The Journal of Immunology

RESEARCH ARTICLE | SEPTEMBER 01 2003

In Vivo, Dendritic Cells Can Cross-Present Virus-Like Particles Using an Endosome-to-Cytosol Pathway [1] FREE

Víctor Gabriel Morón; ... et. al

J Immunol (2003) 171 (5): 2242–2250.
https://doi.org/10.4049/jimmunol.171.5.2242

In Vivo, Dendritic Cells Can Cross-Present Virus-Like Particles Using an Endosome-to-Cytosol Pathway[1]

Víctor Gabriel Morón,* Paloma Rueda,† Christine Sedlik,‡ and Claude Leclerc[2]*

Recombinant parvovirus-like particles (PPV-VLPs) are particulate exogenous Ags that induce strong CTL response in the absence of adjuvant. In the present report to decipher the mechanisms responsible for CTL activation by such exogenous Ag, we analyzed ex vivo and in vitro the mechanisms of capture and processing of PPV-VLPs by dendritic cells (DCs). In vivo, PPV-VLPs are very efficiently captured by CD8α^- and CD8α^+ DCs and then localize in late endosomes of DCs. Macropinocytosis and lipid rafts participate in PPV-VLPs capture. Processing of PPV-VLPs does not depend upon recycling of MHC class I molecules, but requires vacuolar acidification as well as proteasome activity, TAP translocation, and neosynthesis of MHC class I molecules. This study therefore shows that in vivo DCs can cross-present PPV-VLPs using an endosome-to-cytosol processing pathway. ***The Journal of Immunology,*** **2003, 171: 2242–2250.**

The priming of T cell responses relies on the presentation of Ag-derived peptides by MHC molecules. MHC class I-restricted T cell responses were, until recently, thought to target only Ags synthesized within the APC, for instance when they are infected by a pathogen. Thus, Ags that do not reach the cytosol of APCs should not elicit a CTL response. However, not all pathogens are capable of infecting APCs, and moreover, this mechanism cannot explain how CTL responses can be induced against tumor cells. It is now well established that at least some exogenous, noncytosolic Ags can gain access to the MHC class I pathway of APCs by cross-presentation (1) through several alternative pathways of processing (2). Both macrophages (Mϕ)[3] (3) and dendritic cells (DCs) (4) cross-present Ags, but only DCs are capable of stimulating naive CD8$^+$ T cells (5). Two main routes of cross-presentation have been proposed. One route involves the passage of Ags from endosomes to cytosol (also named cytosolic diversion) (6), while in the other route the Ags do not escape from endosomes, but are processed inside these vesicles (7, 8). The first route seems to be used mostly by DCs, and the second one by Mϕ (9). A third route, which uses the properties of certain viruses and bacterial toxins to gain direct access to cytosol, is based on the translocation of Ag from cell surface to cytosol (10, 11).

We have developed an Ag delivery system based on nonreplicative, recombinant parvovirus-virus-like particles (PPV-VLPs) formed by the self-assembly of the VP2 capsid protein of porcine parvovirus (PPV) (12, 13). The VP2 protein (14), carrying foreign CD8$^+$ T cell epitopes, self-assembles into 25-nm VLPs after expression in insect cells (13). Mice immunized with PPV-VLPs carrying a CD8$^+$ T cell epitope, in the absence of an adjuvant, develop a strong and specific MHC class I-restricted CTL response (13, 15). This CTL response protects against a viral challenge and is based on the induction of a high frequency of high avidity CTLs (16). PPV-VLPs target DCs with very high efficiency, whereas Mϕ and B cells have a poor capacity to capture these particles. Following an in vivo injection of PPV-VLPs, both CD8α^- and CD8α^+ DCs capture and process these particles, although they have different kinetics and T cell requirements (15). DC stimulation by PPV-VLPs induces phenotypic changes on CD8α^- DCs, leading to the acquisition of CD8α and CD205 and the loss of CD4 molecules as well as expression of costimulatory molecules on both DC subsets (15).

Most studies that aimed at studying the different steps of processing of exogenous Ags into the MHC class I pathway have been conducted using unpurified peritoneal exudate cells (17) (which contain various cell populations, including Mϕ and DCs) or bone-marrow derived DCs (18–20), which do not represent the whole spectrum of peripheral DCs. In the present report to decipher the mechanisms responsible for CTL activation by exogenous Ags, we analyze ex vivo and in vitro the mechanisms of capture and processing of PPV-VLPs by DCs, using DCs freshly purified from spleen. This study shows that DCs can use the endosome-to-cytosol pathway in vivo to cross-present exogenous Ags such as PPV-VLPs.

Materials and Methods

Mice

Six- to 8-wk-old female C57BL/6 (H-2^b) mice were obtained from Janvier (Le Genet St. Isle, France). Female TAP1 knockout mice, bred onto a C57BL/6 background, were a gift from Dr. A. Bandeira (Institut Pasteur, Paris, France). All animals were maintained under specific pathogen-free conditions.

*Unité de Biologie des Régulations Immunitaires, Institut National de la Santé et de la Recherche Médicale, E352, Institut Pasteur, Paris, France; †Inmunología y Genética Aplicada S.A., Madrid, Spain; and ‡Institut National de la Santé et de la Recherche Médicale Unité 520, Institut Curie, Paris, France

Received for publication December 31, 2002. Accepted for publication June 24, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This project is a collaborative work between Pasteur Institute and INGENASA, supported by European Commission Grant QLK2-CT-1999-00318. G.M. was supported by a postdoctoral fellowship from the Consejo Nacional de Investigaciones Científicas y Tecnológicas (Argentine), European Economic Community Grant QLK2-CT-1999-00429, and the Fondation de la Recherche Medicale (France). C.S. was supported by grants from the European Union (Contract BMH4-CT 98-3703).

[2] Address correspondence and reprint requests to Dr. Claude Leclerc, Departement d'Immunologie, Institut Pasteur, 25 rue du Docteur Roux 75724, Paris Cedex 15, France. E-mail address: cleclerc@pasteur.fr

[3] Abbreviations used in this paper: Mϕ, macrophages; BFA, brefeldin A; CCB, cytochalasin B; CHX, cycloheximide; DC, dendritic cell; DMA, dimethylamiloride; ER, endoplasmic reticulum; LLmL, *N*-acetyl-L-leucinal-L-methioninal; LLnL, *N*-acetyl-L-leucinal-L-norleucinal; OVA$_{257–264}$, a K^b-restricted epitope corresponding to aa 257–264 of chicken egg albumin; PPV-VLP, porcine parvovirus-virus-like particle; PPV-VLPs-OVA, porcine parvovirus-virus-like particles carrying the OVA$_{257–264}$ epitope; WGA, wheat-germ agglutinin; VLP, virus-like particle; HBsAg, hepatitis B virus small envelope protein.

0022-1767/03/$02.00

Peptides and cell lines

The peptide SIINFEKL, corresponding to aa 257–264 from chicken OVA ($OVA_{257–264}$ peptide), an immunodominant H-2^b-restricted CTL epitope, was purchased from Neosystem (Strasbourg, France). B3Z, a $CD8^+$ T cell hybridoma specific for the $OVA_{257–264}$ epitope in the context of K^b (21), was a gift from Dr. N. Shastri (University of California, Berkeley, CA).

PPV particles

The construction, characterization, and purification of recombinant and control PPV-VLPs were previously described in detail (13, 15). Briefly, the VP2 gene was expressed with the $OVA_{257–264}$ peptide plus natural flanking sequences (LEQLESIINFEKLTE) (the underlined sequence corresponds to the CTL epitope, and the nonunderlined sequences correspond to flanking sequences to the CTL epitope) from chicken egg OVA in its 5′ end (PPV-VLPs-OVA) or without this sequence (PPV-VLPs) using a baculovirus vector system. After infection of Sf9 insect cells, the recombinant VLPs were purified by salt precipitation with 20% ammonium sulfate, followed by dialysis. Characterization of PPV-VLPs-OVA and PPV-VLPs by CsCl sedimentation analysis and electron microscopy revealed properties identical to those of native PPV virions. In some experiments PPV-VLPs-OVA were labeled with the fluorochrome Alexa488, using the Alexa Fluor 488 Protein Labeling Kit (Molecular Probes Europe, Leiden, The Netherlands) following the manufacturer's instructions.

The concentration of PPV-VLPs-OVA was determined by densitometry and by double-Ab sandwich ELISA. The densitometric assay was conducted with 1D Image Analysis software 2.0.1. (Eastman Kodak, Rochester, NY) using BSA as reference. The double-Ab sandwich ELISA was performed as previously described (22), using as capture Ab the anti-PPV mAb 15C5 and as detection Ab the anti-PPV biotinylated mAb 13C6 (23). Highly purified PPV-VLPs from size exclusion chromatography were used as the standard reference.

Endotoxin values were determined in each sample of VLPs using the *Limulus* amebocyte lysate test (BioWhittaker, Walkersville, MD). For PPV-VLPs, endotoxin values were <0.5 ng/mg of protein (5 endotoxin units/mg), and for PPV-VLPs-OVA, they were <10 ng/mg (100 endotoxin units/mg). PPV-VLPs preparations contained minimal traces of DNA, as determined after DNAzol (Invitrogen, Paisley, U.K.) or phenol extractions and electrophoresis in agarose gel, although not enough to be quantified by spectrophotometry at $A_{260/280}$.

