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Double-layered rotavirus-like particles are efficient carriers to elicit strong CTL responses to delivered heterologous antigens

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ABSTRACT

The first 92 amino acids of VP2 of rotavirus are dispensable for VLP assembly and can be replaced by heterologous reporter proteins without affecting either self-assembly of chimeric VP2 or the interaction with VP6 to render chimeric VP2/6 VLPs. In this study, we constructed recombinant baculoviruses expressing GFP or OVA fused to the amino terminus of a truncated VP2 sequence and produced chimeric VLPs by co-infection with a baculovirus expressing VP6. The results showed that these chimeric VLPs were efficiently uptaken by murine dendritic cells and that the heterologous sequences contained in the core of these VLPs seemed to be able to reach the MHC-I pathway as they elicited strong and specific CTL responses. Therefore, the data presented here suggest that chimeric VLPs could be used as excellent carriers to elicit CTL responses to antigens transported inside the VLPs.

1. Introduction

Virus-like particles (VLPs) are macromolecular structures resembling virus particles that are non-infectious, since they completely lack genetic material. Recombinant VLPs expressed in different systems have been assayed as vaccines against different human and veterinary diseases such as Hepatitis C virus, Human papilloma virus or Hepatitis B virus produced in yeast [1–3]. They have also been assessed for Norwalk virus, rotavirus and simian immunodeficiency virus in insect cells [4–7] as well as and Dengue virus 2 and Hepatitis delta virus produced in mammalian cells [8,9]. Recombinant vaccines based on VLPs can produce protective immune responses without the risk of infection, mostly due to the higher immunogenicity respect to those obtained with individual proteins [10,11]. This enhanced response is in part explained by the repetitive array of surface components that facilitates B cell recognition, a high density of Th epitopes in a unique particle that increases MHC-II presentation and the particulate nature that facilitates uptake by dendritic cells [12].

VLPs have also been used to display heterologous sequences. For this purpose, researches focused their studies on the insertion of foreign peptides into the VLP, expecting to not affect it formation and efficacy. In this way, several chimeric VLPs have been developed carrying heterologous sequences in rotavirus backbones [11], Hepatitis B core and e antigens [13] as well as porcine parvovirus [14,15]. However, in most of the studies the size of the heterologous insertions was limited to 15–25 amino acids, which restricts the use of VLPs as carriers [16,17]. Unfortunately, in some cases the immunodominant epitope of a protein is not certainly known, especially mainly regarding the development of cellular responses. For this reason, the design of vaccines must be done with the whole proteins or with important parts that may be responsible for eliciting an immunological response.

Short sequences of heterologous peptides have been previously displayed on the surface of double layer rotavirus VLPs (DLPs) by inserting these foreign sequences in the residue 171 of VP6 [11]. However, when sequences longer than 20 amino acids were inserted, the chimeric VP6 failed to assemble into VLPs (Peralta, unpublished results).

Charpilienne and colleagues [18] fused the entire sequences coding for the green fluorescent protein (GFP) or DsRed by replacing

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the first 92 amino acids of rotavirus VP2. In their study, the deletion of the first 92 amino acids resulted in the formation of pseudo-core particles when expressed in insect cells. Chimeric VP2 pseudo-core particles can interact with VP6 and the heterologous sequence fits into the central space of the core-like particle. With this strategy, sequences longer than 15–20 amino acids can be accommodated in chimeric VLPs, so that these VLPs can be easily produced in insect cells infected with recombinant baculoviruses at appropriate ratios. Despite the advances in this area, few researchers have assessed the potential of chimeric VLPs based on rotavirus subunits to stimulate cellular immune responses.

Given the ability of VP2 to accommodate large foreign sequences, we propose the use of chimeric rotavirus-derived VLPs as a strategy to stimulate CD8 cytotoxic T lymphocytes (CTLs) by delivering antigens to the MHC class I molecules. Here, we constructed recombinant VLPs bearing GFP or ovalbumin (OVA), and assessed their ability to be uptaken by dendritic cells and to elicit specific cellular immune responses in mice. The findings of this study show that rotavirus VLPs are promising carriers for the design of recombinant vaccines that elicit cellular immune responses.

