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Authors

Juliette ANGEL[1,2], Laurence CHAPEROT[1,2], Jean-Paul MOLENS[1,2], Paulette MEZIN[2], Mario AMACKER[4], Rinaldo ZURBRIGGEN[4], Alexei GRICHINE[1], Joël PLUMAS[1,2*]

Affiliations

[1]Inserm, U823, Centre de Recherche Albert Bonniot, La Tronche, F-38706 France ; Univ Joseph Fourier, Grenoble, F-38041 France ;
[2]EFS Rhône-Alpes, Grenoble, F-38701 LA TRONCHE, France ;
[3]CHU Grenoble, Hopital Michallon, Laboratoire de Pathologie Cellulaire, Grenoble, F-38043, France
[4] Pevion Biotech Ltd, Bern, CH-3018, Switzerland

Corresponding author
* Dr Joël Plumas, Etablissement Français du Sang, Department of Research and Development, EFS Rhône-Alpes Grenoble, BP35, 38701 La Tronche France.
Tel : +33 4 76 42 43 44
Fax : +33 4 76 42 94 49
eMail: joel.plumas@efs.sante.fr
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Abstract

Cytotoxic T lymphocytes (CTL) are crucial in viral clearance and tumor growth control. Thus the induction of CTL activity is an important aim in vaccine development. We investigate an innovative delivery system for peptide transfer to the MHC class-I processing pathway of APC with the aim to trigger CTL in the context of an anti-tumoral response. The strategy relies on a novel antigen delivery system termed “chimeric immunopotentiating reconstituted influenza virosomes” (CIRIV) targeting plasmacytoid dendritic cells (PDC). By using virosomes containing encapsulated Melan-A peptide and a PDC line developed in our laboratory, we evaluated the response of Melan-A specific T cells. Virosomes have the capacity to bind PDC and are endocyted within vesicles in the cytosol. This endocytosis is inhibited by neuraminidase, suggesting that it is mediated by sialic acid present on cell surface. Furthermore, PDC loaded with Melan-A virosomes can induce a Melan-A specific T cell activation. Interestingly, they activate T cells with a better efficiency than PDC loaded with a free peptide and when PDC where previously activated by a TLR ligand. These results indicate that virosomes could be a suitable delivery system for tumor peptide in immunotherapy of cancer.

1. Introduction

Cytotoxic T lymphocytes (CTL) play a key role in the clearance of viruses and in the control of tumor development. They are firstly able to identify viral or cancer-associated peptides presented by MHC class I molecules, expressed at the surface of infected or transformed cells, and secondly to kill these cells. Thus, the induction of CTL activity is a major goal for vaccine development.

In this context, the professional antigen presenting cells (APC) are essential (1). Indeed, dendritic cells (DC) are the only cells able to stimulate naive T cells (2). Usually, exogenous proteins are presented by class II MHC molecules, resulting in induction of $CD4^+$ T lymphocytes activity. To induce a CD8+ T cell activation, exogenous antigens have to be translocated into the APC cytosol, where they join class I pathway. This phenomenon is referred to as cross-presentation (3).

To efficiently load antigenic peptides onto MHC class I molecules of APC for a vaccination strategy, several different pathways exist (4). The first type of approach is based on the transfection of RNA in APC, or the active delivery of proteins into the cytoplasm of these cells and exploit the 'classical' endogenous cytosolic MHC class I pathway. The second type is based on the capacity of cross-presentation of APC, and uses cell-associated exogenous antigens. Among these various strategies, virosomes represent good candidates as a vaccine to generate T cell responses. Chimeric immunopotentiating reconstituted *influenza* virosomes (CIRIV) are spherical unilamellar vesicles generated from two different strains of *Influenza* virus (5, 6). These reconstituted viral envelopes of about 150 nm diameter contain the cell binding and fusion proteins of the native *influenza* virus, but do not contain the virus core and its genetic material. Heamagglutinin (HA) and neuraminidase from *Influenza* virus are intercalated within the phospholipid bilayer membrane and endows CIRIV with cell entry and membrane fusion