CyaA-E5-OVA

CyaA-E5-OVA is a genetically detoxified CyaA toxin carrying the $OVA_{257–264}$ peptide plus natural flanking sequences (PASIINFEKLGT) between Arg^{224} and Ala^{225} of the amino acid sequence (11).

Preparation of splenic DCs

Spleens were removed from mice and treated for 45 min at 37°C with 400 U/ml of collagenase type IV and 50 μg/ml of DNase I (Roche, Mannheim, Germany) in RPMI 1640. After inhibition of collagenase activity with 6 mM EDTA in PBS, spleens were dissociated in Ca^{2+}- and Mg^{2+}-free PBS in the presence of 2.5 mM EDTA and 0.5% FCS (Life Technologies, Paisley, Scotland). In all assays involving DCs, the same batch of endotoxin-free FCS (as determined by *Limulus* amebocyte lysate test) was used. Furthermore, all reagents were also tested for endotoxins. Single spleen cell suspensions were prepared and incubated with anti-CD16/32 (2.4G2 clone; BD PharMingen, San Diego, CA) and with colloidal superparamagnetic microbeads conjugated to anti-CD11c mAb (MACS-anti-CD11c, N418 clone; Miltenyi Biotec, Bergisch-Gladbach, Germany) following the manufacturer's instructions. $CD11c^+$ cells were positively selected with high speed magnetic cell sorting (program posseld, AutoMACS; Miltenyi Biotec). The purified DC preparations contained 3–10% autofluorescent cells (defined as double-positive cells in a FL2 vs FL3 dot plot, without Ab labeling). The purity of DC preparations (excluding autofluorescent cells) was always 95–99% (Fig. 1*A*). $CD11c^+$ cells were H-2 K^{b+}, I-$A^{b\,low}$, $CD40^{low}$, $CD80^{low}$, and $CD86^-$. Twenty-five to 30% were $CD8\alpha^+$, and 60–70% were $CD8\alpha^-$ $CD11b^+$.

Ag presentation assay

$CD11c^+$ spleen cells (10^5 cells/well) were first pulsed with Ag (PPV-VLPs-OVA, PPV-VLPs, or $OVA_{257–264}$ peptide) for 4 h in 96-well culture microplates in a final volume of 0.2 ml of RPMI 1640 Glutamax-I plus 5×10^{-5} M 2-ME, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (RPMI 10%; all from Life Technologies). The Ag concentration used in each experiment is indicated in the figure legends. Then DCs were washed three times with RPMI 10%, and 10^5 cells/well of B3Z hybridoma were added and incubated overnight at 37°C in 95% CO_2. The stimulation of B3Z cells was monitored by IL-2 release in the supernatants, which was measured using the CTLL-2 bioassay. Cells (10^4 /well) of the CTLL-2 cell line were cultured with 100 μl of supernatant in a final volume of 200 μl. Two days later, [^{3}H]thymidine (NEN Life Science, Boston, MA) was added, and the cells were harvested 18 h later with an automated cell harvester (Skatron, Lier, Norway). Incorporated thymidine was detected by cell scintillation counting. In all experiments each point was determined in duplicate. Results are expressed as counts per minute. In some experiments APCs were fixed before or after Ag pulse with 0.05% glutaraldehyde/PBS, then treated with 0.2 M lysine/RPMI and washed three times with RPMI 10%.

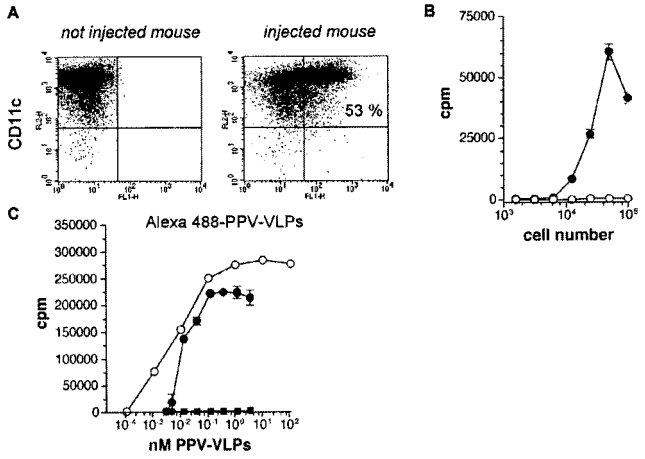

FIGURE 1. In vivo, PPV-VLPs-OVA are captured and presented by DCs, delivering the $OVA_{257–264}$ epitope into the MHC class I pathway. *A*, One C57BL/6 mouse was i.v. injected with 50 μg of Alexa488-PPV-VLPs. One PBS-injected mouse was used as a control. Ninety minutes later, $CD11c^+$ cells were purified from spleen by magnetic sorting, stained with PE-anti-CD11c, and analyzed on a FACStar cytometer. A representative dot-plot analysis of cells purified from PPV-VLP-injected or control mice is shown. *B*, C57BL/6 mice were i.v. injected with 100 μg of PPV-VLPs-OVA (●) or PPV-VLPs (○), and 90 min later, various numbers of DCs purified from their spleens were cocultured overnight with 1×10^5 B3Z cells/well. The presentation of $OVA_{257–264}$ peptide to B3Z cells was monitored by IL-2 production, measured by a CTLL-2 proliferation assay, and expressed as the mean ± SEM counts per minute of duplicate wells. *C*, Purified splenic DCs (10^5 cells/well) were incubated in vitro with PPV-VLPs-OVA (●), control PPV-VLPs (■), or $OVA_{257–264}$ peptide (○) for 4 h, washed, and cultured overnight with 1×10^5 cells/well of B3Z cells. The presentation of $OVA_{257–264}$ peptide to B3Z cells was monitored by IL-2 production, measured by a CTLL-2 proliferation assay, and expressed as the mean ± SEM counts per minute of duplicate wells.

Inhibition studies

For most inhibition studies, APCs (10^5 cells/well) were first incubated in 0.1 ml with each drug for 1 h. Then Ag diluted in 0.1 ml was added to the wells (0.2 ml final volume) at the final concentration indicated in the continuous presence of inhibitors for 4 h. APC were then washed three times and fixed with 0.05% glutaraldehyde, and the experiments were continued as described above. The inhibitors used were brefeldin A (BFA), *N*-acetyl-L-leucinal-L-norleucinal (LLnL), N-acetyl-L-leucinal-L-methioninal (LLmL), dimethylamiloride (DMA), chloroquin, cytochalasin B (CCB), primaquin, cycloheximide (CHX), chlorpromazine, filipin III (all from Sigma-Aldrich, St. Louis, MO), lactacystin (BIOMOL, Plymouth Meeting, PA), leupeptin, and pepstatin (Roche, Indianapolis, IN).

For inhibition of clathrin-mediated endocytosis by K^+ depletion following hypotonic shock, DCs (10^5 cells/well) were preincubated for 30 min in the presence of serum-free synthetic OptiMEM medium (Life Technologies), supplemented with 5×10^{-5} M 2-ME, 100 IU/ml penicillin, and 100 μg/ml streptomycin. DCs were then incubated for 5 min in hypotonic medium (OptiMEM mixed with Ultrapure H_2O, 50/50) and finally for 30 min in isotonic K^+-free (140 mM NaCl, 20 mM HEPES-NaOH, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml glucose, and 0.5% BSA) or K^+-containing (10 mM KCl, 130 mM NaCl, 20 mM HEPES-NaOH, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.5% BSA) buffer. Then 1.23 nM PPV-VLPs-OVA or 1.1 nM

$OVA_{257–264}$ were added. DCs were incubated for 1 h in the continued presence of the drugs and Ags. Then DCs were washed and cocultured overnight with 1×10^5 cells/well of OVA-specific B3Z cells in RPMI 10% . As control, cells were maintained in OptiMEM medium and incubated with Ag before washing and culture with B3Z cells in RPMI 10% as indicated above.

Confocal microscopy and in vitro internalization assay

$CD11c^+$ spleen cells from PPV-VLP-injected mice were allowed to adhere to glass slides coated with poly-L-lysine (Sigma-Aldrich). Cells were then fixed with 4% paraformaldehyde and in some cases labeled with wheat-germ agglutinin (WGA; Molecular Probes Europe,) directly conjugated to Alexa Fluor 488 for 30 min without permeabilization of the cells to detect plasma membrane. Intracellular immunofluorescence was performed on cells permeabilized in PBS containing 1% saponin and 5% BSA with Abs against CD8α (53-6.7 clone; BD PharMingen), Lamp2 (BD PharMingen), H2-M (DM5 clone) (24), Rab7 (supplied by P. Chavrier, Institut Curie, Paris, France) (24, 25), Rab11 (26), or biotinylated anti-VP2 Ab (mixture of 11D1, 13C4, 13C5, 13C6, and 15G4 mAbs) (23). After incubation with the appropriate secondary Abs, cells were mounted and analyzed by confocal microscopy using a Leica TCS SP2 microscope (Leica, Deerfield, IL) equipped with a $\times$100 1.4 NA HCX PL APO oil immersion objective.

For in vitro internalization assay, $CD11c^+$ spleen cells from noninjected mice were incubated with Alexa488-PPV-VLPs for 1 h at 37°C, followed by rhodamine-WGA (Molecular Probes Europe) staining as described above.