2. Materials and methods

2.1. Cell lines and mice

Recombinant baculoviruses and VLPs were produced in *Spodoptera frugiperda* Sf9 cells (Invitrogen) grown at 27 °C with Sf-900 II serum free medium (Life Technologies, Buenos Aires, Argentina). Six to eight week-old female C57BL/6 mice (H-2b) were purchased from Fundación Facultad de Ciencias Veterinarias (UNLP, La Plata, Argentina) and maintained under specific pathogen-free conditions. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

2.2. Recombinant baculovirus construction

Plasmid pFB-ΔVP2 was constructed for the expression of fusions to VP2 by deleting the first 92 amino acids of VP2. A PCR product was obtained from cDNA of MA104 cells infected with rotavirus strain SA11 using primers ΔVP2for (A**TCTAGAGGATCC**AAAACGATACCAACATTT) and ΔVP2rev (ACCCGGGTTACAACTCGTTCAT). An XbaI site (underlined) coding for a small flexible linker SRGS in ΔVP2for (in bold). This linker has been proven to be functional and to allow flexibility between polypeptides [18]. An XmaI site was added in ΔVP2rev (underlined). The PCR product was cloned into pFastBacI (Invitrogen) XmaI/XbaI-digested.

Coding sequences for GFP and OVA were amplified with using specific primers to allow in-frame fusions to the N-terminus of ΔVP2. Forward primer TTCTAGAGT**ATG**GTGAGCAAGGGC (specific sequence for nt 1–15 of gfp, start codon in bold, XbaI site underlined) and reverse primer TTCTAGACTTGTACAGCTCGTC (specific sequence for nt 705–717 of gfp, XbaI site underlined) together with, forward primer AGGATCCAAT**ATG**CCTTTCAGAGTGACT (specific sequence for nt 653–671 of ova, start codon in bold, BamHI site underlined) and reverse primer ATCTAGAAGGGGAAACACATCTGCC (specific sequence for nt 1206–1223 of ova, XbaI site underlined) were used. The amplified fragments were cloned into the XbaI (gfp) or XbaI/BamHI (ova) sites of the pFB-ΔVP2 or XbaI/BamHI-pFB-ΔVP2, respectively. The resulting plasmids were named pFBGFPΔVP2 or pFBOVAΔVP2. The constructs pFBGFPΔVP2 or pFBOVAΔVP2 were individually transformed into *E. coli* DH10Bac cells following the protocols recommended by Bac-to-Bac manufacturers (Life Technologies, Buenos Aires, Argentina) in order to generate the corresponding recombinant bacmids.

Sf9 were transfected with the recombinant bacmid DNA with Cellfectin II (Invitrogen), and the recombinant baculoviruses were amplified by repeated passages. The resulting recombinant viruses were named AcGFPΔVP2 and AcOVAΔVP2. AcVP6 was obtained as we previously described [11]. Infectious virus titers were calculated by end point dilution assay and converted to PFU ml^{-1} as described elsewhere [19]. Virus stocks were considered free of endotoxin (<0.01 endotoxin U ml^{-1}) by the Limulus amebocyte lysate test (E-TOXATE, Sigma).

2.3. VLP purification

Four 175 cm^2 flasks of monolayers of Sf9 cells were co-infected with AcVP6 at a multiplicity of infection (moi) of 5 along with $AcOVAVP_2$ or $AcGFPVP_2$ at a moi of 8. Five days later, the supernatants of the co-infections were collected and centrifuged for 10 min at 1600 × *g* to eliminate cell debris, and the VLPs were pelleted by ultracentrifugation at 70,000 × *g* for 90 min at 10 °C through a 30% (w/w) sucrose cushion. Pellets were resuspended in TNMC buffer (10 mM Tris–HCl pH 7, 150 mM NaCl, 1 mM $MgCl_2$, 10 mM $CaCl_2$) and CsCl was added to a final density of 1.32 g ml^{-1}. After centrifugation at 134,000 × *g* for 18 h at 10 °C, the VLPs fraction was collected, dialyzed against TMNC buffer and re-centrifuged at 70,000 × *g* for 90 min at 10 °C. Finally, VLPs were resuspended in TNMC buffer and stored at 4 °C. VLPs were then analyzed by SDS-PAGE and Western blot with a rabbit polyclonal serum anti-rotavirus SA11, a rabbit polyclonal serum anti-OVA and a monoclonal antibody to VP2. VLP quantification was estimated by comparing bands density of VP2 and VP6 with a BSA curve in a gel stained with Coomassie blue.

