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# Cross-presentation of a CMV pp65 epitope by human dendritic cells using bee venom $PLA_2$ as a membrane-binding vector

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**Abstract We have used bee venom phospholipase $A_2$ as a vector to load human dendritic cells ex vivo with a major histocompatibility complex (MHC) class I-restricted epitope fused to its C-terminus. The fusion protein bound to human monocyte-derived dendritic cells and was internalized into early endosomes. In vitro immunization experiments showed that these dendritic cells were able to generate specific CD8 T cell lines against the epitope carried by the fusion protein. Cross-presentation did not require proteasome, transporter associated with antigen processing, or endosome proteases, but required newly synthesized MHC molecules. Comparison of the antigen presentation pathway observed in this study to that followed by other toxins used as vectors is discussed.**


*Keywords:* Bee venom phospholipase $A_2$; Cross-presentation; Cytomegalovirus; Dendritic cell vaccine

## 1. Introduction

Many studies have shown the extraordinary potential of dendritic cells (DCs) prepared ex vivo for anti-cancer or anti-viral immunotherapy [1,2]. DCs loaded with tumor or viral antigens are able to present major histocompatibility complex (MHC) class I and class II peptide epitopes to CD8 and CD4 T lymphocytes, generating an immune response against these antigens. Many ways of loading DCs with antigens exist, but no method is ideal and each has its own advantages and drawbacks. The use of antigenic peptide epitopes or lipopeptides is limited by MHC restriction, a given peptide being immunogenic in a limited set of individuals. Fusion of tumor cells with DCs [3], incubation with cell lysates [4], apoptotic bodies [5], heat shock protein-associated tumor proteins [6] and tumor messenger RNA transfection [7] provide poorly characterized antigens and may lead to auto-immunity [8]. The use of large vectors such as viruses or large bacterial toxins for the delivery of antigen DNA or protein into DCs may lead to competition between epitopes from the vector and the antigen for presentation, and to a strong immune response against the vector, precluding further vaccination with that vector. Moreover, viral vectors still face safety and regulatory problems [9,10].

This work describes a new vector to deliver protein antigens to DCs, enabling efficient cross-presentation of MHC class I-restricted epitopes. It is based on the use of bee venom phospholipase $A_2$ ($bvPLA_2$) as a cell membrane-binding vector for antigens fused to its C-terminus. $bvPLA_2$ is a small protein of 15 kDa, which binds tightly to phospholipid membranes to gain access to its phospholipid substrate [11]. Although $bvPLA_2$ is not very toxic to cells, the mutation H34Q in the catalytic site abolishes enzymatic activity and toxicity without affecting membrane binding [12]. We have fused model HLA-A2 restricted epitopes from the lower matrix 65 kDa phosphoprotein (pp65) of the cytomegalovirus (CMV) [13,14] to the C-terminus of the H34Q mutant of $bvPLA_2$ ($bvPLA_2$-H34Q). We have studied the binding of this fusion protein to human DCs, its internalization, and the cross-presentation of the CMV-derived epitopes it contained to CD8 T cells.

## 2. Materials and methods

### 2.1. Culture medium, cytokines, reagents, peptides and cell lines

IMDM (Gibco Invitrogen, Cergy Pontoise, France) was supplemented with 10% human AB serum (Biowest, AbCys, Paris, France), 0.24 mM aspartate, 0.55 mM arginine, 1.5 mM glutamine (Sigma, St. Quentin Fallavier, France) and 1% v/v penicillin–streptomycin (Gibco Invitrogen). Cytokines were from R&D (Lille, France), except IL-15, which was from Peprotech (Rocky Hill, NJ). Human albumin was from LFB (Courtaboeuf, France). Clasto-lactacystin β-lactone, chloroquine and brefeldin A (BFA) were from Sigma. Conjugates for flow cytometry were from BD Biosciences (Pont de Claix, France). Peptides N9V and M9V were from Neosystem (Strasbourg, France). The recombinant US6 protein was the kind gift of Dr. Tampé and Dr. Kyritsis (Institute of Physiological Chemistry, Marburg, Germany) [15]. Oligonucleotides were from Eurobio (Les Ulis, France). Cell lines T1 and T2 were obtained from the ATCC (Manassas, VA). T3 cells were kindly provided by Dr. Arnaud Morris (Pasteur Institute, Paris, France).