properties. Regarding *influenza* virus, HA binding to sialic acid-containing glycoproteins on APC surface leads to receptor-mediated endocytosis of the virus. Into the late endosomes, triggered by low pH, HA-mediated fusion occurs between the viral envelope and the endosomal membrane. As a result, the ribonucleoproteins are delivered into the cell cytosol and move to the nucleus (7). Virosomes are devoid of viral nucleoproteins, but during their preparation, antigens can be encapsulated into the CIRIV and thus, like viral genome, could be released into the cytosol of APC. This leads to their presentation in association with MHC class I molecules, and therefore to induction of CD8+ T cells (8-10). In addition to their capacity to deliver compounds into the cytosol of target cells, virosomes physically protect soluble antigens from extracellular protease degradation (11, 12).

In human, there are two main DC subsets : the myeloid and the plasmacytoid DCs. During viral infection, plasmacytoid dendritic cells (PDC) are known to be highly efficient producers of type I IFN (13, 14). They also produce a large amount of cytokines, leading to an inflammation and linking innate to adaptive immunity (15). Recently, PDC have been described to trigger CTL and T helper activation following processing of viral antigens, in the context of influenza infection (16). Because of this specialization of PDC in virus recognition, we decided to exploit the potential of virosomes loaded with a tumor peptide to deliver antigen into cell and induce its presentation to tumor-specific T cells. By using virosomes loaded with Melan-A peptide and a PDC line developed in our laboratory (17), we evaluated the response of Melan-A specific T cells.

2. Materials and Methods

Reagents

Mouse fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, Phycoerythrin-Cy7 (PC7)- or allophycocyanin (APC)-labelled anti-CD3 (UCHT1[23]), -CD8 (B9.11), -CD14 (RMO52), -CD40 (mAb89), -CD80(MAB104), FITC-labeled goat anti-rabbit immunoglobulins (polyclonal) and isotype control antibodies, were purchased from Immunotech (Beckman Coulter, Marseille, France). Rabbit anti-haemagglutinin was purchased from Virology Laboratory (Lyon, France). FITC-CD1a (HI149) were purchased from BD Pharmingen (San Diego, USA). FITC-anti-Human IFN-γ (25723.11) were purchased from BD Biosciences (San jose, USA).

HLA-A*0201-Mart-1/Melan-A and HLA-A*0201-*Influenza* matrix PE-labelled tetramers were purchased from Beckman Coulter (Fullerton, CA, USA).

Immunophenotypes were performed by flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA) and the results were analysed using Cellquestpro software.

Peptides MART-1/Melan-A_{27L-35} (ELAGIGILTV) and *influenza* matrix protein Flu_{58-66} (GILGFVFTL) were purchased from Neosystem (Strasbourg, France) and dissolved in DMSO (SIGMA, St Louis, USA).

Preparation of CIRIVs

Immunopotentiating reconstituted *influenza* virosomes were prepared by Pevion Biotech Ltd. (Bern, Switzerland) as previously described (5). Native inactivated viruses were first dissolved in detergent and the viral nucleocapsids were removed. Surface glycoproteins from *Influenza* virus, were then intercalated within the phospholipid vesicles formed by detergent removal with a total HA content of 2 mg/mL. Peptide-containing CIRIV were prepared as described by encapsulating

the HLA-A2.1-binding Melan-A_{27L-35} peptide into the lumen of CIRIV (6). For 1 µg/mL of fusion-active HA, these Melan-A-CIRIV contained 0.075 µM of encapsulated Melan-A peptide.

Cells

The T2 cell line (ATCC-CRL-1992) is a TAP deficient TB cell hybrid expressing HLA-A*0201 heavy chains non-covalently associated with β2-microglobulin and devoid of peptide. The PDC cell line called GEN2.2 was generated in the laboratory from leukemic PDC (17, 18). GEN2.2 cell line is cultured on a monolayer of irradiated (60 Gy) murine stromal MS-5 cells.