Results

In vivo and in vitro, DCs capture and process PPV-VLPs-OVA

To characterize the in vivo and in vitro processing of PPV-VLPs, we first studied the in vivo capture of PPV-VLPs by DCs. Naive C57BL/6 mice were injected with PPV-VLPs-OVA coupled to Alexa488, a strong green fluorescent dye, which fluorescence does not extinguish at low pH. Alexa488-PPV-VLPs-OVA preserved their biological activity after labeling, as demonstrated by their capacity to be processed and presented by DCs to B3Z cells (data not shown). Ninety minutes after injection, the $CD11c^+$ spleen cells were sorted out and labeled with an anti-CD11c mAb. As shown in a representative experiment depicted in Fig. 1*A*, 53% of $CD11c^+$ cells were $Alexa488^+$ showing that in vivo, DCs captured PPV-VLPs-OVA very efficiently. Fifteen hours after injection, DCs remained strongly positive for Alexa488 (52% of $Alexa488^+$ DCs; data not shown).

We then examined whether DCs can process PPV-VLPs in vivo, using an ex vivo Ag presentation assay. C57BL/6 mice were i.v. injected with 50 μg (13 pmol/mouse) of PPV-VLPs-OVA or PPV-VLPs. Ninety minutes later, splenic $CD11c^+$ cells were purified and cocultured with B3Z $CD8^+$ T cell hybridoma. DCs purified from PPV-VLPs-OVA-injected mice were capable of presenting the $OVA_{257–264}$ epitope to B3Z cells, whereas DCs purified from control PPV-VLP-injected mice failed to stimulate this hybridoma (Fig. 1*B*). Thus, in vivo, DCs efficiently capture and process PPV-VLPs-OVA.

To evaluate the capacity of DCs in vitro to process PPV-VLPs-OVA, splenic $CD11c^+$ spleen cells purified from naive mice were incubated with PPV-VLPs-OVA or PPV-VLPs or the $OVA_{257–264}$ peptide for 4 h and then cocultured overnight with the B3Z hybridoma. DCs incubated with PPV-VLPs-OVA or the $OVA_{257–264}$ peptide efficiently presented the $OVA_{257–264}$ epitope, whereas DCs incubated with PPV-VLPs did not stimulate B3Z cells (Fig. 1*C*). Moreover, presentation of PPV-VLPs-OVA requires cellular processing, because DCs fixed with glutaraldehyde before incubation with PPV-VLPs-OVA did not stimulate B3Z. In contrast, DCs fixed after PPV-VLPs-OVA incubation were fully capable of stimulating this hybridoma (data not shown).

After capture, PPV-VLPs are localized in late endosomes of DCs

To analyze in detail the capture of PPV-VLPs by DCs, we studied by confocal microscopy the intracellular localization of PPV-VLPs

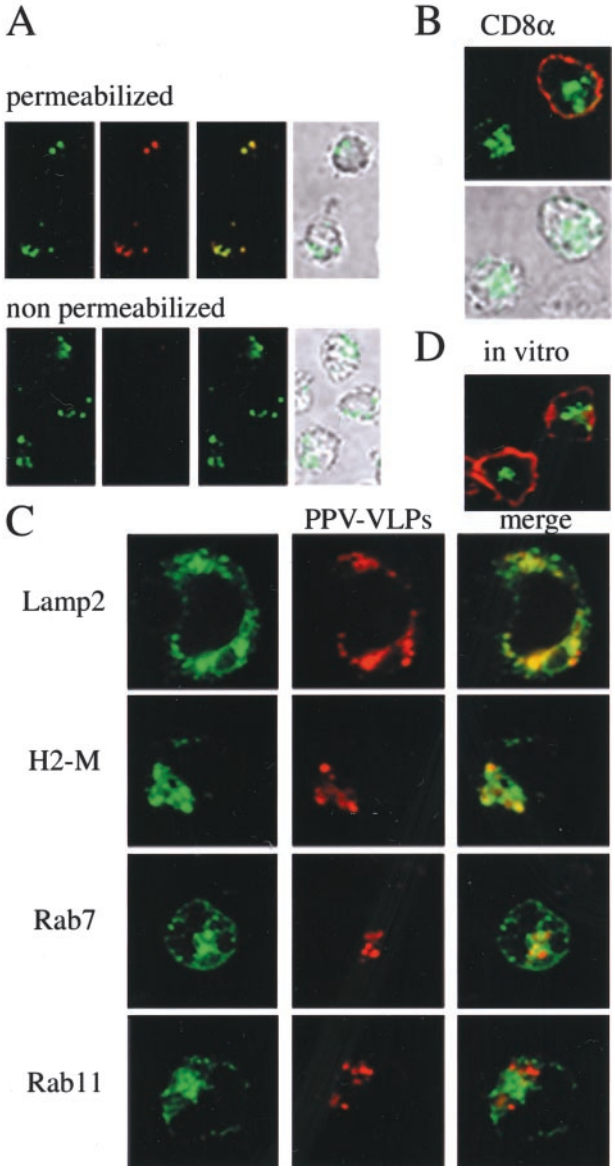

FIGURE 2. In vivo, captured PPV-VLPs-OVA are found in late endosomes/lysosomes in DCs. *A*, Intracellular location of PPV-VLPs. Mice were injected with 50 μg of Alexa488-PPV-VLPs, and 90 min later, splenic DCs were purified. Cells were then stained in the presence or the absence of saponin to permeabilize the cells, with biotinylated anti-PPV-VLPs mAbs detected with Cy3-streptavidin. *Far left* figures show Alexa488 fluorescence, *left* images show Cy3 fluorescence, while *right* figures show the merged images. *Far right* figures show the transmission image. *B*, Capture of PPV-VLPs by $CD8\alpha^+$ and $CD8\alpha^-$ DCs. Mice were injected with 50 μg of Alexa488-PPV-VLPs, and 90 min later, splenic DCs were purified. Cells were then permeabilized and labeled with an anti-CD8α mAb (in red), which was detected with a Cy3-anti-rat secondary Ab. The transmission light image shows the body of the cell. *C*, Mice were injected with unlabeled PPV-VLPs, and 90 min later, splenic DCs were fixed, and different intracellular markers were identified by specific antibodies detected with Alexa488-conjugated secondary Abs. Intracellular location of PPV-VLPs was detected on permeabilized cells with anti-PPV mAb, followed by Cy3-streptavidin. *D*, Purified splenic DCs from naive mice were incubated with 5 μg/ml of Alexa488-PPV-VLPs for 1 h at 37°C, stained with rhodamine-WGA, and analyzed by confocal microscopy to visualize the plasma membrane.

in DCs. Mice were injected with Alexa488-PPV-VLPs-OVA, and their splenic DCs were purified, permeabilized or not with saponin, and stained with a mixture of anti-VP2 mAbs. This mix of mAbs colocalized with Alexa488 labeling in saponin-permeabilized DCs from mice injected with Alexa488-PPV-VLPs, whereas no colocalization was observed in nonpermeabilized DCs from the same mice (Fig. 2*A*), clearly showing that PPV-VLPs were effectively found inside DCs and demonstrating that in vivo, DCs endocytose PPV-VLPs. Furthermore, PPV-VLPs were found inside both CD8α^- and CD8α^+ DCs (Fig. 2*B*). Inside DCs, PPV-VLPs were observed in vesicle-shaped structures of very different sizes (Fig. 2, *A–C*). Therefore, to characterize these vesicles, DCs from mice injected with PPV-VLPs were stained with anti-VP2 mAbs and with mAbs against several molecules recognized as markers of endosomal compartments. The confocal microscopy revealed that PPV-VLPs colocalized with Lamp2$^+$, H2-M$^+$, and rab7$^+$ labeling (Fig. 2*C*), which are associated with late endosomes (27–29). Furthermore, no colocalization of PPV-VLPs was observed with rab11, a GTPase of the Rab family that is a crucial element in the control of traffic through the recycling endosome (30, 31) (Fig. 2*C*). Therefore, after endocytosis, PPV-VLPs reach late endosomes. To determine whether this capture can be also observed in vitro, purified splenic DCs from naive mice were incubated with Alexa488-PPV-VLPs. The confocal analysis of DCs stained with WGA (which recognizes $(GlcNAc)_2$ residues in cell surface glycoproteins) clearly showed that PPV-VLPs were effectively found inside DCs (Fig. 2D), demonstrating that in vitro, DCs can also endocytose PPV-VLPs. Moreover, in vitro endocytosed PPV-VLPs were also found inside Lamp2$^+$, H2-M$^+$, and rab7$^+$ vesicles, confirming the in vivo results (data not shown).

Capture of PPV-VLPs requires macropinocytosis, lipids rafts, and actin polymerization, but not clathrin-coated pits

Endocytic pathways may include clathrin-coated pits, macropinocytosis, phagocytosis, or lipid rafts containing or not containing caveolin. To define the pathways involved in PPV-VLP capture, we used several drugs that are known to inhibit with a relative specificity a particular step of these various pathways. Clathrin-mediated endocytosis was assessed by K^+ depletion following hypotonic shock and by inhibition with chlorpromazine. K^+ depletion following hypotonic shock was performed by cell exposure to hypotonic medium, followed by incubation in the absence of extracellular potassium (32–34). This treatment results in dissociation of clathrin coats from the plasma membrane and nonproductive assembly of clathrin cages in the cytoplasm adjacent to coated pits. As consequence, internalization is impaired for all membrane proteins carrying cytoplasmic amino acid sequences that interact with the clathrin adapter complex AP2. Upon this treatment, DCs incubated with PPV-VLPs-OVA or $OVA_{257–264}$ peptide did not show any inhibition of $OVA_{257–264}$ presentation (Fig. 3*A*).