### 2.2. Fusion protein

The expression plasmid for the fusion protein P30–34 was derived from plasmid pQE-WT $PLA_2$ [16]. A *Bam*HI restriction site was introduced after the last codon of the $bvPLA_2$ sequence to introduce a syn-

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*Abbreviations:* APCs, antigen presenting cells; BFA, brefeldin A; $bvPLA_2$, bee venom phospholipase $A_2$; CMV, cytomegalovirus; DCs, dendritic cells; MHC, major histocompatibility complex; PBMCs, peripheral blood mononuclear cells; TAP, transporter associated with antigen processing; Tf, transferrin; uP30–34, unfolded P30–34

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thetic sequence encoding the epitopes N9V and M9V, each preceded and followed by the three amino acids found in the native sequence of pp65. A His-tag and a kallikrein cleavage site were present at the $bvPLA_2$ N-terminus. In summary, the native sequence of $bvPLA_2$ was preceded by the sequence MRGSHHHHHHGSPFR and followed by the sequence GSLAR**NLVPMVATV**QGQPLK**MLNIPSINV**H-HYGS (epitopes in bold letters). The H34Q mutation was then introduced by site-directed mutagenesis in the $bvPLA_2$ sequence. The entire coding region of the construct was sequenced. Expression of the protein in *Escherichia coli*, extraction of the inclusion bodies, His-tag mediated purification and in vitro refolding were done as described in [17] with a final yield of 2 mg of protein per liter of culture. The unfolded form of P30–34 (uP30–34) was sulfonated on its cysteines to prevent folding and aggregation [17]. For FACS and confocal microscopy experiments, P30–34 was labeled with Alexa488 carboxylic acid succinimidyl ester using the A-10235 labeling kit (Molecular Probes, Eugene, OR) according to the manufacturer's protocol.

### 2.3. Generation of DCs from monocytes

Human DCs were generated as described in [18,19]. In some experiments, DCs were generated from adherent monocyte precursors cultured in the presence of IL-4 and GM-CSF. Results obtained with both types of DCs were equivalent. DCs were typed by flow cytometry for the following markers: CD1a, CD14, CD16, CD80, CD83, CD86, MHC I, MHC II. HLA-A2 typing of the healthy donors' peripheral blood mononuclear cells (PBMCs) was done using monoclonal antibody BB7.2 (BD Bioscience).

### 2.4. Internalization assay using confocal microscopy

DCs were washed twice with phosphate-buffered saline (PBS) and spread onto poly L-lysine coated coverslips. Cells were allowed to adhere 20 min at room temperature in PBS, before recovering for 40 min at 37 °C in complete medium. Cells were washed once with PBS and incubated with 3 μM of P30–34-Alexa488 in PBS for 1 h at room temperature. They were then washed with RPMI supplemented with 2% fetal bovine serum, then incubated for 1 h in the same medium supplemented with 1 mg/ml dextran–rhodamine (66 kDa), 100 nM transferrin-Cy5 (Tf-Cy5) or 100 nM bafilomycin A1, as indicated. Cells were washed, then fixed with 3.7% paraformaldehyde. For immunofluorescence detection of Lamp-1, they were permeabilized with 0.1% saponin in PBS containing 0.1% bovine serum albumin and incubated with an anti-Lamp-1 monoclonal antibody (DSHB, University of Iowa), and then with rhodamine-labeled goat anti-mouse antibodies (Sigma). Cells were finally mounted in Moviol and examined under a Leica TCS 4D confocal microscope as described in [20]. Medial optical sections were recorded using a 63× objective. Fluorescence levels were equilibrated so that crosstalks between channels were negligible.

### 2.5. Generation of antigen-specific CD8 T cell lines

CD8 T cells were purified from non-adherent PBMCs by negative selection using the CD8 T cell Isolation Kit (Miltenyi Biotec, Paris, France) and were characterized by flow cytometry. Unless stated otherwise, immature DCs were pulsed for 1.5 h at 37 °C in PBS with 3 μM P30–34 or uP30–34 or 3 μM peptide N9V and 5 μg/ml human β2-microglobulin, and washed. CD8 T cells ($1.5 \times 10^5$/well) were co-cultured with autologous antigen-pulsed DCs ($3 \times 10^4$/well) in 96-well U-bottom plates in Iscove medium supplemented with 1000 U/ml IL-6, 5 ng/ml IL-12, 500 U/ml IFNγ and 1 μg/ml bacterial extract for maturation (Ribomunyl, Pierre Fabre Medicament, Boulogne, France) [19]. On day 7, DCs pulsed in the same conditions were used to re-stimulate the T cells in the presence of 20 U/ml IL-2, 10 ng/ml IL-7, 1 μg/ml bacterial extract and 500 U/ml IFNγ. On day 14, T cells were re-stimulated with pulsed DCs in the presence of 25 ng/ml IL-7, 25 ng/ml IL-15, 500 U/ml IFNγ and 1 μg/ml bacterial extract.