2.4. Electron microscopy

VLPs obtained by ultracentrifugation in CsCl gradients were loaded onto cupper grids of a 200 mesh, stained with 2% uranyl acetate and analyzed by electronic microscopy (JEOL SVC-model) at 80 kV.

2.5. Antibody detection assays

Antibodies directed to OVA were detected by ELISA as previously described [20]. Briefly, 96-well flat-bottom plates were coated at 4 °C overnight with OVA (1 μg $well^{-1}$) (Sigma–Aldrich) in 0.1 M sodium carbonate–bicarbonate buffer (pH 9.6). The plates were blocked with 0.5% gelatin in PBS for 1 h at 37 °C. After washing, plasma samples diluted in PBS with 0.05% Tween containing 0.5% gelatin were added, and the plates were incubated for 1 h at 37 °C. The presence of antibodies was then revealed by incubation with an HRP conjugate goat anti-mouse IgG. After addition of H_2O_2 and *o*-phenylendiamine, the plates were read on a Bio-Rad Model 450 microplate reader at 490 nm.

Similarly, the antibodies against VP6 were measured by ELISA as previously described [11].

2.6. Generation of bone marrow-derived dendritic cells (BMDC) and uptake assay

BMDCs were prepared according to Inaba et al. [21], with few modifications as previously described by Molinari et al. [22]. Briefly, bone marrow cells were collected from the femurs and tibias, and then cultured (2.5×10^6) in 100 mm plates containing complete RPMI medium supplemented with 7.5% of GM-CSF from supernatant of the stably transfected GM-CSF-J558 cell line. Additional GM-CSF supernatant was added on days 3 and 7 of culture. Then, non-adherent and loosely adherent BMDC were used at day 8 (>90% of the harvested cells expressed CD11c).

For VLP uptake assay, BMDCs were incubated (1.7×10^5 cells well^{-1}) for 2 h in the presence of GFP-VLPs (0–250 μg) in a CO_2 atmosphere at 37 or 4 °C in culture medium. Cells were stained with anti-CD11c mAb and analyzed by flow cytometry.

For VLP uptake competition assay, BMDCs were co-incubated with a fixed amount of GFP-VLPs (4 μg ml^{-1}) and increasing amounts of wt VLPs (0–200 μg ml^{-1}) for 2 h at 37 °C.

In both cases, the geometric Mean of the Fluorescent Intensity (MFI) of the FL1 channel in total BMDCs as well as the percentage of BMDCs positive in FL1 channel were determined. A minimum of 2×10^5 events were acquired on a FACSCanto II (BD).

2.7. Mice immunization and in vivo killing assay

Groups of C57BL/6 mice ($n = 4$) were immunized by a single intravenous (i.v.) injection with OVA-VLPs (10 μg), GFP-VLPs (10 μg), OVA (1 mg) and PBS. After seven days, cytotoxic *in vivo* assay was performed as previously described [22,23]. Briefly, the mice were injected i.v. with a mix of target and control naïve syngenic splenocytes (2×10^7 cells). The target cells were charged with $OVA_{257-264}$ peptide (SIINFEKL, 10 μg ml^{-1}) and labeled with a high concentration (3 μM) of carboxyfluorescein diacetate succinimidyl ester (CFSE). A non-pulsed control population was labeled with a low concentration (0.5 μM) of CFSE. Then, $CFSE_{high}$- and $CFSE_{low}$-labeled cells were mixed at a 1:1 ratio (1×10^7 cells of each population) and injected i.v. into immunized mice. For the evaluation of the *in vivo* killing response, the number of CFSE positive cells remaining in the spleen after 20 h was determined by flow cytometry. Cytotocixity was expressed as percentage of lysis, which was calculated from $[1 - (R_{immune}/R_{control})] \times 100$, where R is given by the expression of $\%CFSE_{high}/\%CFSE_{low}$ cells for immune and non-immunized (control) mice, respectively.

