high levels of class II molecules, and further characterization identified them as MHC class II compartments. In addition, we show that class I molecules co-localized with class II molecules on purified exosomes, the internal vesicles of multivesicular endosomes that are secreted upon fusion of these endosomes with the plasma membrane. Finally, dendritic cells, crucial for the induction of primary immune responses, also displayed class I in endosomes and on exosomes.

Key words: B cells, dendritic cells, endosomes, exosomes, MHC class I

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Major histocompatibility complex (MHC) class I and class II molecules have evolved to capture peptides proteolytically derived from endogenous or exogenous protein sources, respectively (1–3). Endogenous antigenic peptides are generated in the cytosol by the proteasome (4), and these peptides enter the lumen of the endoplasmic reticulum (ER) by translocation through the transporter associated with antigen processing (TAP) (5–7). MHC class I molecules, consisting of heavy chain (HC)/ β_2 -microglobulin (β_2m) heterodimers, require stabilization by antigenic peptides to allow transport from the ER to the Golgi complex and plasma membrane for presentation to CD8⁺ T cells. Proteolytic processing of exogenous proteins, as well as binding of generated peptides to MHC class II molecules, occur in the endocytic system (8,9). MHC class II molecules, consisting of two transmembrane glycoproteins α and β , are expressed by antigen-presenting cells (APCs), like B cells, macrophages and dendritic cells (DCs). The majority of intracellular MHC class II molecules is located in endosomal/lysosomal compartments (10), which have been termed MIIcCs, for MHC class II-enriched compartments (11,12). Peptide loading can occur in different types of endocytic compartments, comprising early endosomes (EEs), late endosomes (LEs) and lysosomes, depending on cell type and source of antigen (8,13–15).

In the last few years, several studies have shown that exogenous peptides can also be presented in the context of class I molecules, a phenomenon with major implications for the peptide repertoire that can be presented to cytotoxic T cells (16–18). Antigen, taken up via phagocytosis or macropinocytosis by either macrophages or DCs, can enter the classical pathway for presentation by class I (19–21), either by possible membrane rupture of the phagosome (17) or by a selective transport mechanism (22). This presentation pathway requires the proteasome machinery, TAP, and newly synthesized class I molecules (23,24). On the other hand, there have been several reports on TAP-independent processing of antigens for CD8⁺ T cell recognition (25–27), as demonstrated for peptides derived from exogenously applied inactivated virus particles, virus-like particles and glycopeptides (16,18). In contrast to TAP-dependent presentation, this pathway requires only low-antigen doses to elicit a response and is inhibited by an increase of the endosomal pH, i.e. by the lysosomotropic drug ammoniumchloride (NH_4Cl), and independent of newly synthesized class I molecules. These observations suggest a role for endocytic compartments in peptide binding to recycling class I molecules.

The purpose of the present study was to explore the presence and function of class I molecules in the endocytic system. We used human B-lymphoblastoid cell lines (B-

LCLs) that were infected with measles virus (MV). Recently, in this system, TAP-independent presentation of the viral envelope fusion protein F (MV-F) in the context of class I MHC has been demonstrated (28). In addition, NH_4Cl inhibited presentation of MV-F. Our analysis of the endocytic compartments in B-LCLs by immunoelectron microscopy (IEM) indicated that class I molecules co-localized with MV-F protein throughout the endocytic pathway, most prominently within multivesicular LEs. We did not find clearly distinct endosomal subtypes for either class I or II molecules, indicating that MIICs contain class I molecules. As we have demonstrated for class II molecules (29), one possible pathway for class I molecules to reach the cell surface may be by fusion of multivesicular LEs with the plasma membrane. Here we show that exosomes containing peptide-loaded class I molecules were indeed released by B cells. The presence of functional class I molecules in endosomes and on exosomes may greatly enhance the peptide-repertoire that can be presented to cytotoxic T cells, especially since we found that also blood-derived DCs, the most potent APCs, expressed class I molecules in endosomes and on exosomes.

Results

TAP-independent presentation of MV protein F by MHC class I molecules and sensitivity to the lysosomotropic drug NH_4Cl

As a model system to study the subcellular compartments involved in TAP-independent presentation of exogenously acquired antigens by class I molecules, we used the presentation of MV-F protein from MV. B-LCLs BM28.7 (parent cell line to BM36.1), TAP2 mutant BM36.1, and the TAP2 recon-

stituted BM36.1 were all transfected for stable expression of HLA-B27. Cytolytic T lymphocyte (CTL) clone WH-F40 specifically recognizes peptides from MV-F in the context of class I human leukocyte antigen (HLA)-B27 molecules (30). When pulsed with the specific peptide, all three cell types were killed with equal efficiency by CTL clone WH-F40, as shown for different effector: target ratios (Figure 1). Interestingly, a significant percentage of BM36.1 cells were lysed after infection with MV, despite the fact that they lack TAP2 (Figure 1B). In TAP2 reconstituted BM36.1 cells, the lysis percentage was similar to the parent cell line (Figure 1C). These results suggest that, apart from TAP-dependent presentation, an alternative pathway, independent of TAP, exists to present MV-F to CTL.

To investigate whether endocytic compartments were involved in the presentation of MV-F, we examined the effect of the lysosomotropic drug NH_4Cl on presentation efficiency in a proliferative assay. This drug neutralizes endosomal pH, thereby affecting protein recycling from endosomes and disturbing endosomal antigen processing (31). Because NH_4Cl prevents killing by CTLs, and thus cannot be used in the killing assay described above, we had to monitor T cell activation in a proliferation assay. The B-LCLs 8.16, 95.3, and JP were infected with MV in the presence or absence of NH_4Cl and, after fixation, used to stimulate the T cell clones WH-F24 and JP11.8. MV infection of cells occurred at neutral pH and was not affected by NH_4Cl (32, 33, and data not shown). Table 1 shows that NH_4Cl inhibited presentation of MV-F protein to class I-restricted T cells in different B cell lines, most dramatically in JP cells with 84% inhibition of T cell proliferation. We found a similar effect on presentation in

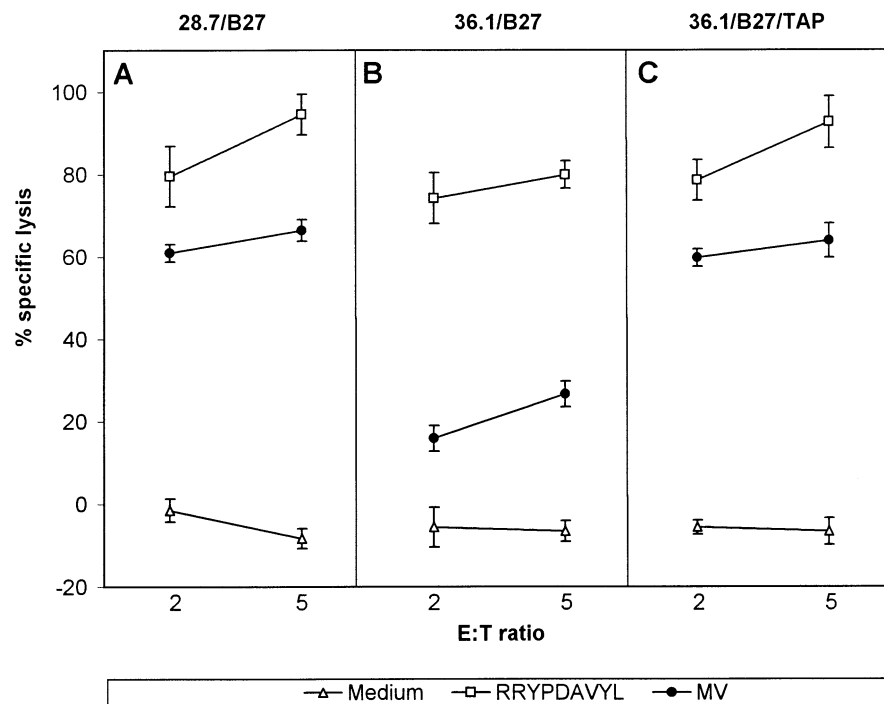


Figure 1: TAP-independent presentation of the MV-F protein by HLA-B27 molecules to human CTL. Parent cell line BM28.7 (A), TAP2 mutant BM36.1 (B), as well as the TAP2 reconstituted BM36.1 (C), all transfected for stable expression of HLA-B27, were infected with MV (closed circles), mock-infected (triangles), or pulsed with MV-F peptide (squares) as described. Cells were subsequently labeled with ^{51}Cr and used as targets for killing by the MV-F-specific and HLA-B27-restricted CTL clone WH-F40 at effector:target (E:T) ratios of 2:5. On the y-axis, the percentages of specific lysis (\pm SD) are depicted.