### 2.6. Stimulation of CD8 T cell lines by APCs loaded with antigens

Stimulation experiments of established specific CD8 T cell lines by antigen presenting cells (APCs) loaded with antigens were performed by conventional IFNγ ELISPOT assay [21]. APCs (DCs or T1, T2 or T3 cells) ($10^4$/well) pulsed with the antigen were mixed with CD8 T cells ($10^3$/well). Controls included APCs alone and T cells in presence of APCs and 10 μg/ml phytohemaglutinin. IFNγ ELISPOT assays were done after overnight incubation at 37 °C. Spots were counted with an AID ELISPOT reader (Straßberg, Germany). For experiments with inhibitors, DCs were treated with the inhibitor during the antigen pulse, washed and cultured with the same doses of inhibitor for 5 h to allow eventual processing prior to incubation with the T cells in the ELISPOT plates. When treated with chloroquine, $NH_4Cl$, leupeptine and BFA, DCs were fixed with glutaraldehyde before incubation with the T cells because the effect of these drugs is reversible.

## 3. Results

### 3.1. Recombinant protein P30–34

A recombinant protein (referred to as P30–34 thereafter) was produced in which the immuno-dominant epitope NLVPMVATV (N9V) [13,14] and the sub-dominant epitope MLNIPSINV (M9V) of pp65 from CMV [13] were fused to the C-terminus of $bvPLA_2$-H34Q. These epitopes correspond, respectively, to positions 495–503 and 120–128 of pp65. Both are HLA-A2-restricted. Each epitope sequence was preceded and followed by the three amino acids found in the native sequence of pp65 (see Section 2). These extra residues were included to favor proper processing.

The protein structure was analyzed by circular dichroism in the far- and near-UV. Spectra were compared to those obtained with recombinant $bvPLA_2$ and $bvPLA_2$ purified from venom (Fig. 1A). The spectra in the far-UV revealed that P30–34 contained secondary structures similar to those of the natural $bvPLA_2$ plus some additional random structures,

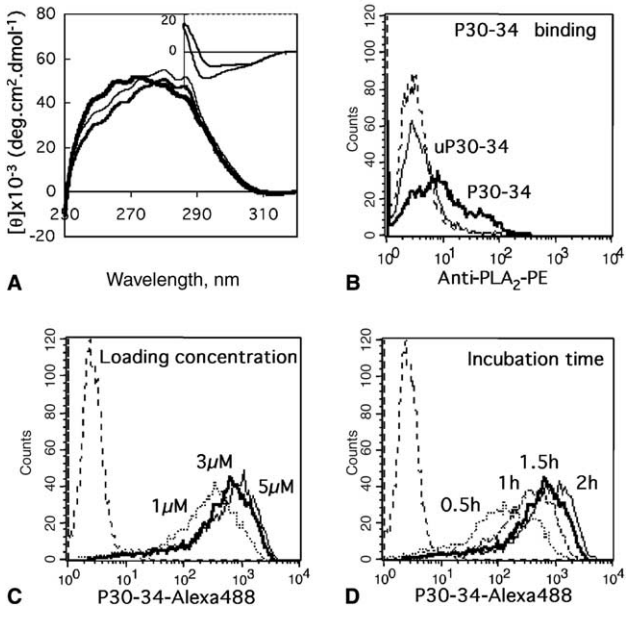


Fig. 1. (A) Near-UV circular dichroism spectra of $bvPLA_2$ from venom (thin line), recombinant $bvPLA_2$ (middle line) and P30–34 (thick line). Inset, far-UV (from 195 to 250 nm) circular dichroism spectra of $bvPLA_2$ from venom and recombinant $bvPLA_2$ (overlapping on the upper trace) and P30–34 (lower trace). (B–D) FACS analysis of P30–34 binding to DCs. (B) DCs were incubated with 3 μM of the indicated proteins for 1.5 h. DCs were then incubated with a rabbit anti-$bvPLA_2$ serum and with a phycoerythrin anti-rabbit IgG antibody. The dotted line corresponds to control DCs incubated only with the serum and the labeled antibody and the dashed line to DCs only. (C) DCs were incubated with the indicated concentration of P30–34-Alexa488 for 1.5 h in PBS at 37 °C. (D) DCs were incubated with 3 μM of P30–34-Alexa488 for the indicated time in PBS at 37 °C. The dashed line corresponds to unlabeled DCs.

likely contributed by the His-tag at the N-terminus and the epitope sequence at the C-terminus of the protein. The spectra in the near-UV were very similar for the three proteins, indicating the presence of a native-like tertiary structure for the $bvPLA_2$ part of P30–34.