As control, we used a second vector developed in our laboratory, the adenylate cyclase (CyaA) toxoid from *Bordetella pertussis* (for review, see El Azami El Idrissi et al. (35)). CyaA is a 1706-aa protein that targets DCs, macrophages, neutrophils, and NK cells through interaction with CD11b (11, 36, 37). Recombinant CyaA toxin, carrying foreign CD8$^+$ T cell epitopes, can induce a strong CTL response after injection in mice (38). In general, CD11b binding is followed by the formation of clathrin-coated pits (39). Indeed, spleen DCs K^+-depleted following hypotonic shock and coincubated with a recombinant CyaA containing the

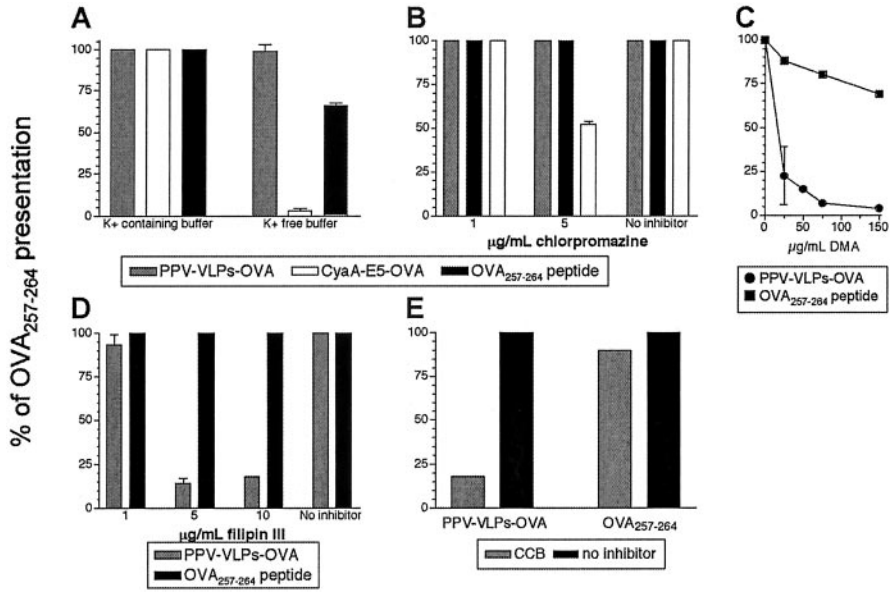

FIGURE 3. PPV-VLP uptake involves macropinocytosis, lipid rafts, and actin cytoskeleton polymerization, but not clathrin-coated pits. *A*, Purified splenic DCs (10^5 cells/well) were preincubated for 30 min in the presence of serum-free synthetic OptiMEM, then for 5 min in hypotonic medium and finally for 30 min in isotonic K^+-free or K^+-containing buffer. Then, 1.2 nM PPV-VLPs-OVA, 27 nM CyaA-E5-MalE-OVA, or 1.1 nM $OVA_{257–264}$ was added. DCs were then incubated for 1 h in the continued presence of the drugs and Ags. Then DCs were washed and cocultured overnight with 1×10^5 cells/well of OVA-specific B3Z cells in RPMI 10% . As control, cells were incubated with OptiMEM and the above-indicated Ags before washing and culture of B3Z cells. *B*, Purified splenic DCs (10^5 cells/well) were preincubated for 1 h in the presence of the indicated concentrations of chlorpromazine. Then 1.2 nM PPV-VLPs-OVA, 27 nM CyaA-E5-MalE-OVA, or 1.1 nM $OVA_{257–264}$ was added. DCs were incubated for 1 h in the continued presence of the drugs and Ags. *C–E*, Purified splenic DCs (10^5 cells/well) were preincubated for 1 h in the presence of the indicated concentrations of DMA (*C*), filipin III (*D*), or 10 μg/ml cytochalasin B (*E*). Then 1.2 nM PPV-VLPs-OVA or 1.1 nM $OVA_{257–264}$ was added. DCs were incubated for 4 h in the continued presence of the drugs and Ags. The DCs were cocultured overnight with 10^5 cells/well of B3Z-specific hybridoma. The presentation of $OVA_{257–264}$ epitope to B3Z cells was monitored by IL-2 production and expressed as the percentage of [^{3}H]thymidine incorporation compared with DCs incubated with PPV-VLPs-OVA or $OVA_{257–264}$ peptide in the absence of inhibitor.

$OVA_{257–264}$ epitope (CyaA-E5-OVA) were incapable of presenting the OVA epitope to the hybridoma cells (Fig. 3*A*).

To confirm these results, we used chlorpromazine as an inhibitor of clathrin-coated pit formation. Chlorpromazine is a cationic amphiphilic drug that has been extensively used to analyze the role of clathrin-mediated endocytosis in virus entry (40, 41). $OVA_{257–264}$ epitope presentation by DCs pretreated with chlorpromazine and then incubated with PPV-VLPs-OVA was not altered, whereas CyaA-E5-OVA presentation was reduced by 50% compared with untreated control DCs (Fig. 3*B*). This result confirmed that clathrin-coated pits do not play a significant role in the capture of PPV-VLPs.

To study whether PPV-VLP uptake is mediated by macropinocytosis, DCs were preincubated with DMA, which inhibits Na^+/H^+ exchange (42) and, therefore, macropinocytosis (43). As shown in Fig. 3*C*, DMA strongly inhibited PPV-VLPs-OVA presentation, whereas it slightly affected the $OVA_{257–264}$ peptide presentation at a very high concentration of DMA.

Lipid rafts also participate in PPV-VLPs uptake, as revealed by the use of filipin III inhibitor. Filipin III is a sterol-binding agent that binds to cholesterol, disrupting lipid raft formation, including caveolin-containing rafts, without affecting clathrin-mediated endocytosis (44). $OVA_{257–264}$ epitope presentation by DCs pretreated with filipin III and then incubated with PPV-VLPs-OVA was severely reduced, whereas this treatment did not affect the presentation of the $OVA_{257–264}$ peptide (Fig. 3*D*).

Finally, we studied PPV-VLPs processing by DCs preincubated with CCB, a known inhibitor of actin filament polymerization (45), which, in turn, can alter macropinocytosis, phagocytosis, as well as caveolae-mediated endocytosis. DCs incubated with PPV-VLPs-OVA in the presence of CCB were not able to present the $OVA_{257–264}$ epitope (Fig. 3*E*), whereas DCs incubated with the $OVA_{257–264}$ peptide in the presence of CCB were fully able to activate B3Z cells.

The $OVA_{257–264}$ epitope processed from PPV-VLPs-OVA does not bind to recycling MHC class I molecules

Most endocytosed molecules, including recycling receptors, are delivered to early endosomes, where efficient sorting occurs (31). Receptors and some ligands segregate to recycling compartments, whereas other molecules are delivered into late endosomes-lysosomes. Recycling of endocytosed MHC class I molecules back to the cell surface has been observed (46). Some of the recycling MHC class I molecules can be loaded into endosomes with peptide derived from endocytosed molecules (20, 47). Therefore, to establish whether PPV-VLP processing involves MHC class I recycling, we studied PPV-VLPs-OVA presentation by DCs incubated in the presence of primaquin, which inhibits the recycling of MHC class I and II molecules (46). DCs incubated in the presence of this drug presented efficiently both PPV-VLPs-OVA and the $OVA_{257–264}$ peptide (Fig. 4*A*). This result is in accord with the absence of colocalization of PPV-VLPs with rab11 by confocal microscopy (Fig. 2*C*). Therefore, PPV-VLPs would not enter into recycling compartments. In contrast, $OVA_{257–264}$ presentation was fully inhibited when DCs were incubated with PPV-VLPs-OVA in the presence of cycloheximide (Fig. 4*B*), a potent inhibitor of protein synthesis, suggesting that newly synthesized proteins, particularly MHC class I molecules, are required for efficient presentation of PPV-VLPs-OVA to B3Z cells.

PPV-VLPs processing needs vacuolar acidification and protease hydrolysis

Purified DCs were incubated with PPV-VLPs-OVA in the presence of chloroquin, a known inhibitor of acidification of late endosomes and lysosomes. Chloroquin strongly inhibited PPV-VLPs-OVA presentation, without altering $OVA_{257–264}$ peptide presentation, showing that acidification of late endosomes is necessary for PPV-VLPs processing (Fig. 5*A*).

To analyze the participation of proteases in the processing of PPV-VLPs, two different proteases inhibitors, leupeptin (which is an inhibitor of serine and cysteine proteases, including cathepsin B) and pepstatin (a inhibitor of aspartate proteases, including cathepsins D and E) (48, 49), were used. Incubation of DCs with PPV-VLPs in the presence of leupeptin did not block PPV-VLP-OVA presentation (Fig. 5*B*), whereas it inhibited the MHC class II-restricted presentation of exogenous proteins (data not shown). Incubation in the presence of pepstatin resulted in a partial inhibition of this presentation (Fig. 5*B*). These inhibitors did not significantly influence $OVA_{257–264}$ peptide presentation, suggesting that PPV-VLP processing required the participation of some proteases.