MS-5, GEN2.2 PDC and T2 cell line were grown in RPMI 1640 Glutamax (Gibco, Paisley, UK) supplemented with 1 mmol/L of sodium pyruvate (Sigma Cell Culture, Irvine, UK), 200 µg/mL gentamycin, non essential amino acids (Gibco) (referred to as complete medium) and 10% Fetal Calf Serum (FCS, Gibco).

Melan-A specific $CD8^+$ T cells

Based on the method described by Fonteneau (19), $CD8^+$ T cells and monocytes were purified from peripheral blood mononuclear cells (PBMC) of HLA-A*0201^+ healthy volunteers by EasySep and RosetteSep technology kit respectively (StemCell Technologies, Vancouver, BC), according to the manufacturer's instructions. The purity of $CD8^+$ T cells suspension was higher than 90% ; and in monocytes preparation, the percentage of $CD14^+$ cells was above 95%.

Mature myeloid dendritic cells (MDC) were generated by culturing monocytes in complete medium supplemented with 10 % FCS in the presence of GM-CSF (500 U/mL, Leucomax, Schering-Plough, France) and IL-4 (10 ng/mL, Tebu Bio, Le Perray-en-Yvelines, France) for 6 days. At the fourth days of culture IL-4 (10 ng/mL), and on day six, soluble CD40L and Enhancer (100 ng/mL and 1µg/mL, respectively, Alexis, Lausen, Switzerland) were added. After

seven days of culture, CD1a, CD40, and CD80 expression were checked, usually around 90%, and MDC were cryopreserved.

In order to generate Melan-A specific $CD8^+$ T cells, MDC were incubated with 10 µM of Melan-A peptide at 37°C for 3 hours in complete medium without FCS and then washed twice. Melan-A pulsed MDC (3.10^5 cells) were co-cultured with autologous $CD8^+$ T cells at the ratio 1:10 in 24-well culture plates in 2 mL of complete medium supplemented with 10 % FCS in the presence of IL-6 (1000 U/mL, Peprotech, Rocky Hill, NJ, USA) and IL-12 (5 ng/mL, R&D Systems, Abingdon, UK). T cells were restimulated weekly with Melan-A pulsed MDC at the ratio 1:10 in presence of IL-2 (10 U/mL, Roussel-Uclaf, Romainville, France) and IL-7 (5 ng/mL, TEBU). Six days after the second restimulation, the percentage of Melan-A-specific T cells (between 40 to 60 %) was determined by tetramer labelling, and cells were cryopreserved.

Binding and endocytosis of virosomes

Binding of CIRIV to GEN2.2 PDC was analysed by incubation of GEN2.2 PDC with increasing amounts of empty CIRIV at 37°C or 4°C for 1 hour. Binding of CIRIV to T2 cells was performed with 1 µg/mL of empty virosome at 37°C for 1 hour. Then cells were washed twice and labelled with a rabbit anti-HA antibody revealed by a goat anti-rabbit-FITC antibody. In some experiments, cells were pre-incubated with neuraminidase from *Vibrio cholerae* (5 mU/mL, Roche, Germany) for 1 h at 37°C before incubation with CIRIV. Cells were then stained as previously described.

In order to determine the location of virosomes, GEN2.2 PDC were incubated with empty CIRIV (1 µg/mL HA) at 37°C for 1 hour, washed and attached to glass slides by cyto-centrifugation (500 rpm, 5 min). Cells were labelled with a rabbit anti-HA antibody followed by a goat anti-rabbit-

FITC antibody, and nucleus were counterstained in blue by slowfade (Molecular Probes, Eugene, USA). Cells were observed with optical microscope.

GEN2.2 PDC incubated with CIRIV (10 µg/mL HA) or *Influenza* virus strain A/New Caledonia/20/99 IVR116 (Aventis Pasteur, Val de Reuil, France) for 1 hour were also analyzed by transmission electron microscopy.