We also produced an uP30–34 by sulfonation of its cysteines to prevent folding and aggregation. It was recognized on a Western blot by a polyclonal rabbit serum against $bvPLA_2$ (not shown) and was expected to be unable to bind to cell membranes.

### 3.2. Binding and internalization of P30–34 by DCs

Binding of P30–34 and uP30–34 to DCs was evaluated by flow cytometry. Binding was analyzed for both forms of the protein using a polyclonal rabbit serum against $bvPLA_2$ and a phycoerythrin-anti-rabbit secondary antibody. P30–34 bound to DCs was detected by the antibodies while no binding of uP30–34 to DCs was found (Fig. 1B). This confirmed that binding of P30–34 to the membrane of DCs was dependant on proper folding of the protein. To determine the best binding conditions, P30–34 was labeled with the fluorescent probe Alexa488 and incubated with the cells in PBS for various lengths of time and concentrations. Figs. 1C and D show that maximal binding was reached after 1.5 h for a P30–34 concentration of 3 μM. Higher protein concentrations or longer incubation times did not significantly increase binding.

We studied the internalization of P30–34 by DCs using confocal microscopy. Cells were first exposed to P30–34-Alexa488 at 20 °C before incubation for 1 h at 37 °C with Tf-Cy5 to label early endosomes, and with 66 kDa dextran-rhodamine, a tracer endocytosed by fluid phase uptake and directed to endosomes and then lysosomes [22]. As shown in Fig. 2, DCs efficiently internalized P30–34. Dextran extensively colocalized with Tf (~60%) indicating that in the absence of chase a large fraction of dextran remained in early endosomes. Accumulation of P30–34 could be observed within these early endosomes ($Tf^+$ and $dextran^+$ structures) and to some extent within late endosomes ($Tf^-$ and $dextran^+$ structures) (Fig. 2). Delivery of P30–34 to late endocytic elements was confirmed using the late endosome/lysosome marker Lamp-1 [22]. Endocytosed P30–34 was found within Lamp-1 positive structures (Fig. 2) and P30–34 transport to lysosomes was inhibited in the presence of bafilomycin, which neutralizes endosomes [22]. This finding is in agreement with previous studies showing that bafilomycin blocks cargo delivery to lysosomes [23].

Taken together, these data indicate that P30–34 was able to bind to the surface of DCs and was then efficiently internalized within early endosomes and routed toward late endosomes/lysosomes during the first hour following exposure of DCs.

### 3.3. Generation of specific CD8 T cell lines against epitopes N9V and M9V by DCs loaded with protein P30–34

In order to study the capacity of human DCs to present epitopes contained in the P30–34 protein, we performed in vitro immunization experiments. Purified HLA-A2 CD8 T cells from healthy donors were stimulated with autologous DCs loaded with P30–34. The uP30–34 as well as synthetic 9-mer peptides N9V and M9V were used as controls. In particular, uP30–34 was used to determine whether binding of P30–34 enhanced epitope presentation. DCs were incubated with the proteins or the peptides at a concentration of 3 μM in PBS for 1.5 h and then mixed with the CD8 T cells in 96-well plates. After three such stimulations over three weeks, the cell content of each well was assayed for specificity against peptides N9V