PPV-VLP processing requires proteasome activity

To evaluate whether the proteosomal complex also participates in PPV-VLPs processing, $CD11c^+$ spleen cells were treated with two 20S proteasome inhibitors, lactacystin (50, 51) and LLnL (52). $OVA_{257–264}$ presentation by DCs incubated with PPV-VLPs-OVA

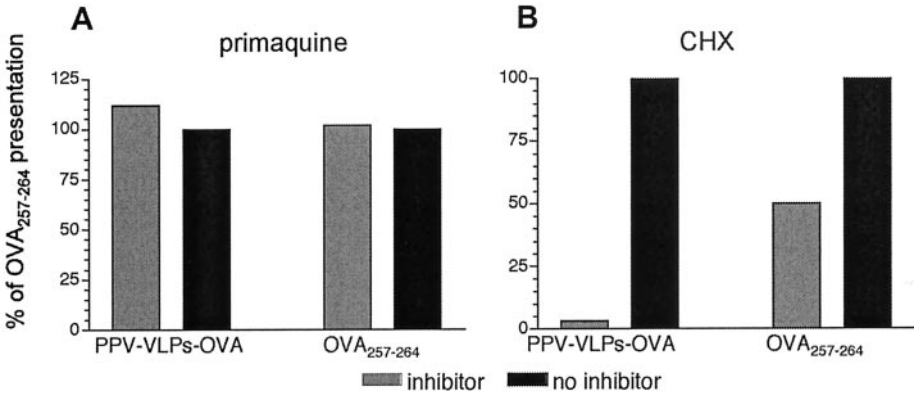

FIGURE 4. Presentation of PPV-VLPs-OVA requires the neosynthesis of MHC class I molecules. Purified splenic DCs (10^5 cells/well) were preincubated for 1 h with medium alone or in the presence of 0.2 nM primaquin (*A*) or 4 μg/ml cycloheximide (*B*). Then 1.2 nM PPV-VLPs-OVA or 110 nM $OVA_{257–264}$ was added, and DCs were incubated for 4 h in the continued presence of the drugs. After glutaraldehyde fixation, DCs were cocultured overnight with 10^5 cells/well of B3Z hybridoma. The presentation of $OVA_{257–264}$ epitope to B3Z cells was monitored by IL-2 production and expressed as the percentage of [^{3}H]thymidine incorporation compared with DCs incubated with PPV-VLPs-OVA or $OVA_{257–264}$ peptide in the absence of inhibitor.

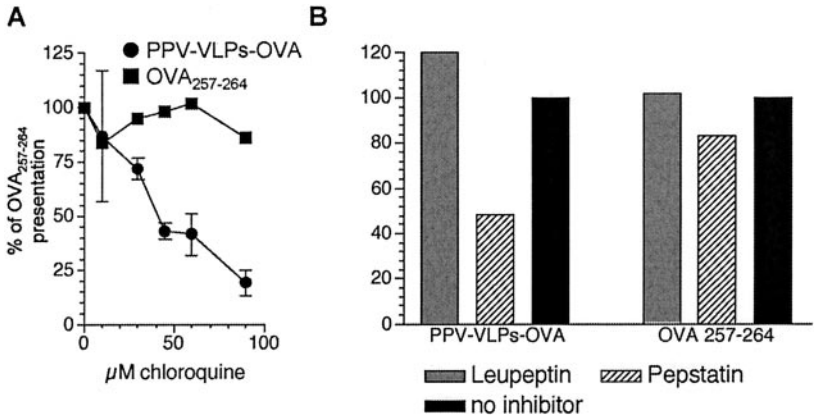

FIGURE 5. PPV-VLP processing depends on vacuolar acidification and protease activity. *A*, Purified splenic DCs (10^5 cells/well) were preincubated for 1 h with medium alone or in the presence of various amounts of chloroquin. Then 1.2 nM PPV-VLPs-OVA (●) or 110 nM $OVA_{257–264}$ peptide (■) was added; DCs were incubated for 4 h in the continued presence of the drugs. *B*, DCs (10^5 cells/well) were preincubated for 1 h with medium alone, in the presence of 50 μg/ml of leupeptin or pepstatin, or in the absence of drugs. Then 1.2 nM PPV-VLPs-OVA (*left*) or 110 nM $OVA_{257–264}$ (*right*) was added; DCs were incubated for 4 h in the continued presence of the drugs. After glutaraldehyde fixation, DCs were cocultured overnight with 1×10^5 cells/well of B3Z hybridoma. The presentation of $OVA_{257–264}$ epitope to B3Z cells was monitored by IL-2 production and expressed as the percentage of [^{3}H]thymidine incorporation compared with DCs incubated with PPV-VLPs-OVA or $OVA_{257–264}$ peptide in the absence of inhibitor.

in the presence of these inhibitors was severely diminished (Fig. 6, *A* and *B*), demonstrating that PPV-VLPs or derived peptides translocate to the cytosol of DCs and are degraded by the proteasome. The lack of inhibition of PPV-VLPs-OVA presentation in the presence of LLmL (a calpain I inhibitor) confirmed the specificity of the inhibition observed with LLnL (Fig. 6*C*). DCs incubated with the $OVA_{257–264}$ peptide in the presence of these inhibitors fully stimulated B3Z cells, showing that these inhibitors did not block steps of the MHC class I processing pathway (such as peptide transport, synthesis of MHC molecules, or exocytosis of peptide-MHC complexes) other than proteasome activity.

PPV-VLPs-OVA are presented in vitro by DCs in a TAP-dependent way

We then analyzed whether translocation to endoplasmic reticulum (ER)-Golgi complex is necessary for PPV-VLPs processing. We thus analyzed the in vitro PPV-VLP-OVA presentation by DCs from $TAP1^{-/-}$ mice, which do not have a functional TAP system (53). DCs from $TAP1^{-/-}$ mice incubated with PPV-VLPs-OVA were unable to present the $OVA_{257–264}$ epitope, in contrast to $TAP1^{+/+}$ DCs (Fig. 7*A*). Although $TAP1^{-/-}$ cells have a diminished expression of class I MHC molecules (53), their capacity to present $OVA_{257–264}$-K^b complexes was not affected, as shown for DCs incubated with the $OVA_{257–264}$ peptide (Fig. 7*B*). This result clearly shows that the processing of PPV-VLPs depends upon the translocation of derived peptides from cytosol to endoplasmic reticulum through TAP molecules, confirming previous in vivo results (15).

PPV-VLPs processing is sensitive to BFA

BFA disorganizes the Golgi complex and then inhibits the exocytosis of proteins, blocking the secretion of newly synthesized proteins (54, 55). Using this drug, we therefore determined whether

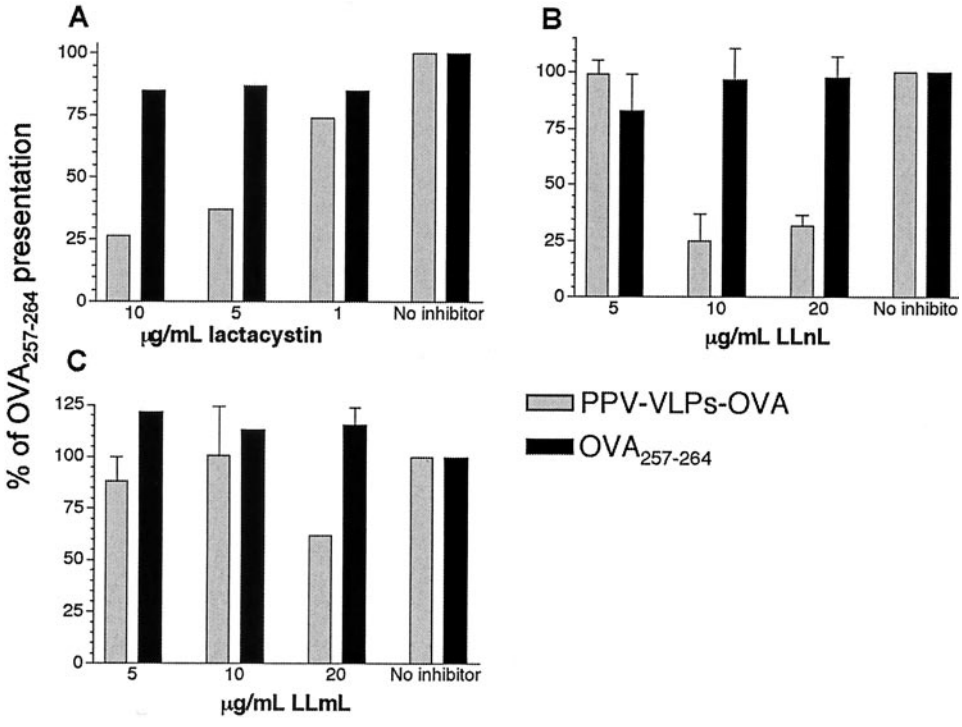

FIGURE 6. PPV-VLP processing requires proteasome activity. Purified splenic DCs (10^5 cells/well) were preincubated for 1 h with medium alone or in the presence of lactacystin (*A*), LLnL (*B*), or LLmL (*C*). Then 1.2 nM PPV-VLPs-OVA or 110 nM $OVA_{257–264}$ was added and incubated for 4 h in the continued presence of the drugs. After glutaraldehyde fixation, the DCs were cocultured overnight with 10^5 cells/well of B3Z hybridoma. The presentation of $OVA_{257–264}$ epitope to B3Z cells was monitored by IL-2 production and is expressed as the percentage of [^{3}H]thymidine incorporation compared with DCs incubated with PPV-VLPs-OVA or $OVA_{257–264}$ peptide in the absence of inhibitor.