In addition, 1×10^6 splenocytes from these mice were culture with OVA (1 mg ml^{-1}), $OVA_{257-264}$ peptide (1 μg ml^{-1}) and PBS as control in duplicated. Then, we assessed the production of IFNγ by ELISA, and evaluated the presence of CD8 T cell producing IFNγ by intracellular staining.

Groups of Balb/c mice ($n = 4$) were immunized by a single intraperitoneal (i.p.) injection with OVA-VLPs (10 μg), GFP-VLPs (10 μg), OVA (1 mg) and PBS. Twenty days post-injection, serum samples were taken for antibody detection.

2.8. Cytokine detection assays

Levels of IFNγ were measured in culture supernatants by standard double-Ab sandwich ELISA (Becton Dickinson Argentina) following the instructions from the manufacturer. All assays were standardized with recombinant murine cytokines (Becton Dickinson Argentina). The Antibody pairs used were capture, R4-6A2 and biotynilated detection, XMG1.2/(all from Becton Dickinson Argentina). For the intracytoplasmatic staining of IFNγ, stimulated splenocytes were cultured for 6 h with Monensin A (Sigma–Aldrich), to inhibit the transport of protein Golgi Apparatus allowing cytokine accumulation inside the producer cells. Spenocytes were harvested and immunostained with CD4 and CD8 antibodies. Then, these cells were fixed with paraphormaldehid (4%) and permebilized with saponin (0.5%) for the intracitoplasmatic staining of IFNγ.

2.9. Statistics

Statistical analysis was performed with GraphPad Prism (La Jolla, CA) and one way ANOVA analysis with the Bonferroni post-test. Values of $p = 0.05$ were considered significant.

3. Results

3.1. Design of the chimeric DLPs and expression of recombinant proteins in the baculovirus-insect cell system

The first 92 amino acids of VP2 are dispensable for DLP assembly and can be replaced by heterologous sequences [18]. With this in mind, we constructed the recombinant baculoviruses AcGFPΔVP2 and AcOVAΔVP2, which code for GFP or for an OVA fragment, respectively, spanning amino acids 197–386 fused to the amino terminus of ΔVP2 (Fig. 1A). A flexible peptide (SRGS) between the heterologous sequences and ΔVP2 was introduced to avoid steric constrains and to allow flexibility between fragments. The cell extracts from co-infections with AcGFPΔVP2 and AcVP6 or AcOVAΔVP2 and AcVP6 were assessed for recombinant expression of the chimeric proteins by Western blot revealed with an anti-rotavirus hyperimmune rabbit serum. Bands of electrophoretic mobilities of 110 kDa, 101 kDa and 45 kDa were observed. These bands, correspond to the expected molecular weight of GFPΔVP2, OVAΔVP2 and VP6, respectively (Fig. 1B). This result indicates proper expression of the fusion proteins.

3.2. Assembly of the chimeric VLPs

For the assessment of the assembly of the chimeric DLPs, insect cells were co-infected with AcGFPΔVP2 and AcVP6 or AcOVAΔVP2 and AcVP6 as described in the Material and Methods section. The optimal quantities and qualities of VLPs were obtained when Sf9 cells were infected with MOI 5 for AcVP6 and MOI 8 for AcOVAVP2 or AcGFPVP2 (data not shown). Five dpi, the supernatants were collected and the clarified and putative VLPs were pelleted by ultracentrifugation on sucrose cushions. Pellets were resuspended and purified by density gradient centrifugation in CsCl. Two bands were observed in the tubes illuminated with white light, while only the lower band was visible in the tube containing GFP-VLPs when illuminated with UV light (Fig. 2A). The upper band probably corresponded to budded baculovirus virions and the lower coincided with the VLPs. The lower bands were collected, pelleted and subjected to electrophoresis followed by silver staining, Western blot and electron microscopy. Western blot experiments revealed by an anti-rabbit hyperimmune serum against denatured rotavirus (Fig. 2B) showed that the lower band in density gradients comprised both VP6 and chimeric VP2. Silver staining (Fig. 2C) revealed two major bands. The lower band corresponded to VP6 and the upper bands corresponded to VP2 fusions. The proteins forming the chimeric VLPs showed stoichiometries of about 2.7 in densitometry assays (data not shown). These values are similar to those previously reported for VP2/VP6 VLPs [24]. Finally, electron microscopy revealed DLPs morphologically indistinguishable from wild type VLPs (Fig. 2D). This finding indicates that the heterologous sequences did not interfere with the normal assembly of DLPs.