To check the effect of CIRIV on PDC activation, the expression of maturation markers was determined after incubation of PDC with empty CIRIV (1 µg/mL HA) at 37°C for 24h. As control, GEN2.2 PDC were incubated in medium alone or with *Influenza* virus in the same conditions. After 24h the supernatants were harvested and analysed for cytokine contents (IFN-γ, TNF, IL-6 and IL-8) by Cytokine Bead Array kit (CBA, Becton Dickinson, Pont de Claix, France) and for IFN-α by ELISA (PBL biomedical laboratories, Piscataway, NJ) according to the manufacturer's instructions. CD80 and CD40 expression were analyzed on GEN2.2 PDC.

Antigen presentation assay

For presentation assay we used a protocol set up in our laboratory (Manches et al, submitted) as following. GEN2.2 (0.5 106/mL) were pre-incubated either with IL-3 (10 ng/mL, PeproTech) or with IL-3 and R848 (1 µg/mL, InvivoGen, San Diego, USA) for 24 hours. Then cells were washed, incubated in complete medium without FCS with 2-microglobulin (100 ng/mL, Sigma) for 15 minutes at 37°C, afterwards either Melan-A (10 µM) or Flu peptide (1 µM) or empty CIRIV or Melan-A-CIRIV (1 or 10 µg/mL of HA) were added for 3 hours at 37°C. After two washes, 50 000 GEN2.2 PDC were co-cultured with 50 000 Melan-A specific $CD8^+$ T cells in round-bottom 96 wells culture plates (Becton Dickinson, NJ, USA), in 200 µL of complete medium supplemented with 10 % FCS at 37°C. After 2 days, culture supernatants were recovered for IFN-γ measurement, using CBA kit following the manufacturer's instructions.

In the inhibition experiment, R848 activated GEN2.2 PDC were pre-incubated 30 minutes with Cytochalasin D (10 μM, Sigma) prior to antigen presentation assay. Cytochalasin D was present during the loading of peptide or virosome.

Some experiments were also performed with the T2 cell line, without prior activation.

Intracellular staining of IFN-γ

Melan-A pulsed GEN2.2 PDC were incubated for 5h30 at 37°C with Melan-A specific T cells pre-labelled with Melan-A tetramer. Cells were washed and labelled with CD3 and CD8. After permeabilization, cells were stained with IFN-γ and fixed before flow cytometry analysis.

Statistical analysis

The unpaired Student's t-test was used to determine if the difference in IFN-γ secretion observed between conditions was significant. A value of $p < 0.05$ was considered significant.

3. Results

Uptake of empty CIRIV by GEN2.2 PDC

We first focused on the virosome binding to and capture by DC. To evaluate the binding of CIRIV, GEN2.2 PDC were incubated for 1 hour with increasing amounts of empty CIRIV, and HA expression at the surface of the cells was revealed. In preliminary kinetic experiments, we determined that 1 hour was a sufficient time for CIRIV binding to GEN2.2 cells (data not shown). Barely detectable fluorescence intensity could be observed in GEN2.2 incubated without virosomes. From 100 ng/mL of HA, more than 90 % of PDC expressed HA. However, there was

a dose-dependent increase in fluorescence intensity from 10 to 1000 ng/ml HA of CIRIV (Fig 1A). We chose the highest HA concentration (1 µg/mL) for the following experiments.

We further investigated if virosomes used sialic acid to bind to cells. In order to remove these sugars, cells were treated with neuraminidase for 1h before addition of empty CIRIV. Whereas 97 % of the cells were positive for HA labelling under normal conditions, only 17 % of GEN2.2 PDC treated by neuraminidase captured virosomes (Fig 1B).