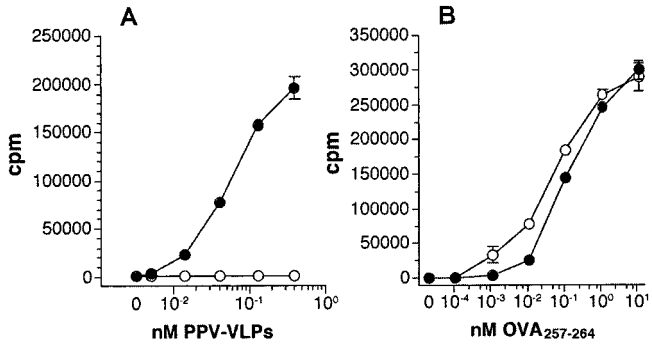

FIGURE 7. PPV-VLPs-OVA are presented in vitro by DCs in a TAP-dependent way. Purified splenic DCs (10^5 cells/well), purified from TAP1$^{+/+}$ (●) or TAP1$^{-/-}$ (○) mice, were incubated with various amounts of PPV-VLPs-OVA (*A*) or $OVA_{257–264}$ (*B*) for 4 h. Then pulsed DCs were washed and cocultured overnight with 1×10^5 cells/well of B3Z cells. The presentation of $OVA_{257–264}$ epitope to B3Z cells was monitored by IL-2 production and is expressed as the mean ± SEM counts per minute of duplicate wells.

PPV-VLPs-OVA processing involves the transport of processed peptides through Golgi complex to the DC membrane. BFA did not block presentation by DCs of the $OVA_{257–264}$ synthetic peptide (Fig. 8) but, as shown in Fig. 8, BFA inhibited B3Z stimulation by DCs incubated with PPV-VLPs-OVA. This strongly suggests that after translocation to ER, PPV-VLP-derived peptides are transported to DC surface through the Golgi complex.

Discussion

VLPs have clearly demonstrated their potential as a vector for vaccination (2, 13, 56–60) due to their capacity to induce a strong CTL response. We have recently shown that in vivo, PPV-VLPs target CD8α^- and CD8α^+ DCs with a high efficiency (15), and DCs are the only APC capable of presenting PPV-VLPs to CD8$^+$ T cells. In the present report we analyzed in vitro and ex vivo the mechanisms used by spleen DCs to process and present PPV-VLPs associated with MHC class I molecules.

The results of this study clearly show that PPV-VLPs can be processed by DCs through an endosome-to-cytosol pathway. Indeed, after i.v. injection, PPV-VLPs are found in late endosomal vesicles of DCs, as revealed by confocal microscopy. The use of protease inhibitors and cloroquin suggests that vacuolar acidification and lysosomal proteases are involved in PPV-VLPs processing. However, proteasome, TAP-mediated translocation, and transport through Golgi complex are also required for processing of these particles, steps that belong to the cytosolic processing pathway. Protein synthesis is strictly necessary for presentation of the $OVA_{257–264}$ epitope contained in PPV-VLPs-OVA.

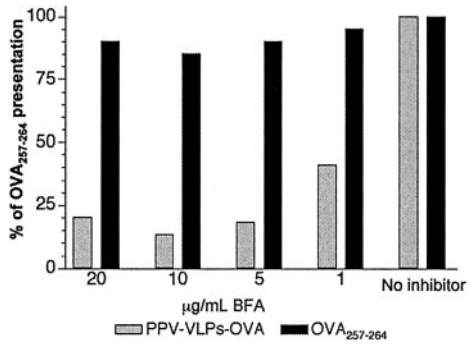

FIGURE 8. PPV-VLP processing is sensitive to BFA. DCs (10^5 cells/well) were preincubated for 1 h with medium alone or in the presence of BFA. Then 0.25 nM PPV-VLPs-OVA or 110 nM $OVA_{257–264}$ was added and incubated for 4 h in the continued presence of the drugs. After glutaraldehyde fixation, the DCs were cocultured overnight with 10^5 cells/well of B3Z-specific hybridoma. The presentation of $OVA_{257–264}$ epitope to B3Z cells was monitored by IL-2 production and is expressed as the percentage of [^{3}H]thymidine incorporation compared with DCs incubated with PPV-VLPs-OVA or $OVA_{257–264}$ peptide in the absence of inhibitor.

The capture of PPV-VLPs seems to be mediated by macropinocytosis rather than by clathrin-coated pits, based on the inhibition of $OVA_{257–264}$ presentation in the presence of DMA and on the lack of inhibition when cells were deprived of K^+ or treated with chlorpromazine. Sequestration of cholesterol in plasma membrane by filipin III, which disrupts lipid rafts, also inhibited PPV-VLPs-OVA presentation. It remains to be determined whether these lipids rafts contain caveolin. However, most vesicles containing PPV-VLPs were large, in contrast to the typical small size of caveolin-containing vesicles (61).

Interestingly, the processing pathway of hepatitis B virus small envelope protein (HBsAg) VLPs, another model of VLPs, has been shown to be completely different. Indeed, HBsAg particles are presented to CD8$^+$ T lymphocytes by several cell types, including mastocytoma cells, fibroblasts, DC lines, Mϕ lines, and B and T lymphocytes, indicating that these particles do not follow a pathway exclusive to DCs (56, 62, 63). In contrast, in vivo, PPV-VLPs are only processed by DCs, whereas Mϕ and B cells can neither present PPV-VLPs nor induce CTL responses (15). The internalization of both HBsAg and PPV particles is inhibited by amiloride-derived drugs, and both types of VLPs are initially processed in acidic compartments (20, 63). However, after this step these particles follow different routes. Indeed, HBsAg follow a BFA-resistant and TAP-independent pathway in both Mϕ and DCs (20, 63). The presentation of HBsAg VLPs involves binding of antigenic peptides derived from endocytosed HBsAg particles to empty L^d molecules in endosomal compartments and recycling to the cell surface of APCs (20, 47). In contrast, PPV-VLPs translocate to the cytosol of DCs, as demonstrated by their lack of presentation by DCs treated with proteasome inhibitors or from TAP1$^{-/-}$ mice (15).

An intermediate model is the processing of HBcAg particles, which are 30-nm particles formed by the HBV core Ag. Indeed, in vitro as well as in vivo, the presentation of HBcAg particles by DCs is partially TAP dependent, whereas their presentation by Mϕs is fully TAP independent, showing that these APCs use different pathways for HBcAg processing (64).

Therefore, DCs can cross-present particulate Ags such as VLPs by different pathways. The translocation from endosome to cytosol remains the key event that determines the pathway used by APCs for presentation to CD8$^+$ T cells. If the exogenous Ag has the ability to cross the endosome barrier, it will follow an intracytosolic processing. If not, it will end its processing within vesicles, and the resulting peptides could bind to recycling MHC class I molecules. The lack of inhibition of PPV-VLPs presentation by primaquin as well as the lack of colocalization of PPV-VLPs and rab11 strongly suggest that PPV-VLPs (or derived peptides) are not delivered into recycling vesicles, but remain in the endosomes to deliver the $OVA_{257–264}$ epitope into the cytosol. This conclusion is consistent with the inhibition of $OVA_{257–264}$ presentation in the presence of BFA, which inhibits protein transport (including nascent MHC class I molecules) from the ER, without affecting recycling MHC class I and II molecules (65), showing that $OVA_{257–264}$ presentation is mediated by neosynthesized MHC class I molecules.

After uptake, PPV-VLPs are localized in late endosomal compartments. Acidification of late endosomes is necessary for receptor recycling, movement/maturation of organelles, activation of endolysosomal hydrolases, and activation of membrane transporters (66). The inhibitory effect of chloroquin as well as the partial inhibition of PPV-VLPs processing by pepstatin (an inhibitor of aspartate proteases, included cathepsins D and E) (48, 49) indicates that proteolytic degradation could be necessary in the initial steps of PPV-VLP degradation. However, acidification could also play a role in PPV-VLP translocation to cytosol. Indeed, translocation from endosomes to cytosol is a crucial event for the entry of some viruses. Porcine parvovirus is a nonenveloped virus, and the mechanisms by which it enters the host cell are unknown, as they are for most nonenveloped viruses. However, it seems that interactions between hydrophobic portions of capside proteins participate in the entry. Furthermore, the entry is pH dependent, because endosome acidification blocks virus infection and replication (67).

Translocation to the cytosol is a key event observed only in DCs during endosome-to-cytosol transport (68). The mechanisms involved in this translocation are still unknown, but such a transport has been also proposed for soluble OVA (69), bacteria (19), DC-targeted liposomes (70), heat shock proteins (71), immune complexes (68, 72), exosomes (73), and apoptotic (4) and necrotic (74) cells. This mechanism seems to be specific for internalized Ags and selective for the size of the transported molecules (68). Therefore, PPV-VLPs may take also advantage of this pathway, which has been described as more efficient than TAP-independent pathways (75). This could explain why PPV-VLPs, which need cytosolic processing to allow $OVA_{257–264}$ presentation, can only be presented by DCs in vivo.

In the cytosol of DCs, PPV-VLPs or, more likely, products of its degradation are processed by proteasome, as shown by experiments on inhibition with lactacystin and LLnL. Peptides generated from proteolysis in the cytosol are then translocated into the lumen of ER by TAP molecules, where they can bind MHC class I molecules, which can then transit to the cell surface for recognition by $CD8^+$ T cells.