3.3. In vitro uptake of the chimeric VLPs by dendritic cells

In order to evaluate of VLPs as carriers for heterologous antigens, we analyzed the ability of BMDCs to uptake the constructed VLPs. BMDCs were incubated with GFP-VLPs at 37 °C for 2 h, washed and analyzed by flow cytometry. We observed a strong uptake of GFP-VLPs by BMDCs which increased with VLP concentration. This increment was revealed by the increase of both the mean fluorescence intensity and the proportion of BMDCs positive for green fluorescence in a VLP concentration-dependent fashion (Fig. 3A and 3B). At a VLP concentration of 10 μg, at least 60% of BMDCs incorporated GFP-VLPs. The same experiment conducted at 4 °C showed a notably lower GFP signal (about five times less MFI). These findings suggest chimeric VLPs are readily internalized by BMDCs.

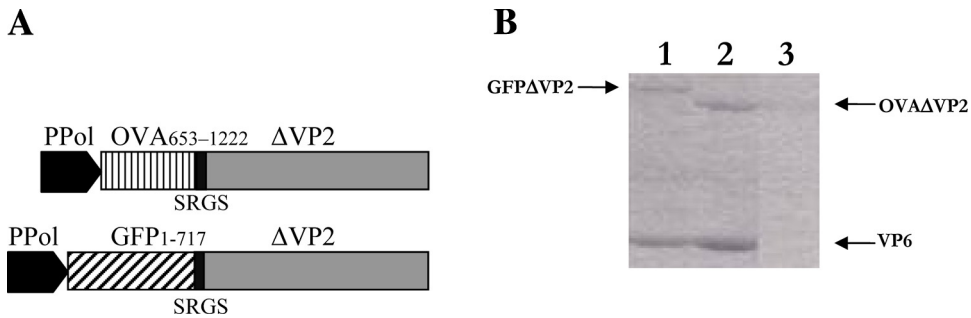

Fig. 1. Expression of the fusion proteins GFPΔVP2 and OVAΔVP2. (A) Scheme of the baculovirus vectors. The ovalbumin (OVA) (653–1222) and enhanced green fluorescent protein (GFP) (1–717) sequences were cloned 5′ of the ΔVP2 sequence. A linker sequence (SRGS) was added in the N-terminus of ΔVP2 to provide distance and flexibility for the N-terminal fusion proteins, so that it could fold correctly. The production of the fusion protein is driven by the strong polyhedrin promoter (PPOL). (B) Western blot of total cell extracts infected with AcGFPΔVP2 (lane 1), AcOVAΔVP2 (lane 2) and AcWT (lane 3) revealed with a rabbit hyperimmune serum to denatured rotavirus.

Next, a competition assay was performed to assess if VLPs uptake by BMDCs is mediated by receptors. For this purpose, BMDCs were incubated with fixed quantities of GFP-VLPs (and increasing amounts of wt VLPs for 2 h). A decrease in the percentage of BMDCs positive for GFP was observed with the increase of wt VLPs concentrations, which suggest that VLPs uptake occurs in part by a receptor-mediated mechanism (Fig. 3C).

3.4. Humoral and cellular immune responses to OVA elicited by OVA-VLPs

It has been previously observed that the injection of GFP-VLPs in mice elicits antibody responses to GFP, even though the heterologous sequence is inside the VP2 core and covered by a VP6 shell [18]. We next evaluated the humoral immunogenic properties of