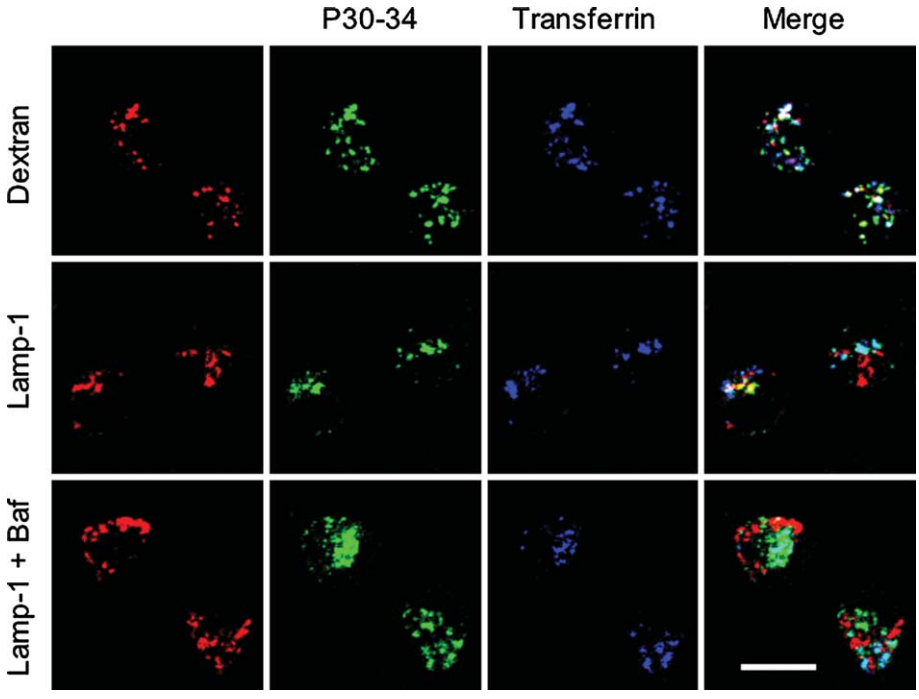


Fig. 2. Internalization of P30–34 by DCs. Upper panel, DCs loaded with P30–34-Alexa488 (green) and then incubated for 1 h with Tf-Cy5 (blue) and dextran-rhodamine (red). Lower panels, DCs loaded with P30–34-Alexa488 (green) and then incubated for 1 h with Tf-Cy5 (blue) in the presence or absence of bafilomycin (Baf). Lamp-1 was revealed by immuno-fluorescence (red). Medial optical sections were obtained with a confocal microscope. Scale bars = 10 μm.

Table 1
Efficiency of generation of N9V-specific CD8 T cell lines using P30–34, uP30–34, or N9V as antigens in in vitro immunizations

| Healthy donor # | N9V-specific cell lines/number of seeded wells | | |
|---|---|---|---|
| | Generated with P30–34 | Generated with uP30–34 | Generated with N9V |
| HD 04 | 13/20 (65%) | | 18/20 (90%) |
| HD 15 | 4/20 (20%) | 1/20 (5%) | 10/20 (50%) |
| HD 29 | 20/20 (100%) | 9/20 (45%) | 20/20 (100%) |
| HD 33 | 24/30 (80%) | | 27/30 (90%) |
| Total | 61/90 (68%) | 10/40 (25%) | 75/90 (83%) |

Specificity was assessed using DCs loaded with peptide N9V.

and M9V by IFNγ ELISPOT (using DCs loaded with peptides N9V or M9V). When DCs were loaded with P30–34, 68% of seeded wells contained T cell lines specific for N9V (Table 1). In contrast, when DCs were loaded with uP30–34, only 25% of wells contained T cell lines specific for N9V. In control experiments with DCs loaded with peptide N9V, 83% of seeded wells contained T cell lines specific for N9V. Very few cell lines were generated against epitope M9V, whether DCs were loaded with P30–34 or peptide M9V. This may be explained by the subdominance of M9V [13], while N9V is dominant. Therefore, we focused the study on the N9V epitope only.

Together, the results show that a dominant epitope linked to bvPLA$_2$-H34Q can be efficiently presented to circulating CD8 T cells by DCs. Also, the results strongly suggest that binding of the vector protein to the surface of DCs enhance presentation of the epitope it carries.

### 3.4. Activation of CD8 T cell lines specific for epitope N9V by DCs loaded with protein P30–34

We studied the capacity of DCs loaded with P30–34 to activate N9V-specific T cell lines established in the previous experiment (T cell lines generated by DCs loaded with either P30–34, uP30–34 or peptide N9V). As controls, we used DCs loaded with uP30–34, bvPLA$_2$, peptide N9V, peptide M9V or PBS alone. All T cell lines were activated by DCs loaded with P30–34 (Fig. 3). They all responded to peptide N9V as well. In sharp contrast, T cells did not respond to DCs incubated with uP30–34. As expected, they did not respond to

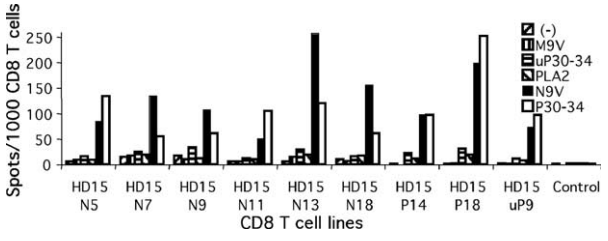


Fig. 3. Response profile of N9V-specific CD8 T cell lines generated with DCs loaded with either peptide N9V, proteins P30–34 or uP30–34. Cell lines were named after healthy donor # (HD#) and the antigen used for their generation (N for peptide N9V, P for P30–34 and uP for uP30–34). After screening of T cell lines for N9V-specificity, their response against PBS, peptides N9V or M9V and proteins P30–34, uP30–34 or bvPLA$_2$ was monitored by IFNγ ELISPOT (see bar motif correspondence on the figure). Peptides and proteins were incubated with DCs at a concentration of 3 μM.