Very recently, the existence of cross-presentation and cross-priming as general, physiological processes to induce $CD8^+$ T cell response has been doubted (76) on the basis of the lack of convincing in vivo solid experimental evidence. Previous studies have clearly showed that exogenous PPV-VLPs can induce a protective antiviral response, based on the induction of a high frequency of CTLs of high affinity (12, 13, 16, 77). The evidence presented in this report clearly establishes that PPV-VLPs processing by DCs involves cross-presentation. Thus, our results show that cross-presentation can occur in vivo under physiological conditions and lead to the activation of protective CTL responses.

Acknowledgments

We thank Daniel Ladant for the gift of CyaA-E5-OVA, and Bruno Goud and Fransesca Senic-Matuglia for the gift of the anti-Rab11 Ab. We thank Sebastián Amigorena for his helpful discussion. We are grateful to Catherine Fayolle for endotoxin dosage, and to Anne Louise, Anne-Marie Balazuc, and Javier Sarraseca for their excellent technical assistance.

References

1. Heath, W. R., and F. R. Carbone. 2001. Cross-presentation, dendritic cells, tolerance and immunity. *Annu. Rev. Immunol. 19:47*.
2. Reimann, J., and R. Schirmbeck. 1999. Alternative pathways for processing exogenous and endogenous antigens that can generate peptides for MHC class I-restricted presentation. *Immunol. Rev. 172:131*.
3. Bellone, M., G. Iezzi, P. Rovere, G. Galati, A. Ronchetti, M. P. Protti, J. Davoust, C. Rugarli, and A. A. Manfredi. 1997. Processing of engulfed apoptotic bodies yields T cell epitopes. *J. Immunol. 159:5391*.
4. Albert, M. L., B. Sauter, and N. Bhardwaj. 1998. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature 392:86*.
5. Ronchetti, A., P. Rovere, G. Iezzi, G. Galati, S. Heltai, M. P. Protti, M. P. Garancini, A. A. Manfredi, C. Rugarli, and M. Bellone. 1999. Immunogenicity of apoptotic cells in vivo: role of antigen load, antigen-presenting cells, and cytokines. *J. Immunol. 163:130*.
6. Heath, W. R., and F. R. Carbone. 2001. Cross-presentation in viral immunity and self-tolerance. *Nat. Rev. Immunol. 1:128*.
7. Gromme, M., F. G. Uytdehaag, H. Janssen, J. Calafat, R. S. van Binnendijk, M. J. Kenter, A. Tulp, D. Verwoerd, and J. Neefjes. 1999. Recycling MHC class I molecules and endosomal peptide loading. *Proc. Natl. Acad. Sci. USA. 96:10326*.
8. den Haan, J. M., and M. J. Bevan. 2001. Antigen presentation to $CD8^+$ T cells: cross-priming in infectious diseases. *Curr. Opin. Immunol. 13:437*.
9. Yrlid, U., M. Svensson, C. Johansson, and M. J. Wick. 2000. *Salmonella* infection of bone marrow-derived macrophages and dendritic cells: influence on antigen presentation and initiating an immune response. *FEMS Immunol. Med. Microbiol. 27:313*.
10. Saron, M. F., C. Fayolle, P. Sebo, D. Ladant, A. Ullmann, and C. Leclerc. 1997. Anti-viral protection conferred by recombinant adenylate cyclase toxins from *Bordetella pertussis* carrying a $CD8^+$ T cell epitope from lymphocytic choriomeningitis virus. *Proc. Natl. Acad. Sci. USA 94:3314*.
11. Guermonprez, P., D. Ladant, G. Karimova, A. Ullmann, and C. Leclerc. 1999. Direct delivery of the *Bordetella pertussis* adenylate cyclase toxin to the MHC class I antigen presentation pathway. *J. Immunol. 162:1910*.
12. Sedlik, C., J. Sarraseca, P. Rueda, C. Leclerc, and I. Casal. 1995. Immunogenicity of poliovirus B and T cell epitopes presented by hybrid porcine parvovirus particles. *J. Gen. Virol. 76:2361*.
13. Sedlik, C., M. Saron, J. Sarraseca, I. Casal, and C. Leclerc. 1997. Recombinant parvovirus-like particles as an antigen carrier: a novel nonreplicative exogenous antigen to elicit protective antiviral cytotoxic T cells. *Proc. Natl. Acad. Sci. USA 94:7503*.
14. Ranz, A. I., J. J. Manclus, E. Diaz-Aroca, and J. I. Casal. 1989. Porcine parvovirus: DNA sequence and genome organization. *J. Gen. Virol. 70:2541*.
15. Moron, G., P. Rueda, I. Casal, and C. Leclerc. 2002. $CD8\alpha^-$ $CD11b^+$ dendritic cells present exogenous virus-like particles to $CD8^+$ T cells and subsequently express $CD8\alpha$ and CD205 molecules. *J. Exp. Med. 195:1233*.
16. Sedlik, C., G. Dadaglio, M. F. Saron, E. Deriaud, M. Rojas, S. I. Casal, and C. Leclerc. 2000. In vivo induction of a high-avidity, high-frequency cytotoxic T-lymphocyte response is associated with antiviral protective immunity. *J. Virol. 74:5769*.
17. Kovacsovics-Bankowski, M., and K. L. Rock. 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science 267:243*.
18. Norbury, C. C., B. J. Chambers, A. R. Prescott, H. G. Ljunggren, and C. Watts. 1997. 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*.
19. Svensson, M., and M. J. Wick. 1999. Classical MHC class I peptide presentation of a bacterial fusion protein by bone marrow-derived dendritic cells. *Eur. J. Immunol. 29:180*.
20. Stober, D., Z. Trobonjaca, J. Reimann, and R. Schirmbeck. 2002. Dendritic cells pulsed with exogenous hepatitis B surface antigen particles efficiently present epitopes to MHC class I-restricted cytotoxic T cells. *Eur. J. Immunol. 32:1099*.
21. Karttunen, J., S. Sanderson, and N. Shastri. 1992. Detection of rare antigen-presenting cells by the *lacZ* T-cell activation assay suggests an expression cloning strategy for T-cell antigens. *Proc. Natl. Acad. Sci. USA 89:6020*.
22. Rueda, P., J. Fominaya, J. P. Langeveld, C. Bruschke, C. Vela, and J. I. Casal. 2000. Effect of different baculovirus inactivation procedures on the integrity and immunogenicity of porcine parvovirus-like particles. *Vaccine 19:726*.
23. Casal, J. I., E. Cortés, J. A. López de Turiso, and C. Vela. 1992. Production of porcine and canine parvovirus-like particles using recombinant baculovirus. In *Baculovirus and Recombinant Protein Production Processes.* J. M. Vlak, E. S. Schlaeger, and A. Bernard, eds. Editiones Roche, Basel, Switzerland, p. 76.
24. Lankar, D., H. Vincent-Schneider, V. Briken, T. Yokozeki, G. Raposo, and C. Bonnerot. 2002. Dynamics of major histocompatibility complex class II compartments during B cell receptor-mediated cell activation. *J. Exp. Med. 195:461*.
25. Meresse, S., J. P. Gorvel, and P. Chavrier. 1995. The rab7 GTPase resides on a vesicular compartment connected to lysosomes. *J. Cell Sci. 108:3349*.
26. Wilcke, M., L. Johannes, T. Galli, V. Mayau, B. Goud, and J. Salamero. 2000. Rab11 regulates the compartmentalization of early endosomes required for efficient transport from early endosomes to the trans-golgi network. *J. Cell Biol. 151:1207*.
27. Chen, J. W., T. L. Murphy, M. C. Willingham, I. Pastan, and J. T. August. 1985. Identification of two lysosomal membrane glycoproteins. *J. Cell Biol. 101:85*.
28. Chavrier, P., R. G. Parton, H. P. Hauri, K. Simons, and M. Zerial. 1990. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell 62:317*.
29. Harding, C. V. 1995. Intracellular organelles involved in antigen processing and the binding of peptides to class II MHC molecules. *Semin. Immunol. 7:355*.
30. Ullrich, O., S. Reinsch, S. Urbe, M. Zerial, and R. G. Parton. 1996. Rab11 regulates recycling through the pericentriolar recycling endosome. *J. Cell Biol. 135:913*.
31. Gruenberg, J. 2001. The endocytic pathway: a mosaic of domains. *Nat. Rev. Mol. Cell. Biol. 2:721*.
32. Larkin, J. M., M. S. Brown, J. L. Goldstein, and R. G. Anderson. 1983. Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. *Cell 33:273*.