The internalization of the virosomes by GEN2.2 cells was then evaluated. GEN2.2 PDC were incubated with empty virosomes for one hour at 4°C or 37°C, cells were observed after HA and DAPI labelling, in order to localize CIRIV. GEN2.2 incubated with virosomes at 4°C showed a membranous fluorescence (Fig 2A). Conversely, cells incubated with virosomes at 37°C displayed a vesicular fluorescence into the cytosol, suggesting an endosomal localization of CIRIV-associated HA. By transmission electron microscopy, we confirmed that CIRIV were located within vesicles in the cytoplasm of GEN2.2 cells. As shown in figure 2B, one endosome usually contained more than one virosome. Altogether, these experiments suggest that virosomes bind to cells through sialic acid residue and are further endocyted into the cytoplasm.

Effect of CIRIV on PDC maturation

We then wondered whether CIRIV induced a maturation of GEN2.2 PDC. After a 24h-culture of GEN2.2 PDC with virosomes, cell activation was measured by expression of CD80 and CD40, and by cytokine production. As positive control, cells were incubated with *Influenza* virus, a TLR7-ligand able to induce the expression of maturation markers in GEN2.2 PDC. Direct addition of CIRIV to GEN2.2 PDC did not up-regulate the expression of CD80 and CD40 (Fig 3A), whereas with virus more than 75% of the cells were positive for both molecules. Similarly, CIRIV did not induce inflammatory cytokine production, contrary to virus, that triggered the

secretion of TNF-α, IL-6, IL-8 and IFN-α (Fig 3B and 3C). These data show that virosomes did not induce the maturation of GEN2.2 PDC *in vitro*, as expected since virosomes do not contain viral RNA material.

Endocytosis of Melan-A-CIRIV allows efficient cross-presentation of Melan-A peptide by PDC cell line to Melan-A specific CTL

We generated Melan-A specific CTL by culturing $CD8^+$ T cells in the presence of Melan-A pulsed dendritic cells and cytokines. After a 21-day culture, we obtained a cell population containing Melan-A specific T cells as shown in figure 4A (left panel). Moreover, these tetramer positive cells were the only one responsible for the secretion of IFN-γ in response to Melan-A pulsed GEN2.2, as demonstrated by intracellular staining (Fig 4B, right panel). In further experiments, the specific activation of Melan-A specific T cells was monitored by measuring the release of soluble IFN-γ.

Cross-presentation of Melan-A peptide by the PDC cell line was then evaluated after activation or not by a TLR-7/8 ligand, R848. Indeed, like normal PDC, GEN2.2 express TLR-7 and TLR-9 , and mature upon activation by TLR-7 or -9 ligands (20, 21) (Fig 4B). We show that GEN2.2 PDC incubated with empty CIRIV were as inefficient in stimulating Melan-A specific T cells as the negative control ie Flu pulsed GEN2.2. Conversely, T cells efficiently produced IFN-γ when they were in the presence of GEN2.2 cells pre-incubated with Melan-A-CIRIV. Interestingly, when PDC were pre-incubated with R848, they induced a higher activation of Melan-A specific CTL, revealing the importance of DC maturation for T cell response. Moreover, as 1 μg/mL of fusion-active HA Melan-A-CIRIV correspond to 0.075 μM of Melan-A peptide, the experiment

showed a similar efficiency between 0.75 μM of encapsulated Melan-A and 10 μM of free peptide in the absence of R848.

We then confirmed that endocytosis of virosomes was necessary for the presentation of encapsulated peptides. As shown in figure 4C, the pre-treatment of GEN2.2 cells with cytochalasin D (a drug inhibiting endocytosis by disrupting actin cytoskeleton) led to a 60% inhibition of T cell activation.

We also evaluated virosome-driven antigen presentation by the TAP-deficient HLA-A*0201 T2 cell line to check the absence of free peptide in virosome preparation. As shown in figure 4D, T2 cells efficiently bind to virosome since they became positive for heamagglutinin after a 1-hour incubation with virosomes. T2 cells were then incubated with free Melan-A peptide or with Melan-A-CIRIV and co-cultured with Melan-A specific CTL. Whereas free peptide incubated with T2 cells induced a strong IFN-γ production, Melan-A-CIRIV did not (Fig 4E). Besides confirming the absence of free Melan-A peptide in Melan-A-CIRIV preparation and the integrity of the virosomes, this result also suggests the implication of TAP in the processing of Melan-A peptide delivered by CIRIV.