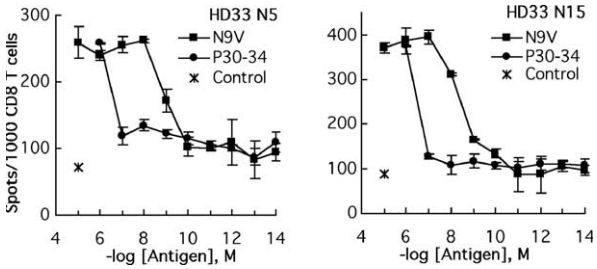


Fig. 4. Effect of the concentration of peptide N9V or protein P30–34 used for the loading of DCs on the response of N9V-specific CD8 T cell lines. The level of stimulation was monitored by IFNγ ELISPOT. Unloaded DCs were used as controls.

bvPLA$_2$ or peptide M9V either. These results further assess the specificity of the N9V-specific T cell lines and do not reveal any difference in specificity among cell lines generated with the peptide or the protein carrying the N9V epitope. These data also show that DCs do not efficiently present the epitope carried by uP30–34. This confirms that P30–34 must bind to the surface of DCs for the epitope to be efficiently presented.

To get a rough estimate of the efficiency of epitope generation using the P30–34 protein, we compared the stimulation of N9V-specific cell lines by DCs incubated with decreasing doses of protein or peptide. The results in Fig. 4 show that presentation of epitope N9V required 100-fold more protein than peptide, suggesting that roughly 1 epitope was produced and presented from 100 protein copies. Maximum stimulation was reached when DCs were incubated with P30–34 at a concentration of 3 μM, the concentration giving maximum binding to the DCs.

### 3.5. Cross-presentation of epitope N9V from protein P30–34 did not require proteasome nor TAP activities, was chloroquine insensitive but BFA sensitive

In an attempt to analyze the processing pathway followed by P30–34, we studied the role played by the proteasome, the transporter associated with antigen processing (TAP), the endosome acidity and the Golgi in the presentation of P30–34.

DCs were treated with clasto-lactacystin β-lactone, an irreversible inhibitor of proteasome function [24]. Ten μM of inhibitor did not affect presentation of epitope N9V carried by P30–34 or as a free peptide (Fig. 5A). In contrast, clasto-lactacystin β-lactone was able to inhibit by 63% the presentation of the intracellular tumor antigen NY-ESO-1 by SK-MEL-37 tumor cells, but not of its epitope S9C as a peptide incubated with the cells [25] (data not shown). These results indicate that the protein P30–34 was not processed by the proteasome of DCs for presentation of its N9V epitope to T cells.

The cell lines T1 and T2 were used as APCs to study the role of TAP in the presentation of epitope N9V from P30–34. T1 is a T cell-B cell hybridoma capable of cross-presentation. T2 is a TAP-deficient derivative of T1 generated by mutagenesis. T1 and T2 cells were incubated with the protein P30–34 or the peptide N9V under the same conditions as DCs. T1 and T2 incubated with P30–34 had the same capacity to stimulate N9V-specific T cells (Fig. 5B). Similar results were obtained using the cell line T3 instead of T1, which was derived from T2 by TAP reconstitution following gene transfer (Fig. 5C). In another experiment, DCs were incubated with recombinant US6 protein, a potent TAP inhibitor expressed by CMV [15]