Table 1: Inhibition of MV-F presentation by NH₄Cl

	Medium cpm × 10 ³	MV cpm × 10 ³	MV+NH ₄ Cl cpm × 10 ³	% Inhibition
9.5.3	1.1	14.3 ± 0.6	9.3 ± 1.2	34
8.1.6	1.0	17.4 ± 0.3	8.1 ± 3.6	53
JP	0.1	18.8 ± 2.7	2.9 ± 0.3	84

Different HLA-B27⁺ B-LCLs were infected with MV in the absence or presence of NH₄Cl. After fixation, cells were used as APCs in a proliferative assay with T cell clones WH-F24, WH-F40, or JP111.8. Numbers represent ³H-thymidine uptake (cpm × 10³) of representative experiments. The right column shows the percentage of inhibition of T cell proliferation by NH₄Cl.

the presence of the lysosomotropic drug chloroquine (data not shown), and together, these data demonstrate that endosomes play an important role in the presentation of MV-F protein by class I. Control experiments showed that NH₄Cl did not have any effect on the viability of the APCs, nor on the expression of class I at the cell surface as measured by flow cytometry analysis (not shown). Similarly, total DNA and protein synthesis as measured by ³[H]-TdR and L-[4,5-³H]leucine incorporation, respectively, were not affected (data not shown).

Co-localization of MV-F and class I in endosomes

To study the endosomal distribution of class I and MV-F, we used IEM on JP cells, which showed the most dramatic reduction of MV-F presentation by NH₄Cl (Table 1). JP cells, infected with MV for 24 h, were fixed and ultrathin cryosections were labeled with antibodies for class I HC and MV-F. We found that class I and MV-F co-localized at the cell surface (Figure 2A) and in multivesicular endosomes (Figure 2B,C). Also, class I molecules in the TAP mutant cells BM36.1 were located in endosomes (data not shown), suggesting that these compartments may indeed play a role in the generation of MV-F peptides and their loading onto class I molecules. Since class I was frequently observed in clathrin-coated pits and vesicles in close proximity to the cell surface (Figure 3A), the endosomal class I molecules might be internalized from the plasma membrane. To investigate the origin of endosomal class I further, JP cells were treated with cycloheximide for 6 h to block the supply of newly synthesized class I molecules (16,18). Quantitation of class I immunogold labeling revealed that the number of gold particles for class I in the endocytic tract did not change upon cycloheximide treatment. Both in control and cycloheximide-treated cells, the average number of gold particles per LE/lysosome was eight, whereas the biosynthetic pathway in cycloheximide-treated cells was devoid of class I labeling, and cell surface expression had decreased by 40%. In contrast, class II labeling (illustrated for non-treated JP cells in Figure 3B) in the same cell samples had decreased by 50% in endosomal/lysosomal compartments. These data suggest that, unlike class II molecules, the majority of endosomal class I molecules did not derive from *de novo* synthesis and represented recycling molecules.

Characterization of class I-positive endosomes

In a previous IEM study on the human B-LCL JY, we were unable to detect appreciable amounts of class I molecules in late endocytic compartments. In the light of the present data, we further explored the occurrence of class I molecules in endocytic compartments of the B cell lines JP and RN, and reinvestigated JY cells. By morphological criteria and marker distribution, three major types of endocytic compartments were distinguished: EEs, LEs, and lysosomes (see 12 for detailed characterization). The most prominent class I-positive structure in JP (Figure 2B,C, Figure 3B) and RN cells (Figures 4 and 5) were multivesicular LEs, but in addition EEs, identified by double-labeling for the EE marker transferrin receptor (TfR) (Figure 4A), and lysosomes (Figure 4C) contained significant class I labeling (Table 2). Importantly, the labeling patterns of β₂m and HC were similar (Figure 5A, inset), suggesting that functional class I molecules could be formed in endosomes. In JY cells, the labeling of class I in endosomes was very low, of which most was found in EEs (Table 2). The data confirm our previous findings in these cells (11). Multivesicular LEs were distinguished from lysosomes mainly by morphological criteria, i.e. the presence of many internal membrane vesicles or membrane sheets (12), respectively, and the arrival time of endocytic tracers (12,29). Furthermore, the lysosomal membrane proteins LAMP-1 and CD63, although present in both compartments, were more abundant on lysosomal membranes (Figure 4B,C). The relative distribution of class I molecules over plasma membrane, biosynthetic organelles, and endosomal compartments indicated that, in the RN cells, 5% of the total gold particles were present in endosomes and lysosomes, while 3% were located in ER and Golgi membranes. As expected, the majority (92%) were present at the cell surface. In contrast, countings on BM36.1 showed that 61% of the total class I was present on membranes of the ER and Golgi complex, due to insufficient peptide loading and retention of class I in the biosynthetic pathway in the absence of TAP. Still, we found that 34% of the labeling was present on the plasma membrane and 3% in endosomes and lysosomes, indicating that also in TAP mutant cells, class I molecules can reach endosomes.

Presence of class I molecules in MIICs

Endocytic compartments in human B cells are enriched in class II molecules, and are termed MIICs (11,12). The observation that many of the endocytic compartments in B cells were class I-positive prompted us to examine whether class I and II molecules localized to the same compartments or if subtypes existed. As indicated above, in JY cells, only EEs displayed significant class I labeling and the overlap with class II labeling was minute. However, in JP (Figure 3B) and RN cells (Figure 5), class I and II molecules co-localized in all types of endocytic compartments, including EEs, LEs, and lysosomes. Class I molecules also co-localized with invariant chain, a chaperone that mediates transport of newly synthesized class II molecules to the endocytic pathway (Figure 5B), suggesting that class I and II molecules come together in the same endocytic compartments.

Transport of class I from endosomes to the extracellular milieu via exocytosis of LEs

We have previously described that in B cells, multivesicular LEs can fuse with the plasma membrane (29). As a consequence, class II molecules present in the limiting membrane of LEs are incorporated in the plasma membrane, whereas the internal vesicles of LEs, which also contain functional class II molecules, are released into the extracellular medium as so-called exosomes (29,34). To determine whether endosomal class I molecules follow a similar exocytic pathway, we analyzed the class I content of exosomes by IEM and biochemically. The term exosomes was first described in reticulocytes, which release 50–80-nm small-membrane vesicles during their maturation into red blood cells (35). In

reticulocytes secretion of these vesicles, which originate from multivesicular compartments of the endocytic pathway, is a way to eliminate internalized plasma membrane proteins, such as TfR (36,37). Recent studies show that exosomes of B-LCLs and DCs, as well as the internal vesicles of LEs, are enriched in tetraspan proteins (CD37, CD53, CD63, CD81, CD82), class II molecules, and heat-shock protein hsc73, but are poor in LAMP-1 and HLA-DM, which are primarily located at the limiting membranes of LEs and lysosomes (34, 38, 39, unpublished observations, M.J. Kleijmeer and M. Marsh, MRC Laboratory for Molecular Cell Biology, UCL, London). Recently, a study by Zitvogel et al. (40) showed that exosomes from mouse DCs have strong anti-tumor effects, probably mediated by class I presentation to cytotoxic T cells.