4. Discussion

In this paper, we evaluated the uptake of antigens encapsulated into virosomes and their further cross-presentation by plasmacytoid dendritic cells in the context of anti-tumoral immune response. Despite the fact that virosomes are already used as vaccine to generate an anti-hepatitis A or Influenza virus immunity (22, 23), little is known regarding their ability to develop T cell responses against tumor antigen. Because PDC are thought to be involved in virus detection and antiviral responses, we focused on the interactions between PDC and virosomes, and we asked whether PDC could be a target for inducing antitumoral response.

By using virosomes loaded with tumor-associated peptide, we demonstrated that PDC can bind virosomes and internalize these particules into vesicular cytoplasmic compartments with a great efficiency. By using the same machinery as influenza virus, the fusion between viral envelope and endosomal membrane is supposed to occur, and is required to have an efficient presentation of antigen encapsulated into CIRIV (9).

Here, because the antigen is delivered into the cytosol in the form of an optimized peptide, one can suggest that it will easily enter the RE through TAP. Our data showing the inability of the TAP-deficient T2 cell line to present Melan-A packed into CIRIV, while they can capture virosome, suggest the involvement of TAP in the presentation of peptides encapsulated into virosomes. Altogether, in our model, CIRIV enter into PDC by an active endocytosis pathway, and the peptide delivered into the cytosol is further translocated in the RE thanks to TAP.

Interestingly, quite the same efficiency of T cell activation was observed with CIRIV compared to free peptide while the virosome contained less peptide. This better efficiency could rely either on a protection of the peptide contained into virosomes from degradation in the medium, or on a more efficient presentation driven by virosomes. Indeed, the delivery of the antigen directly into

the cytosol could allow a long lasting presentation of the peptide, compared to the pulse with exogenous peptide. Because the T2 cell line did not present the antigen delivered by means of CIRIV, we were able to rule out the possible contamination of virosome suspension by free peptide.

In the case of infection by *influenza* virus, the response of plasmacytoid dendritic cells to the virus is driven by the recognition of viral ssRNA by TLR7 and PKR (24). Because during the synthesis of CIRIV, viral genetic material is removed, thus, these vesicles made of lipids, heamagglutinin and neuraminidase should not contain any danger signal. Indeed, we did not find any activation of the GEN2.2 cells by CIRIV, on the contrary of Bungener's work (25), who observed an increase of activation markers after incubation of mouse MDC with virosomes. This difference could come from specific interaction of mice DC with virosomes. Indeed, from an immunological point there are many differences between human and mouse (26). The mouse dendritic cells perhaps contain a particular receptor, such as lectine, that could recognize protein present in the virosome envelope (heamagglutinin, neuraminidase) and that could deliver an activation signal. In order to efficiently prime specific T cells, human DC need to express costimularory molecules and to secrete appropriate Th1 polarizing cytokines such as IFN-γ or IL-12. Regarding virosomes as vaccine to trigger T cell responses, the addition of adjuvant molecules such as TLR7 or TLR9 ligands with, on or into CIRIV could enhance their immunogenicity. Interestingly, molecules such as imiquimod (27) or CpG ODN (28) are already used in clinical trials to trigger anti-tumor or anti-viral immunity. In our study, in the absence of adjuvant, GEN2.2 cells did not trigger a very efficient activation of specific T cells, either when the antigen was provided as free peptide, or encapsulated into CIRIV. Conversely, after maturation with R848, a TLR7/8 ligand, they became highly efficient stimulators for T cell response, and they efficiently presented the Melan-A peptide delivered by CIRIV.