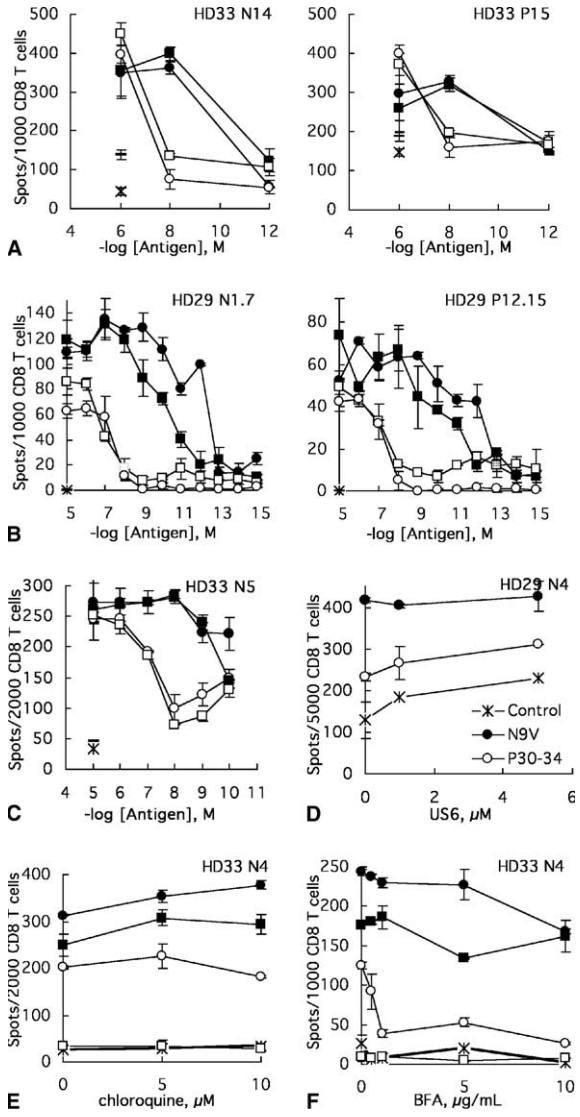


Fig. 5. Role of proteasome, TAP, endosome acidification and Golgi in the presentation of P30–34. (A) DCs were treated with (squares) or without (circles) 10 μM clasto-lactacystin β-lactone to block proteasome during loading with the indicated concentrations of N9V (closed symbols) or P30–34 (open symbols). Unloaded DCs treated with (dash) or without (cross) 10 μM clasto-lactacystin β-lactone were used as controls. (B) The cell lines T1 (squares) or T2 (circles) were used as APCs and loaded with the indicated concentrations of N9V (closed symbols) or P30–34 (open symbols). Unloaded T1 (cross) or T2 (dash) were used as controls. (C) Same as B except that T3 cells (squares) were used in place of T1. Unloaded T3 (cross) or T2 (dash) were used as controls. (D) DCs were treated with the indicated concentrations of US6 during loading with 3 μM of the indicated antigen. (E,F) DCs were treated with the indicated concentrations of chloroquine or BFA during loading with 3 μM (circles) or 0.3 μM (squares) of N9V (closed symbols) or P30–34 (open symbols). Unloaded DCs were used as controls (cross). In all panels, the level of stimulation was monitored by IFNγ ELISPOT. Each panel is named after the CD8 T cell line used (see Fig. 3 legend).

before incubation with P30–34. US6 did not prevent the presentation of epitope N9V from P30–34 (Fig. 5D). Altogether, these results indicate that cross-presentation of the N9V epitope processed from P30–34 was not dependent on TAP function. As expected, presentation of the peptide N9V used as a control, which binds directly to HLA-A2 at the surface of the presenting cells without need for processing, was not dependent on TAP.

In order to study the role of endosomal proteases in the processing of N9V contained in the P30–34 protein, DCs were treated with chloroquine (Fig. 5E) or $NH_4Cl$ (not shown), which are inhibitors of endosome acidification (and thus of endosomal proteases) and with leupeptine (not shown), which is an inhibitor of some endosomal proteases [26]. None of these inhibitors affected presentation of epitope N9V carried by P30–34. As expected, presentation of the control peptide N9V was not affected either. These data indicate that processing of P30–34 did not involve endosomal proteases.

The possibility that P30–34 was processed by extracellular proteases once bound to the surface of DCs was investigated. We compared the presentation of P30–34 by DCs alive or fixed with glutaraldehyde prior to incubation with the antigen. Glutaraldehyde blocks intracellular trafficking and thus intracellular processing, but not processing by extracellular proteases [27]. Presentation of epitope N9V from P30–34 was strongly decreased using fixed DCs (66–78% decrease in presentation). In contrast, presentation of peptide N9V was almost as efficient using fixed or untreated DCs (17–21% decrease in presentation). uP30–34 was not presented by DCs fixed or untreated. These results suggest that P30–34 was not processed by extracellular proteases.

Finally, DCs were treated with BFA, a fungal metabolite that disassemble the Golgi apparatus, blocking the transport of neo-synthesized MHC class I molecules from the endoplasmic reticulum to the surface [26]. Two μg/ml of BFA completely abolished presentation of epitope N9V from P30–34, but not of peptide N9V (Fig. 5F). The level of HLA-A2 on the cell surface was moderately reduced (not shown). These results show that an intact, functional Golgi is required for cross-presentation of epitope N9V from P30–34. Therefore, they suggest that binding of the epitope to MHC class I molecules occurs in intracellular compartments involved in the secretory pathway containing newly synthesized MHC molecules and not in recycling vesicles.