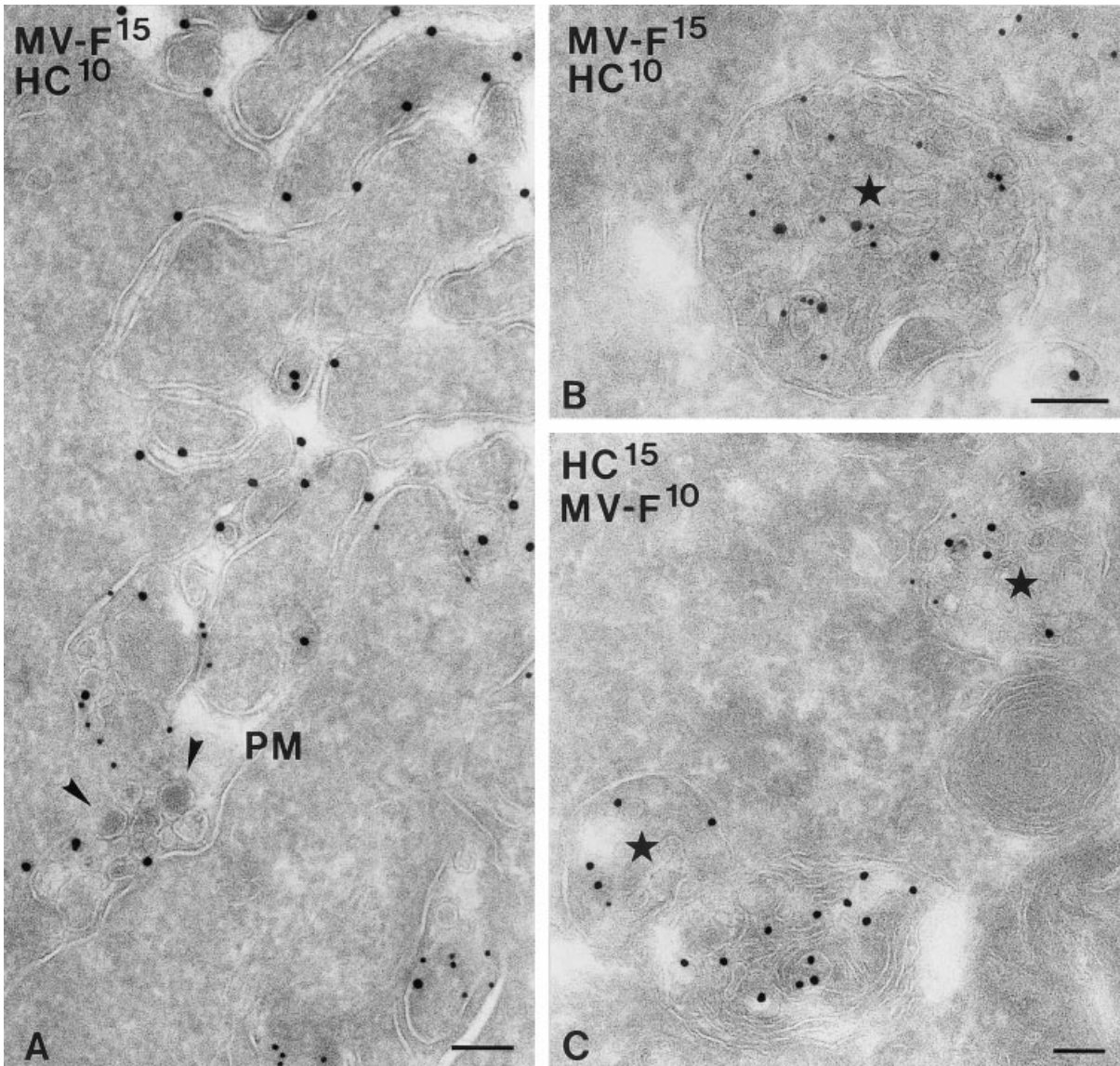


Figure 2: Subcellular localization of MV-F and class I heavy chain (HC) in JP cells. Ultrathin cryosections of MV-infected JP cells were double-immunolabeled for class I HC and MV-F with 10- and 15-nm gold particles, as indicated in the figures. A) Class I HC and MV-F are both present on the plasma membrane (PM). The arrowheads point at virus particles present at the extracellular face of the PM. B and C) Co-localization of MV-F with class I HC is observed in multivesicular compartments (stars). A and B 10 nm gold particle labels Class 1 HC, 15 nm gold particle labels MV-F. C 15 nm gold particle labels MV-F, 10 nm gold particle labels Class 1 HC. Bars, 100 nm.