Virosomes represent ingenious vectors to deliver tumoral antigens into APC for the following reasons, that are of great interest for further efficient antigen presentation to trigger antitumor immunity. First, the antigen is encapsulated into the virosome, and hence is protected from extracellular proteases (12) that are in part responsible for the short half-life of free peptides (29). Secondly, the fusion of the virosome with the endosomal membranes probably occurs in late endosomes when the pH is near 5.5 (30), and thus before the fusion of endosomes with lysosomes containing proteases. Lastly, the antigen is delivered directly from endosomal vesicles to the cytosol of target cells, where it can follow the 'classical' route of endogenous antigen presentation.

Taken together, our data suggests that in the field of cancer vaccines, virosomes encapsulating tumor-associated antigens represent a promising way to deliver antigen to APC cytosol and thus to activate specific CTLs.

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Figure legends

Figure 1 : Endocytosis of empty CIRIV by PDC cell line.

(A) : Cells were incubated with increasing amounts of CIRIV (normalized by their content of HA) at the indicated concentrations. The binding of empty CIRIV was assessed using the mean fluorescence intensity of FITC labelling or the percentage of positive cells.

(B) : Inhibition of CIRIV binding was assessed by removal of sialic acid. Cells were treated or not with neuraminidase for 1h before adding CIRIV. The binding of empty CIRIV was assessed using the mean fluorescence intensity of FITC labelling.

Figure 2 : Effect of the CIRIV on the PDC cell line maturation

(A) : Optical microscopy of empty CIRIV endocytosis by PDC cell line. Cells were incubated with medium, with empty CIRIV for 1h at 4°C or at 37°C and prepared for microscopy. Cells were labelled with a rabbit anti-HA followed by a goat anti-rabbit-FITC. Nucleus were stained in blue with slowfade.

(B) : Electron microscopy of empty CIRIV endocytosis by PDC cell line. Black arrowheads show virosomes into a vesicle.

Figure 3 : Effect of the CIRIV on the PDC cell line maturation

Cells were incubated in the absence of stimuli (negative control) or in the presence of CIRIV (1µg/mL HA) or *Influenza* virus (positive control) for 24h. Cells were phenotyped for the

expression of CD80 and CD40 (A) and cytokine secretion was measured in culture supernatants (B and C).

Figure 4 : Endocytosis of Melan-A-CIRIV allows efficient cross-presentation of Melan-A peptide by PDC cell line to Melan-A specific T cells.

(A) Melan A specific T cells were cocultured for 5h30 with GEN2.2 (left) or Melan-A pulsed GEN2.2 PDC (right) and stained for intracellular IFN-γ and Melan-A tetramer.

(B) PDC cell line preincubated for 24 h or not in the presence of R848 was cultured with 10 µM of Melan-A peptide, 1 µg/mL HA or 10 µg/mL HA of empty CIRIV or Melan-A-CIRIV with 0.075 or 0.75 µM Melan-A, respectively. As negative control, cells were cultured in the absence of stimuli or Flu peptide. Then cells were co-cultured with anti-Melan-A specific T cell and IFN-γ secretion was measured in the supernatant. Standard deviations were calculated on 4 experiments. Statistical analysis were performed to compare IFN-γ secretion induced by empty/Melan A CIRIV ; (*) indicates a significant difference ($p < 0.05$). The secretions of IFN-γ in the presence or absence were compared ; (**) indicates a significant difference ($p<0.05$).

(C) PDC cell line were preincubated with Cytochalasin D prior to addition of Melan-A peptide or the Melan-A-CIRIV (and IFN-γ secretion was measured in the supernatant). Standard deviations were calculated on 4 experiments.

(D) The direct binding of free peptide was evaluated with TAP protein deficient cells (T2). These cells were incubated with peptide or virosomes. As previously, cells were co-cultured with anti-Melan-A specific T cell and IFN-γ secretion was measured in the supernatant. Standard deviations were calculated on 4 experiments.

(E) T2 cells were incubated with 1 μg/mL of HA of empty CIRIV. The binding of empty CIRIV was assessed using the mean fluorescence intensity of FITC. Open curve shows isotype control and black curve shows specific staining.