## 4. Discussion

We have shown that the mutant bvPLA$_2$-H34Q can be used as a membrane-binding vector for the attachment of epitopes or antigens to the membrane of DCs ex vivo. The fusion protein P30–34, in which epitopes N9V and M9V were fused to bvPLA$_2$-H34Q, was internalized by the DCs into early endosomes and within 1 h reached the late endosomes/lysosomes. One of the epitopes, N9V, which has been described as dominant [13,14], was efficiently processed and presented to CD8 T cells. DCs loaded with P30–34 were able both to generate efficiently N9V-specific T cell lines and to activate existing N9V-specific cell lines. The membrane-binding activity of bvPLA$_2$ played an essential role in the cross-presentation of the fusion protein. Indeed, the uP30–34, unable to bind to DCs was less efficient to generate N9V-specific T cell lines and totally unable to activate established N9V-specific T cells in IFNγ ELISPOT assays.

The cross-presentation of the epitope N9V from P30–34 was less efficient than the presentation of the free peptide N9V. This is explained by the direct availability of class I

molecules at the cell surface for peptide binding. Nevertheless, the results presented here show the potential of bvPLA$_2$-H34Q as a vector for loading protein antigens onto DCs for presentation of their MHC class I epitopes to T cells. This may have application for the design of vaccines using DCs loaded ex vivo with tumor or viral antigens [1,2]. Antigens as whole proteins are much better immunogens than peptides because they contain a set of epitopes, which usually encompass the polymorphism of MHC class II and class I molecules. Thus, a protein vaccine may protect more individuals than a vaccine containing a single peptide epitope. However, soluble proteins are poorly internalized by DCs, justifying the need for a vector. Preliminary data with a bvPLA$_2$-H34Q-tumor antigen fusion protein showed the presentation of MHC class II and class I epitopes to CD4 and CD8 T cells, respectively (unpublished results).

Another question raised by our study is the presentation pathway followed by antigens vectorized by bvPLA$_2$-H34Q. The data presented here show that cross-presentation of P30–34 is independent from proteasome, TAP or endosome proteases, but requires a functional Golgi apparatus. Antigens captured by DCs may follow different routes of processing for their epitopes to be loaded onto MHC class I molecules and presented to T cells [28]. In a first pathway, internalized antigens are translocated from the phagosome to the cytoplasm where they are chopped into peptides by the proteasome [29,30]. These peptides are transported back into the phagosome by TAP where they bind to newly synthesized MHC class I molecules. Peptide-MHC class I complexes are then targeted to the cell surface. In a second pathway, internalized antigens can be degraded into peptides by proteases present inside the endosomes following acidification of these compartments [26,28]. These peptides can then bind to preformed MHC class I molecules recycled from the cell surface to the endosomes. However, antigens loaded onto DCs using bacterial or plant toxins as vectors can follow other routes of presentation. In particular, pertussis toxin, shiga toxin, *Pseudomonas* exotoxin A or ricin used as vectors direct the antigen from early endosomes to the endoplasmic reticulum using the retrograde transport through the Golgi [31,32]. This route is independent from endosome acidification but is blocked by BFA [33]. Processing of antigens following this pathway can be independent from proteasome, TAP or endosome proteases [31,32,34] but may rely on proteases inside the reticulum such as signal peptidase [32] or endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP) [35]. Then, generated peptide epitopes bind to newly synthesized MHC class I molecules en route to the surface through the Golgi. Together, our data strongly suggest that P30–34 followed the same processing and presentation pathway.

A last possibility would be that processing involved extracellular proteases, the generated epitopes binding directly to cell surface HLA-A2 molecules. If this was the case, the effect of BFA on the presentation of P30–34 should be due to a decrease of cell surface HLA-A2 and should affect the presentation of peptide N9V as well. In contrast, the presentation of peptide N9V was not affected by BFA and the level of cell surface HLA-A2 was only slightly reduced. In addition, P30–34 was not presented by DCs fixed with glutaraldehyde, a treatment known to block intracellular processing but not extracellular processing [27], while the peptide was. uP30–34 was not presented by fixed or untreated DCs, showing also the absence of extracellular processing.

A recent work showed that the enzymatic activity of bvPLA$_2$ induces the maturation of DCs [36]. Thus, the use of wild type bvPLA$_2$ instead of bvPLA$_2$-H34Q as a vector may further increase the efficiency of antigen presentation, a possibility which remains to be tested. Overall, our data show that bvPLA$_2$-H34Q can function as a membrane-binding vector to attach antigens to the membrane of DCs ex vivo, leading to recognition by and activation of CD8 T cell.

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