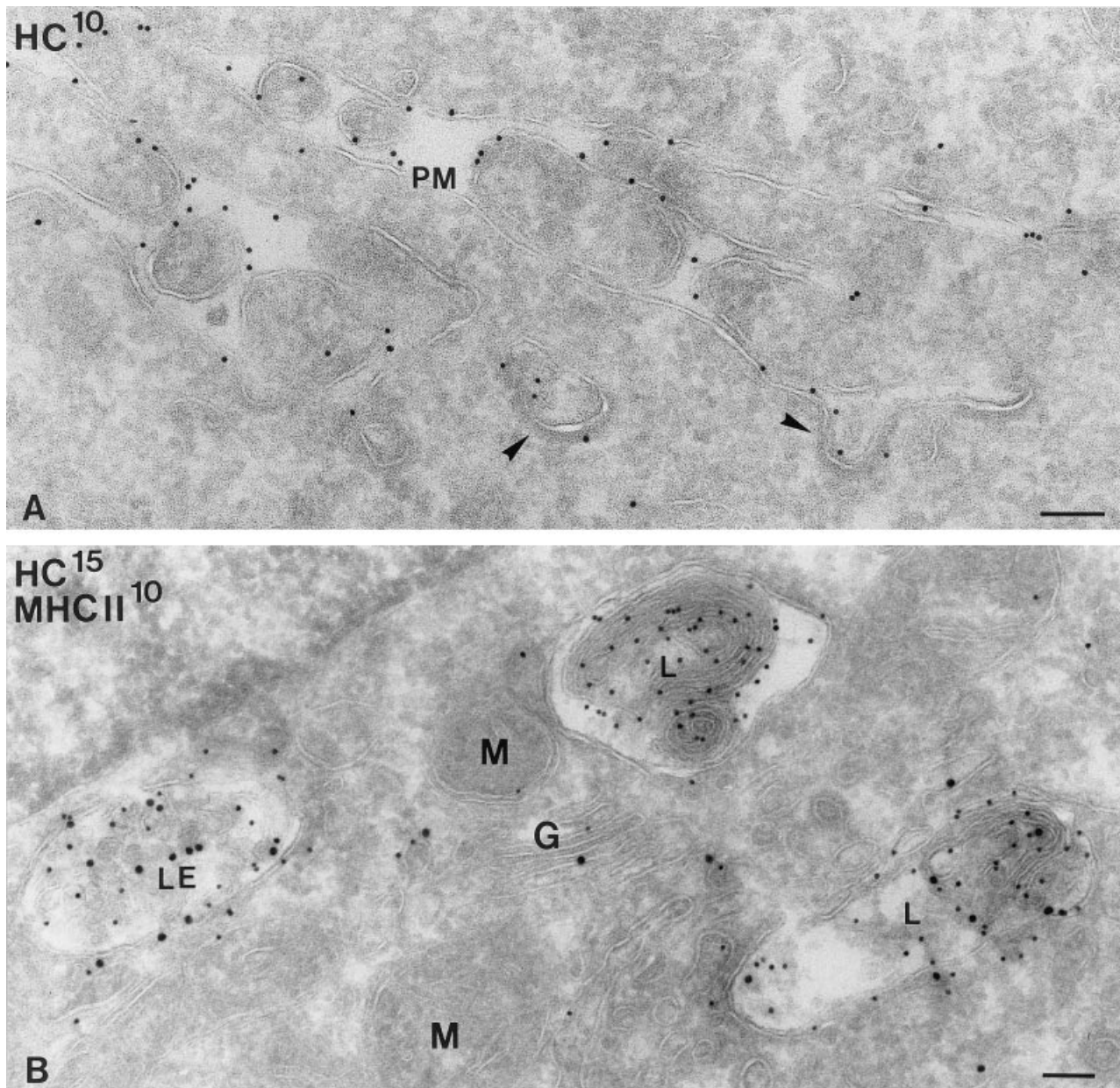


Figure 3: Class I in clathrin-coated pits and co-localization of class I and II in JP cells. A) Single-immunolabeling of class I HC on JP cells shows the presence of HC in a clathrin-coated pit and vesicle (arrowheads). PM, plasma membrane. B) Double-immunolabeling of HC (15 nm) and class II (10 nm) shows abundant labeling for both class I and II in multivesicular LE and multilaminar lysosomal (L) compartments. G, Golgi complex, M, mitochondrion. Bars, 100 nm.

Class I molecules have not been demonstrated on B cell-derived exosomes before, and interestingly, by IEM, RN cells showed many class I-carrying exosomes in exocytic profiles at the plasma membrane (Figure 6A). CD81, one of the exosomal tetraspan proteins (Figure 6A), localized to the same exosomal vesicles. To further explore these findings, exosomes were isolated from culture media of RN cells by differential centrifugation as described before (29,34). The exosome-enriched pellet (P_5 , obtained after a final centrifugation step at $70000 \times g$) was analyzed by whole-mount IEM and biochemically. Double-immunolabeling with antibody

W6/32, specifically recognizing complexed heterodimeric class I molecules (41) and with class II antibody showed the presence of both molecules on typical 50–80-nm exosomes (Figure 6B). These results indicate that exosomes contain functional class I molecules. Next, pellets P_1 (cells) to P_5 (exosomes) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting to study the presence of class I HC, β_2m , and TfR (Figure 7A,B,C, respectively). The amounts of these proteins recovered in P_1 and P_5 were determined and the ratios P_5/P_1 and P_5/P_1 were calculated. The P_5/P_1 value for TfR, an established

marker for plasma membrane and limiting membrane of EEs, but with a low abundance in internal LE vesicles and exosomes, was used as a reference to determine the relative enrichment of class I HC and β_2m in exosomes (15,16). As shown in Table 3, exosomes from RN cells exhibited a 6-fold enrichment of class I HC and a 2.4-fold enrichment for β_2m over TfR (Figure 7, Table 3). The lower enrichment factor for β_2m indicated that either a proportion of β_2m was lost during the experiment or that also free HCs were present in exosomes. As a control, P₁/P₅ values from the media of JY cells, which also secrete exosomes (29), were analyzed for the presence of class I HC. As expected from the low abundance of class I in multivesicular LEs of JY cells, no enrichment for the HC was found (Figure 7A).

Class I and II molecules co-localized to endocytic compartments and exosomes of DCs

An important role for loading of class I molecules with exogenous antigens exists in the event of cross-priming, whereby professional APCs induce cytotoxic T cells against foreign antigenic material *in vivo* (18). Since DCs are the most potent APCs and are likely to be involved in cross-priming (42), we next examined the presence of class I molecules in endocytic compartments of these cells. DCs exist in two states: as immature cells that endocytose very efficiently and process antigens and as mature cells specializing in antigen presentation (42). To obtain DCs, human monocytes were treated with GM-CSF and IL-4 for 12 days. Maturation of these cells was induced by incubation in a mixture of tumor necrosis factor (TNF) α , lipopolysaccharide, and CD40 anti-