33. Madshus, I. H., K. Sandvig, S. Olsnes, and B. van Deurs. 1987. Effect of reduced endocytosis induced by hypotonic shock and potassium depletion on the infection of Hep 2 cells by picornaviruses. *J. Cell Physiol. 131:14.*
34. Bayer, N., D. Schober, M. Huttinger, D. Blaas, and R. Fuchs. 2001. Inhibition of clathrin-dependent endocytosis has multiple effects on human rhinovirus serotype 2 cell entry. *J. Biol. Chem. 276:3952.*
35. El Azami El Idrissi, M., D. Ladant, and C. Leclerc. 2002. The adenylate cyclase of *Bordetella pertussis*: a vector to target antigen presenting cells. *Toxicon 40:1661.*
36. Guermonprez, P., C. Fayolle, M. J. Rojas, M. Rescigno, D. Ladant, and C. Leclerc. 2002. In vivo receptor-mediated delivery of a recombinant invasive bacterial toxoid to $CD11c^{+}CD8\alpha^{-}CD11b^{high}$ dendritic cells. *Eur. J. Immunol. 32:3071.*
37. Guermonprez, P., N. Khelef, E. Blouin, P. Rieu, P. Ricciardi-Castagnoli, N. Guiso, D. Ladant, and C. Leclerc. 2001. The adenylate cyclase toxin of *Bordetella pertussis* binds to target cells via the $\alpha_M\beta_2$ integrin (CD11b/CD18). *J. Exp. Med. 193:1035.*
38. Fayolle, C., P. Sebo, D. Ladant, A. Ullmann, and C. Leclerc. 1996. In vivo induction of CTL responses by recombinant adenylate cyclase of *Bordetella pertussis* carrying viral $CD8^{+}$ T cell epitopes. *J. Immunol. 156:4697.*
39. de la Salle, H., J. Galon, H. Bausinger, D. Spehner, A. Bohbot, J. Cohen, J. P. Cazenave, W. H. Fridman, C. Sautes, and D. Hanau. 1997. Soluble CD16/FcγRIII induces maturation of dendritic cells and production of several cytokines including IL-12. *Adv. Exp. Med. Biol. 417:345.*
40. Krizanova, O., F. Ciampor, and P. Veber. 1982. Influence of chlorpromazine on the replication of influenza virus in chick embryo cells. *Acta Virol. 26:209.*
41. Wang, L. H., K. G. Rothberg, and R. G. Anderson. 1993. Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J. Cell Biol. 123:1107.*
42. West, M. A., M. S. Bretscher, and C. Watts. 1989. Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. *J. Cell Biol. 109:2731.*
43. Sallusto, F., M. Cella, C. Danieli, and A. Lanzavecchia. 1995. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J. Exp. Med. 182:389.*
44. Rothberg, K. G., J. E. Heuser, W. C. Donzell, Y. S. Ying, J. R. Glenney, and R. G. Anderson. 1992. Caveolin, a protein component of caveolae membrane coats. *Cell 68:673.*
45. Gottlieb, T. A., I. E. Ivanov, M. Adesnik, and D. D. Sabatini. 1993. Actin microfilaments play a critical role in endocytosis at the apical but not the basolateral surface of polarized epithelial cells. *J. Cell Biol. 120:695.*
46. Reid, P. A., and C. Watts. 1990. Cycling of cell-surface MHC glycoproteins through primaquine-sensitive intracellular compartments. *Nature 346:655.*
47. Schirmbeck, R., and J. Reimann. 1996. 'Empty' Ld molecules capture peptides from endocytosed hepatitis B surface antigen particles for major histocompatibility complex class I-restricted presentation. *Eur. J. Immunol. 26:2812.*
48. Umezawa, H. 1982. Low-molecular-weight enzyme inhibitors of microbial origin. *Annu. Rev. Microbiol. 36:75.*
49. Mizuochi, T., S. T. Yee, M. Kasai, T. Kakiuchi, D. Muno, and E. Kominami. 1994. Both cathepsin B and cathepsin D are necessary for processing of ovalbumin as well as for degradation of class II MHC invariant chain. *Immunol. Lett. 43:189.*
50. Fenteany, G., R. F. Standaert, W. S. Lane, S. Choi, E. J. Corey, and S. L. Schreiber. 1995. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science 268:726.*
51. Craiu, A., M. Gaczynska, T. Akopian, C. F. Gramm, G. Fenteany, A. L. Goldberg, and K. L. Rock. 1997. Lactacystin and clasto-lactacystin β-lactone modify multiple proteasome β-subunits and inhibit intracellular protein degradation and major histocompatibility complex class I antigen presentation. *J. Biol. Chem. 272:13437.*
52. Rock, K. L., C. Gramm, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang, and A. L. Goldberg. 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell 78:761.*
53. Van Kaer, L., P. G. Ashton-Rickardt, H. L. Ploegh, and S. Tonegawa. 1992. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and $CD4^{-}8^{+}$ T cells. *Cell 71:1205.*
54. Doms, R. W., G. Russ, and J. W. Yewdell. 1989. Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell Biol. 109:61.*
55. Pelham, H. R. 1991. Multiple targets for brefeldin A. *Cell 67:449.*
56. Bohm, W., R. Schirmbeck, A. Elbe, K. Melber, D. Diminky, G. Kraal, N. van Rooijen, Y. Barenholz, and J. Reimann. 1995. Exogenous hepatitis B surface antigen particles processed by dendritic cells or macrophages prime murine MHC class I-restricted cytotoxic T lymphocytes in vivo. *J. Immunol. 155:3313.*
57. Ball, J. M., D. Y. Graham, A. R. Opekun, M. A. Gilger, R. A. Guerrero, and M. K. Estes. 1999. Recombinant Norwalk virus-like particles given orally to volunteers: phase I study. *Gastroenterology 117:40.*
58. Oliveira-Ferreira, J., Y. Miyahira, G. T. Layton, N. Savage, M. Esteban, D. Rodriguez, J. R. Rodriguez, R. S. Nussenzweig, F. Zavala, and Y. Myahira. 2000. Immunogenicity of Ty-VLP bearing a $CD8^{+}$ T cell epitope of the CS protein of *P. yoelii*: enhanced memory response by boosting with recombinant vaccinia virus. *Vaccine 18:1863.*
59. Chackerian, B., D. R. Lowy, and J. T. Schiller. 2001. Conjugation of a self-antigen to papillomavirus-like particles allows for efficient induction of protective autoantibodies. *J. Clin. Invest. 108:415.*
60. Rudolf, M. P., S. C. Fausch, D. M. Da Silva, and W. M. Kast. 2001. Human dendritic cells are activated by chimeric human papillomavirus type-16 virus-like particles and induce epitope-specific human T cell responses in vitro. *J. Immunol. 166:5917.*
61. Harris, J., D. Werling, J. C. Hope, G. Taylor, and C. J. Howard. 2002. Caveolae and caveolin in immune cells: distribution and functions. *Trends Immunol. 23:158.*
62. Schirmbeck, R., K. Melber, and J. Reimann. 1995. Hepatitis B virus small surface antigen particles are processed in a novel endosomal pathway for major histocompatibility complex class I-restricted epitope presentation. *Eur. J. Immunol. 25:1063.*
63. Schirmbeck, R., W. Bohm, K. Melber, and J. Reimann. 1995. Processing of exogenous heat-aggregated (denatured) and particulate (native) hepatitis B surface antigen for class I-restricted epitope presentation. *J. Immunol. 155:4676.*
64. Ruedl, C., T. Storni, F. Lechner, T. Bachi, and M. Bachmann. 2002. Cross-presentation of virus-like particles by skin-derived $CD8^{-}$ dendritic cells: a dispensable role for TAP. *Eur. J. Immunol. 32:818.*
65. Sinnathamby, G., and L. C. Eisenlohr. 2003. Presentation by Recycling MHC class II molecules of an influenza hemagglutinin-derived epitope that is revealed in the early endosome by acidification. *J. Immunol. 170:3504.*
66. Pillay, C. S., E. Elliott, and C. Dennison. 2002. Endolysosomal proteolysis and its regulation. *Biochem. J. 363:417.*
67. Basak, S., and H. Turner. 1992. Infectious entry pathway for canine parvovirus. *Virology 186:368.*
68. Rodriguez, A., A. Regnault, M. Kleijmeer, P. Ricciardi-Castagnoli, and S. Amigorena. 1999. Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat. Cell. Biol. 1:362.*
69. Norbury, C. C., L. J. Hewlett, A. R. Prescott, N. Shastri, and C. Watts. 1995. Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity 3:783.*
70. Machy, P., K. Serre, and L. Leserman. 2000. Class I-restricted presentation of exogenous antigen acquired by Fcγ receptor-mediated endocytosis is regulated in dendritic cells. *Eur. J. Immunol. 30:848.*
71. Srivastava, P. K., H. Udono, N. E. Blachere, and Z. Li. 1994. Heat shock proteins transfer peptides during antigen processing and CTL priming. *Immunogenetics 39:93.*
72. Regnault, A., D. Lankar, V. Lacabanne, A. Rodriguez, C. Thery, M. Rescigno, T. Saito, S. Verbeek, C. Bonnerot, P. Ricciardi-Castagnoli, et al. 1999. Fcγ receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J. Exp. Med. 189:371.*
73. Zitvogel, L., A. Regnault, A. Lozier, J. Wolfers, C. Flament, D. Tenza, P. Ricciardi-Castagnoli, G. Raposo, and S. Amigorena. 1998. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat. Med. 4:594.*
74. Lu, Z., L. Yuan, X. Zhou, E. Sotomayor, H. I. Levitsky, and D. M. Pardoll. 2000. CD40-independent pathways of T cell help for priming of $CD8^{+}$ cytotoxic T lymphocytes. *J. Exp. Med. 191:541.*
75. Sigal, L. J., and K. L. Rock. 2000. Bone marrow-derived antigen-presenting cells are required for the generation of cytotoxic T lymphocyte responses to viruses and use transporter associated with antigen presentation (TAP)-dependent and -independent pathways of antigen presentation. *J. Exp. Med. 192:1143.*
76. Zinkernagel, R. M. 2002. On cross-priming of MHC class I-specific CTL: rule or exception? *Eur. J. Immunol. 32:2385.*
77. Sedlik, C., A. Dridi, E. Deriaud, M. F. Saron, P. Rueda, J. Sarraseca, J. I. Casal, and C. Leclerc. 1999. Intranasal delivery of recombinant parvovirus-like particles elicits cytotoxic T-cell and neutralizing antibody responses. *J. Virol. 73:2739.*