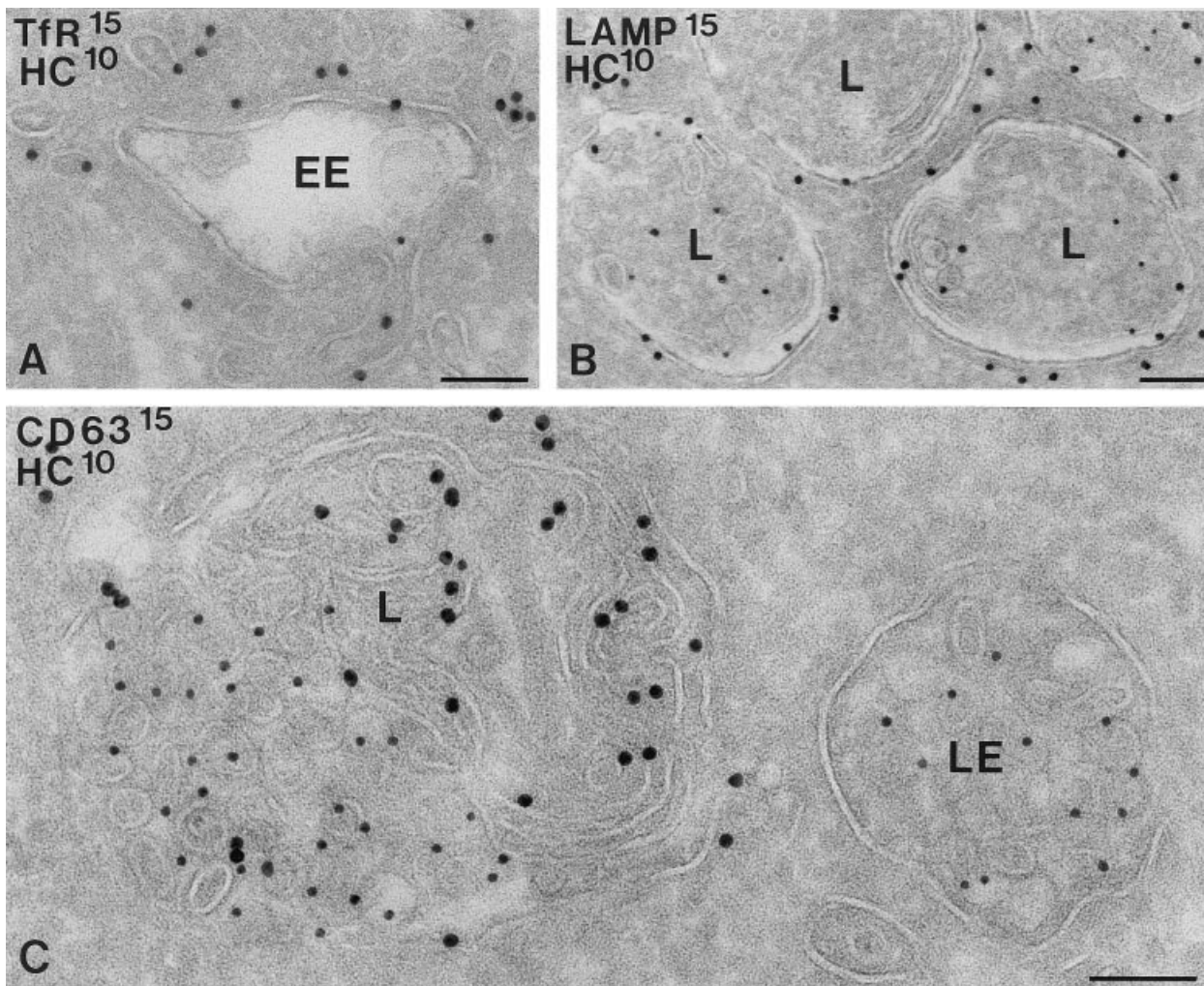


Figure 4: Characterization of class I-positive compartments in JY and RN cells. A) Ultrathin cryosection of JY cells was double-immunogold-labeled for HC (10 nm) and TfR (15 nm), showing abundant TfR labeling in an EE, and few gold particles for HC. B) Cryosection of RN cell double-immunogold-labeled for HC (10 nm) and LAMP-1 (15 nm). Three lysosomes (L), with internal membranes and electron-dense material, show labeling for LAMP-1 primarily at the limiting membrane, and HC labeling enriched on the internal membranes. C) Cryosection of RN cell double-immunogold-labeled for CD63 (15 nm) and HC (10 nm). CD63 is localized to the limiting and internal membrane sheets of the lysosome, whereas HC labeling is enriched on the internal vesicles of the lysosome and the LE on the right. Note that the internal vesicles and sheets are separated within the lysosome, suggestive of a recent fusion event between an LE and lysosome. Bars, 100 nm.

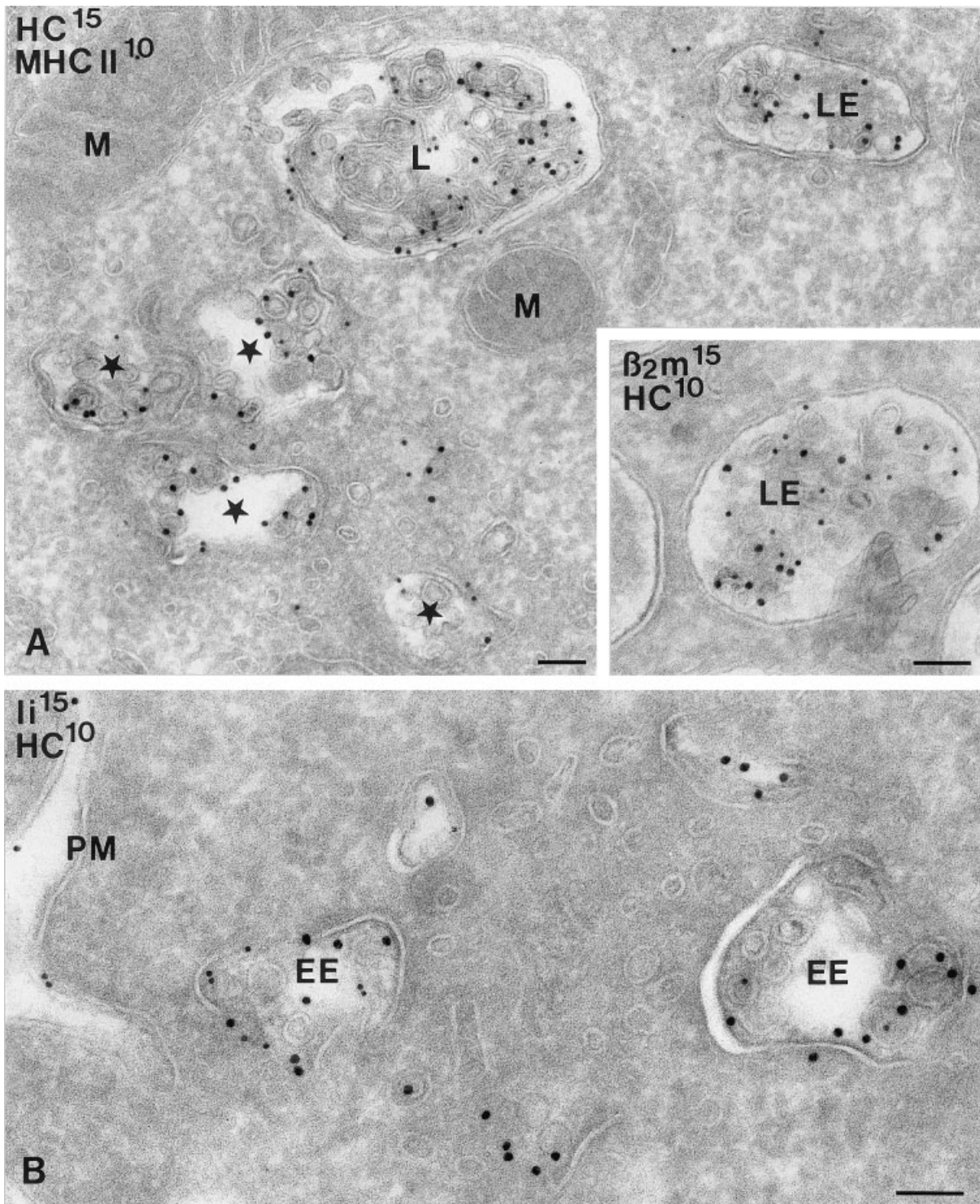


Figure 5: Co-localization of class I HC with β_2m , class II and li in endocytic compartments. Ultrathin cryosections of RN cells were double-immunogold-labeled with antibodies against HC and β -chain of class II (A), HC and β_2m (A, inset), HC and invariant chain (li) (B). A) HC (10-nm gold particles) and class II (15-nm gold particles) co-localize in EEs (stars), LEs, and Lysosomes (Ls). The inset shows a multivesicular LE with HC and β_2m labeling. B) EEs, abundantly labeled for li (15 nm), are also positive for HC (10 nm). PM only contains gold particles for HC. Bars, 100nm.

body for 48 h (43). Immunolabeling showed that class I HC was present in both EEs and multivesicular LEs, together with class II molecules (Figure 8A, Table 2). Of the total class I labeling present in these cells, 9% was located in endosomes. In agreement with the presence of class I in LEs, DCs are able to secrete exosomes (39,40). We show now that class I and II molecules were located together on DC exosomes (Figure 8B). Since class I was also present in the limiting membrane of multivesicular LEs (Figure 8), exocytosis of these compartments represents a pathway of insertion of endosomal class I molecules into the plasma membrane of DCs.

Discussion

TAP-independent peptide loading of class I molecules has been reported to involve endocytic compartments (16,18,25,28,44–48). We have studied the presentation of MV-F via class I by B-LCLs to further explore the role and characteristics of such compartments. Stimulation of MV-F-specific CTL was shown to be partially independent from TAP, and required acidic endocytic compartments. IEM showed that class I molecules were present throughout the endocytic tract, i.e. in EEs, multivesicular LEs, and lysosomes, together with MV-F and class II molecules. Multivesicular LEs, containing both class I and II molecules, were shown to fuse with the plasma membrane and externalize the internal vesicles as exosomes. Secreted exosomes contained class I molecules, as shown by IEM and biochemical analysis. Importantly, also in DCs, the most potent type of APC, class I and II co-localized in endosomes and on exosomes.

MV-F protein is synthesized as a type I transmembrane glycoprotein, and is presented predominantly by class I MHC

Table 2: Distribution of MHC class I heavy chain (HC) in endocytic compartments

	EE		LE		L	
	Gold	%	Gold	%	Gold	%
JP	60	7 ± 1	483	58 ± 4	296	35 ± 4
RN	41	17 ± 1	137	56 ± 3	66	27 ± 4
JY	28	59 ± 1	11	24 ± 4	8	16 ± 4
DC	69	33 ± 0	108	52 ± 4	20	16 ± 5

Endocytic compartments were subdivided in three major types: early endosomes (EE), multivesicular late endosomes (LE) and lysosomes (L). The columns on the left below each compartment show the total number of gold particles counted in 20 cell profiles on this compartment. The respective percentages ± SEM, based on three countings of 20 cell profiles, are displayed in the right hand columns.

molecules to CD8⁺ T cells in a TAP-dependent fashion (30). Using TAP2 mutant cells, we demonstrate here that presentation can also occur independently of TAP, which is in agreement with data obtained on presentation of MV-F by the TAP-deficient cell line T2 (28). The weak base NH₄Cl inhibited class I-restricted MV-F presentation. Furthermore, class I HC, β₂m, and MV-F co-localized in endosomes, suggesting that MV-F could be processed and bound to class I somewhere in the endocytic tract. What fraction of the endosomal class I molecules is functional or destined for degradation (49) remains to be determined. The antibody that we used against class I HC did not allow us to distinguish between free or complexed HCs and antibodies recognizing peptide-loaded class I molecules failed to react in immunolabeling on ultrathin cryosections. However, a subcellular fractionation study on MeJuSo cells (28) has shown that LEs and lysosomes contain mature class I molecules by immunoprecipitation with conformation specific W6/32 antibody, which recognizes mature heterodimeric class I complexes. This antibody also showed reactivity in our whole-mount IEM on exosomes, implying that class I molecules in multivesicular LEs contain peptide. Another strong indication that class I molecules on exosomes are functional comes from a study on DC-derived exosomes, which are capable of stimulating CD8⁺ T cells *in vitro* (40).

Endosomal class I molecules are probably not derived from the biosynthetic pathway (50,51), but represent recycling surface molecules (52–56), since a blockade in protein synthesis for 6 h with cycloheximide did not lower the level of class I in endocytic compartments. In addition, class I was present in clathrin-coated pits of the plasma membrane. This is in agreement with observations that an endosomal pathway of class I-restricted presentation is not affected by cycloheximide, nor by brefeldin A, an agent interfering with transport of newly synthesized proteins from the ER (16,44,47,57). MHC class I loading with MV-F-derived peptides in endosomes most likely involves peptide exchange of pre-loaded class I. Indeed, that class I molecules are able to exchange peptides at acidic pH is indicated by the fact that at pH 5, which corresponds with the pH of LEs and early lysosomal compartments, peptide-receptive 'empty' class I molecules can be generated (28).

LEs and lysosomes play a major role in class II antigen presentation, a reason why these compartments in APCs have been collectively termed MIICs (11). MIICs were first described in the B cell line JY, which harbors almost no endosomal class I molecules, suggesting an exclusive role of these compartments in class II presentation (11). However, our present observations show that JY cells are exceptional among 10 different B cell lines with respect to the low abundance of class I in endosomes (present study, C. Rabouille, unpublished observations). Changes in rates of endocytosis and degradation of class I molecules might be influenced by the cell's state of differentiation, which may explain differences in the amount of endosomal class I molecules (52). Since DCs also contain class I molecules in endosomes (40, present study), it is more likely that endo-

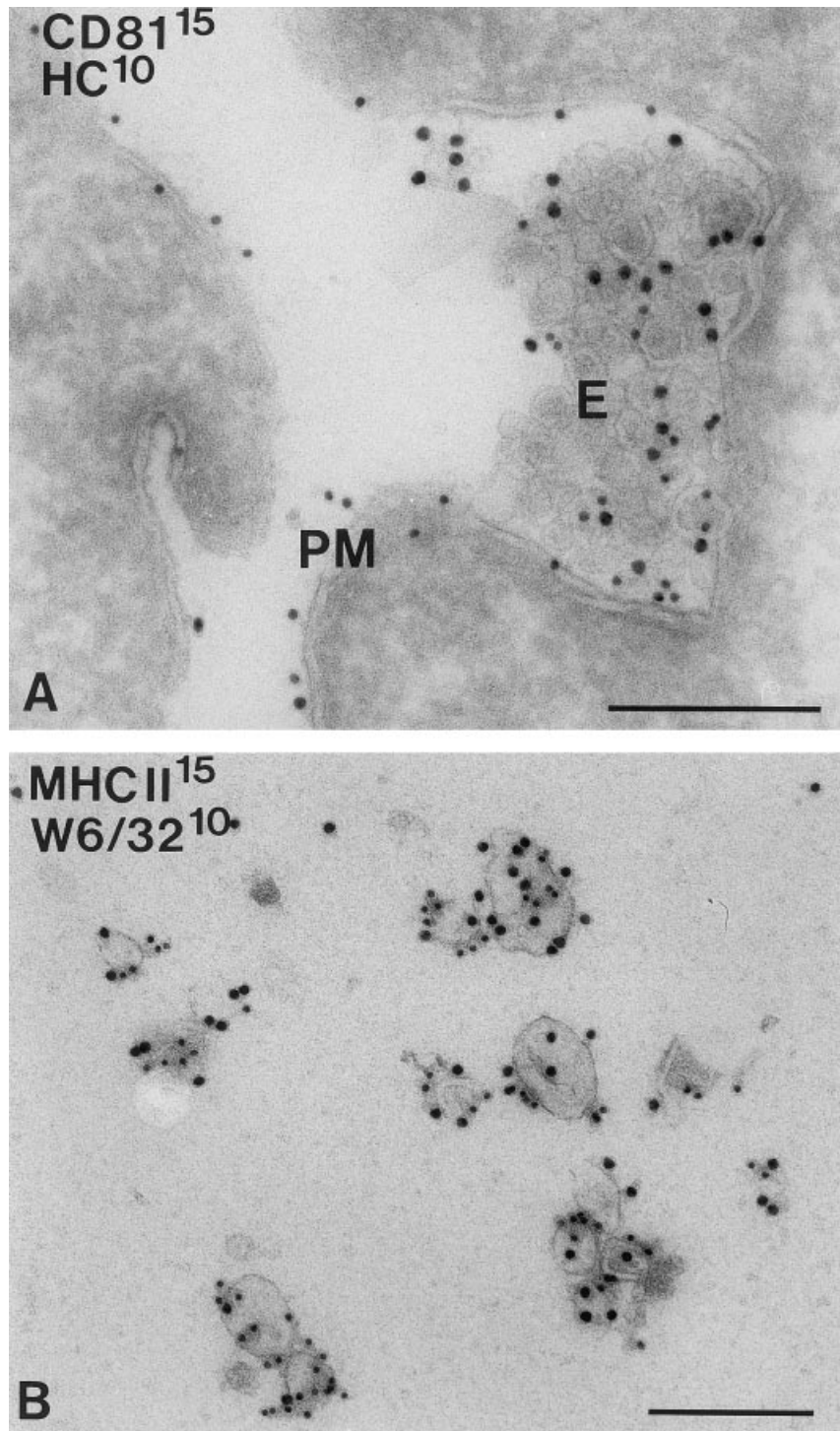


Figure 6: Class I immunolabeling of exosomes. PM of RN cell, with exosomes (E) associated at the external face in a fusion profile. The exosomes are labeled for HC (10 nm) and the tetraspan protein CD81 (15 nm). B) Whole-mount EM of isolated RN exosomes, immunolabeled with class II antibody (15 nm) and W6/32, specific for antigen-loaded class I (10 nm). Bars, 200 nm.

cytic compartments in APCs are not solely involved in class II functioning, but in addition, may serve in the processing and loading of exogenous antigen onto class I.

A physiological role for class I molecules in endosomes may be the event of cross-priming, whereby cytotoxic T lymphocytes are primed for antigens derived from cells in the

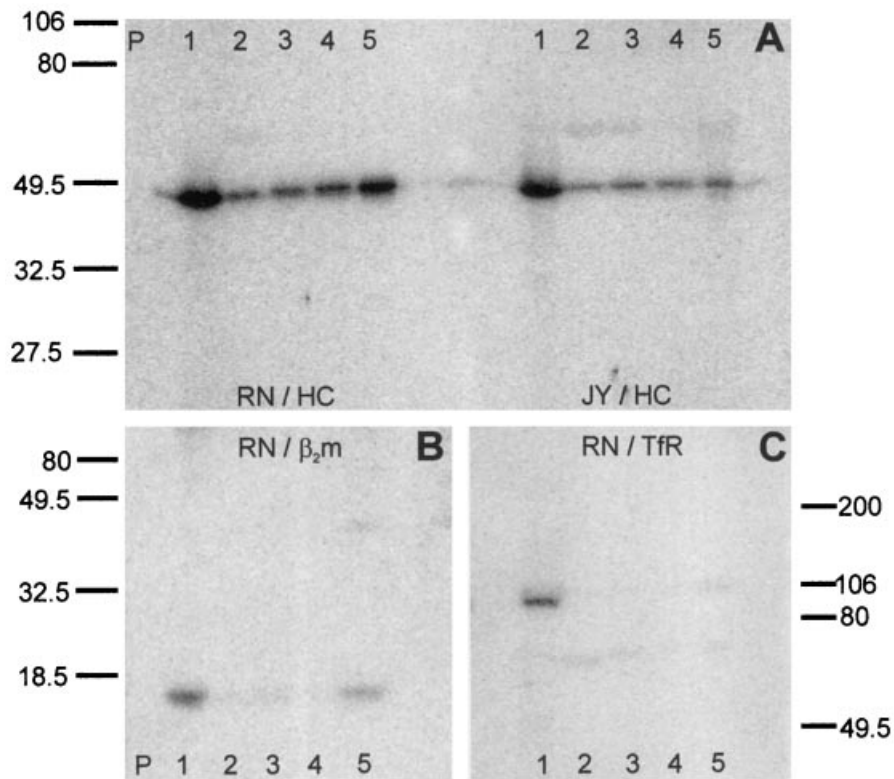


Figure 7: Detection of class I molecules on exosomes by Western blotting. Exosomes were isolated from RN and JY culture media by differential centrifugation, resulting in the pellets P₁ (cells), P₂₋₄, P₅ (exosomes). The pellets were analyzed by SDS-PAGE and Western blotting. HC and β₂m are both present in P₅ of RN cells (A, B), whereas TfR is absent (C). JY cells show no enrichment of HC in P₅ (A).

periphery (58,59). It has been shown that this presentation pathway requires professional APCs and CD4⁺ T helper cells, which indirectly prime cytotoxic T cells (60,61). Recently, it was found that the help provided by these T cells could be replaced by signaling through CD40 (62,63), which implicates that APCs may directly cross-present cellular antigens to CD8⁺ T cells. These cellular antigens, which can be derived from apoptotic bodies (64), can enter the APC via different mechanisms of endocytosis (18,65), and the association of antigenic peptides with class I molecules can occur either via the TAP-dependent pathway, or in endocytic compartments. Since we and others find class I molecules in endosomes of DCs (present study, 40), the latter mechanism could indeed be valid in the most potent APC of all.

The presence of class I molecules on exosomes indicates that peptide-loaded class I may reach the cell surface via fusion of multivesicular LEs with the plasma membrane. Exosomes can be isolated from the culture medium by differential centrifugation (29). The selective enrichment of proteins, like tetraspanins, hsc 73, co-stimulatory and MHC molecules in exosomes (29,34,39) suggests that they serve a role in the immune response. It has been shown that B cell-derived exosomes can activate CD4⁺ T cells *in vitro*, and interestingly exosomes from DCs are able to eradicate

established tumors in mice (40). The mechanism underlying this process is largely unknown, but does require the presence of class I on exosomes. From our biochemical and IEM data, it is clear that exosomes derived from B cells and DCs (40) express abundant mature class I molecules, implying that they have peptide-binding capacity, and could play a role in the class I-restricted immune response.

Table 3: Relative enrichment of class I HC and β₂m over TfR in exosomes

	P ₅ /P ₁	Enrichment over TfR (fold)
Class I HC	0.59 ± 0.16 (6)	6.09 ± 1.62
Class I β ₂ m	0.23 ± 0.13 (6)	2.41 ± 1.41
TfR	0.10 ± 0.01 (3)	1

The signals from P₅ and P₁, obtained as in Figure 7, were quantified with a PhosphorImager and the average ratios (P₅/P₁ ± SD) obtained from independent experiments are presented in the first column (number of determinations in parentheses). The relative enrichment of HC and β₂m was determined by normalizing their ratio P₅/P₁ value to that of TfR.

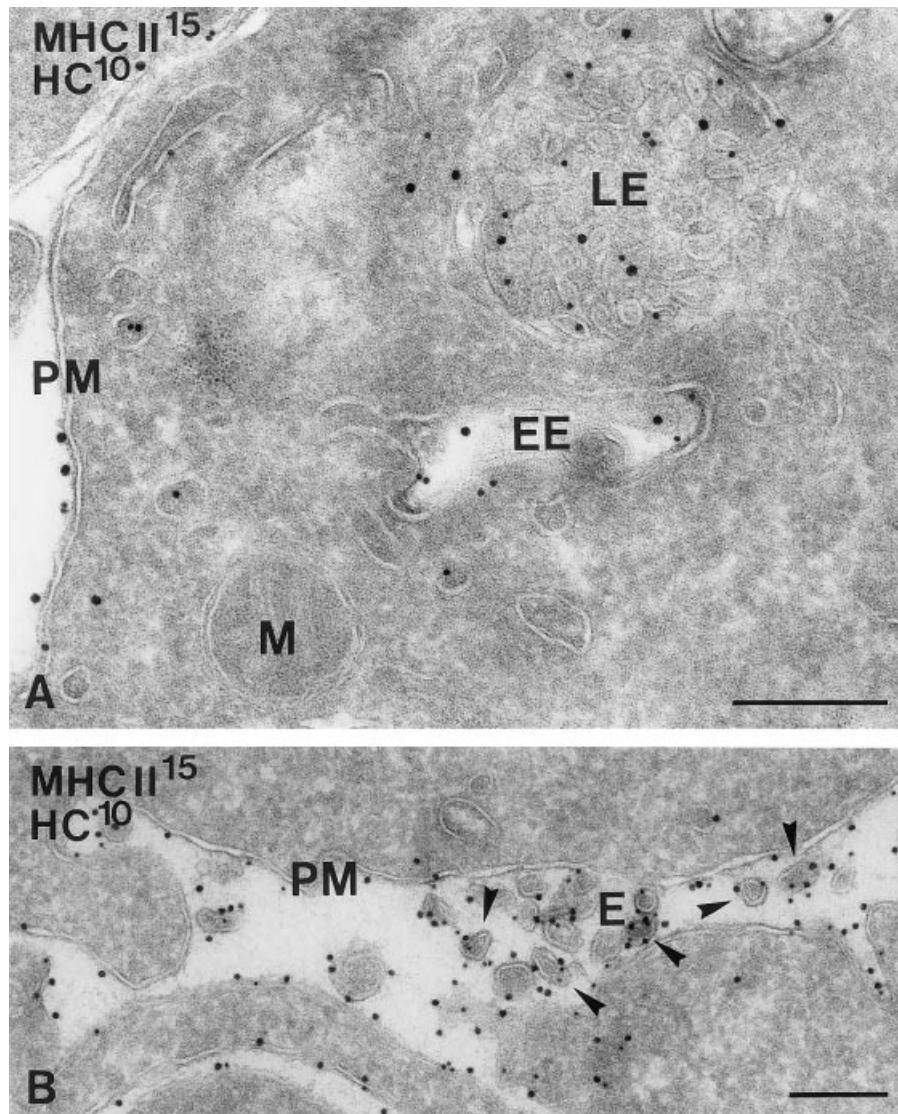


Figure 8: Class I and II in endosomes and on exosomes of human blood-derived DCs. DCs were isolated from blood as described and ultrathin cryosections were immunolabeled for class I HC and class II molecules. A) Class I HC (HC; 10 nm) co-localizes with class II molecules (15 nm) in an EE and multivesicular LE and on exosomes (arrowheads) located at the extracellular site of the PM in B. Bars, 200 nm.

Materials and Methods

Cells

The Epstein–Barr virus-transformed B-LCLs 8.16, 9.53, JP, as well as the TAP2 mutant cell line BM36.1 and its parent cell line 28.7, were used as APCs (30,66,67). RN and JY were maintained as described previously (29). The construction and characterization of HLA-B27 transfectants of BM36.1 and 28.7 have been described previously (30). Reconstitution of HLA-B27⁺ BM36.1 with a functional TAP2 molecule was established by transfection of TAP2 cDNA (gift of Dr J. Trowsdale, Cambridge, UK) ligated in the episomal expression vector pREP8, containing the histidinol resistance gene (InvitroGen).

DCs were generated from human peripheral blood mononuclear cells (PBMC) as described by Romani et al. (43). Briefly, PBMC were prepared from buffy coats of healthy donors by Ficoll–Paque density gradient centrifugation (Pharmacia and Upjohn, Uppsala, Sweden). T

cells were removed by two rounds of rosetting at 4°C with sheep erythrocytes. The remaining cells were resuspended at 3–4 × 10⁶ cells/ml in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Scotland) supplemented with 8% heat-inactivated fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT) and distributed in 6-well tissue-culture plates (Costar, Badhoevedorp, the Netherlands) at 3 ml/well. After 2 h incubation at 37°C, the plates were gently whirled and non-adherent cells were washed away. The remaining and loosely adherent cells were cultured in 3 ml of RPMI containing 8% FCS, 500–1000 U/ml recombinant human interleukin-4 (Intergen, New York, NY) and 800 U/ml recombinant human GM-CSF. Half of this culture medium was replenished every second day. After 12 days in culture, DCs were harvested and incubated with a mixture of 50 ng/ml lipopolysaccharide (Braunswieg), 30 ng/ml TNF α (Genzyme, Cambridge, MA) and 25 μ g/ml anti-CD40 (clone S2C6, Mabtech AB, Sweden) for 48 h and finally fixed for IEM.

Antibodies

The following monoclonal antibodies were used: anti- β_2m BBM-1 (kind gift of Dr J.J. Neefjes, NKI, Amsterdam, the Netherlands), H68.4 against TfR (Zymed Lab., San Francisco, CA), CLB gran 1/2, 435 against CD63 (CLB, Amsterdam, the Netherlands), H4A3 against LAMP-1 (CD107a; Pharmingen, San Diego, CA), and W6/32 (DAKO, Glostrup, Denmark) against MHC class I heterodimeric complexes. The rabbit polyclonal antibodies were: anti-TfR (kind gift of Dr A. Schwartz), 631 69 to β_2m , anti-MHC class I HC (both kind gifts of Dr J.J. Neefjes, NKI), anti-MHC class II (kind gift of Dr H.L. Ploegh, Department of Pathology, Harvard Medical School, Boston, MA), anti-luminal epitope of invariant chain antibody ICC5 (68; kind gift of Dr P.A. Morton, Monsanto, St Louis, MO), K9 against MV-F (obtained from RIVM, Bilthoven, the Netherlands), and anti-IgG from DAKO.

Presentation assays

MV-infected or peptide-pulsed APCs were used as target cells in a ^{51}Cr -release assay or, after fixation, as stimulator cells in a proliferative T cell assay as described (30). The CD8⁺ CTL clones WH-F24 and WH-F40 were HLA-B*2705-restricted and MV-F protein (aa sequence RRYPDVAVYL)-specific (30). JP III.8 was a CD8⁺, class I-restricted MV-F protein-specific T cell clone obtained from a patient with acute measles (30). To investigate the effects of lysosomotropic drugs on MHC class I-restricted presentation of the MV-F protein, the HLA-B27⁺ APCs 8.16, 9.53 and the autologous JP B-LCLs were infected with MV in the presence or absence of NH₄Cl (20 mM in RPMI-1640, 1% FBS) for 1 h, washed twice, and further cultured in RPMI, 10% FBS, and the indicated concentration of NH₄Cl, where after the cells were fixed with paraformaldehyde (30).

IEM

Cells were fixed and prepared for ultrathin cryosectioning and immunolabeling as described previously (69). Briefly, cells were fixed in 2% paraformaldehyde or in a mixture of 2% paraformaldehyde/0.2% glutaraldehyde in phosphate buffer. After washing with phosphate PBS and PBS/50 mM glycine, cell pellets were embedded in 10% gelatin, cut in small blocks, and infiltrated with 2.3 M sucrose at 4°C for 4 h. Finally, the blocks were mounted and frozen in liquid nitrogen. Ultrathin cryosections were indirectly immunolabeled with 10-nm protein A gold particles in single-immunolabeling experiments and with 10 and 15 nm in double-immunolabeling experiments. Sections were embedded in a mixture of 2% methyl cellulose and 0.4% uranyl. When indicated, JP cells were incubated with 100 μ g/ml cycloheximide for 6 h and then fixed and processed for IEM as described.

Semi-quantitative analysis of HC distribution

Ultrathin cryosections of JY and RN cells, and mature cytokine-derived DCs were single-immunolabeled with HC antibody and 10-nm protein A gold particles. In 3 \times 20 cell profiles of each cell type, gold particles present on endocytic compartments were counted when within a 20-nm range of a membrane and designated to EEs, multivesicular LEs, or lysosomes, as detailed in Table 2. To determine the relative distribution of class I molecules, gold particles were counted on membranes of ER, Golgi complex, plasma membrane, and endosomes. Ultrathin cryosections of control and cycloheximide-treated JP cells were immunolabeled with HC antibody and, in each quantitation, 50 endosomes/lysosomes were randomly selected. Plasma membrane length was measured by a point-hit method.

Isolation of exosomes

Exosomes were isolated by differential centrifugation as previously described (29,34). Briefly, RN cells were washed by centrifugation and re-cultured in fresh medium for 18 h. Cell culture media (35 ml)

containing about 5 \times 10⁷ cells were centrifuged once for 10 min at 200 \times g (pellet P₁), twice for 10 min at 500 \times g (pellet P₂), twice for 15 min at 2000 \times g (pellet P₃), once for 30 min at 10000 \times g (pellet P₄), and finally once for 60 min at 70000 \times g (pellet P₅) using a rotor SW27 (Beckman Instruments, Fullerton, CA). P₁ corresponds to cells and P₅ to the fraction enriched in exosomes. P₁-P₅ were directly solubilized in reducing or non-reducing SDS sample buffer, incubated for 5 min at 95°C, and submitted to SDS-PAGE and Western blotting. For whole-mount EM, membranes from P₅ were floated into a sucrose gradient, adhered to a grid, fixed in 2% paraformaldehyde and double-immunolabeled for class I and II molecules.

Immunoblotting

After SDS-PAGE, the proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA). The membranes were then blocked for 90 min in PBS containing 5% (w/v) non-fatty dry milk Protivar (Nutricia, Zoetermeer, the Netherlands) with 0.1% (w/v) Tween 20 (blocking buffer) and reacted for 90 min with the primary antibody, followed by detection with 0.1 μ g/ml of ¹²⁵I-labeled recombinant protein-G (Zymed Lab.) for 90 min. For detection of monoclonal antibodies, a rabbit anti-IgG was used as an intermediate. ¹²⁵I was detected and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

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