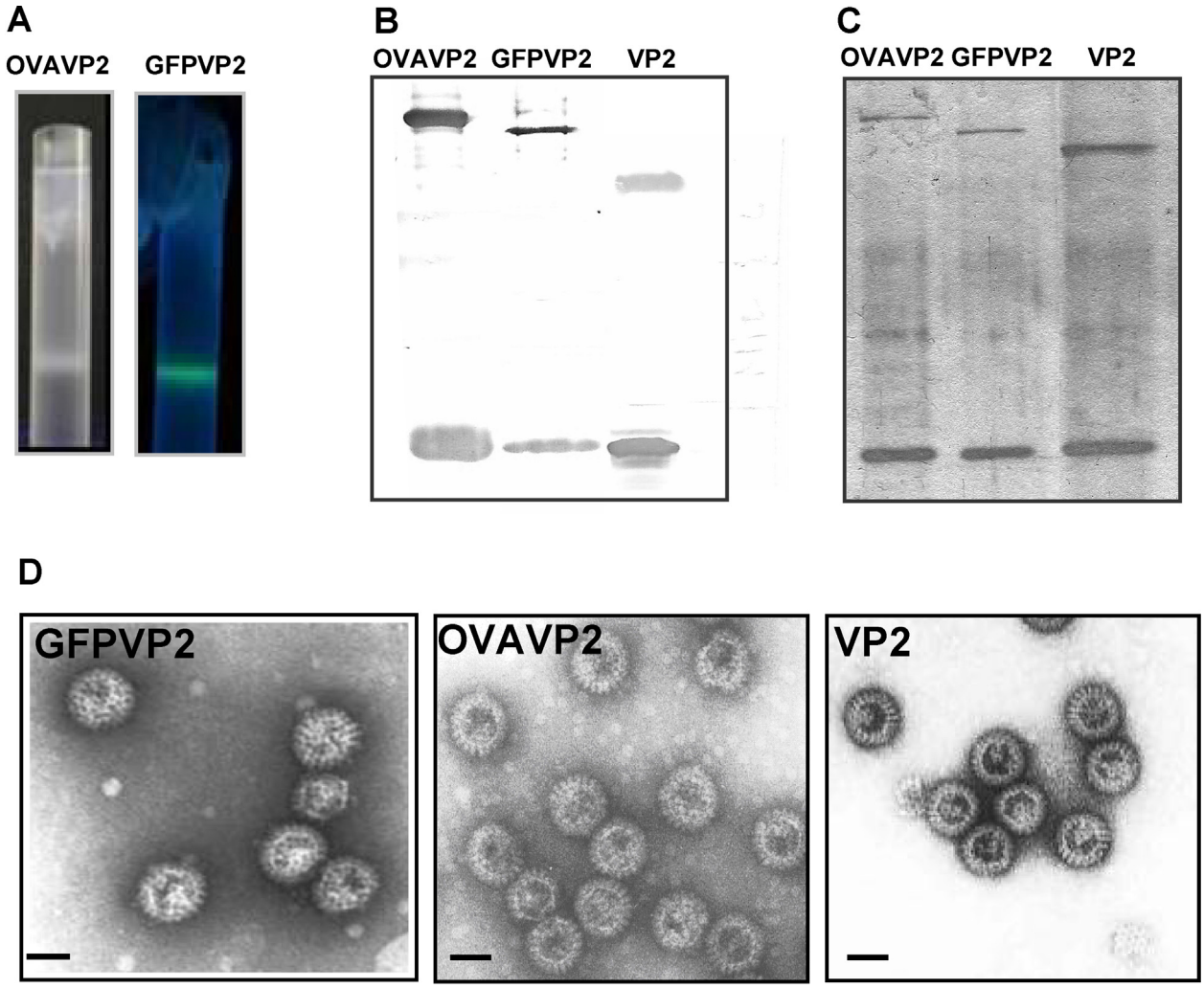

Fig. 2. Assembly of chimeric VLPs. In order to characterize the VLP formation, supernatants of Sf9 cells co-infected with recombinant baculoviruses expressing different chimeric VP2 and wtVP6 were processed as described in Section 2. (A) After ultracentrifugation of CsCl gradients, tubes were illuminated with white light (left) or with a hand-held UV lamp (right) and photographed. (B) Western blot revealed with a rabbit hyperimmune serum to denatured rotavirus and (C) silver staining of purified GFPΔVP2 (lane 1), OVAΔVP2 (lane 2) and ΔVP2 (lane 3). The upper bands correspond to VP2 forms and the lower bands correspond to VP6. (D) Negative staining of particles purified from supernatants of co-infected cells, adsorbed onto carbon-coated grids, stained with 2% uranyl acetate and examined under TEM. The bar indicates 50 nm.

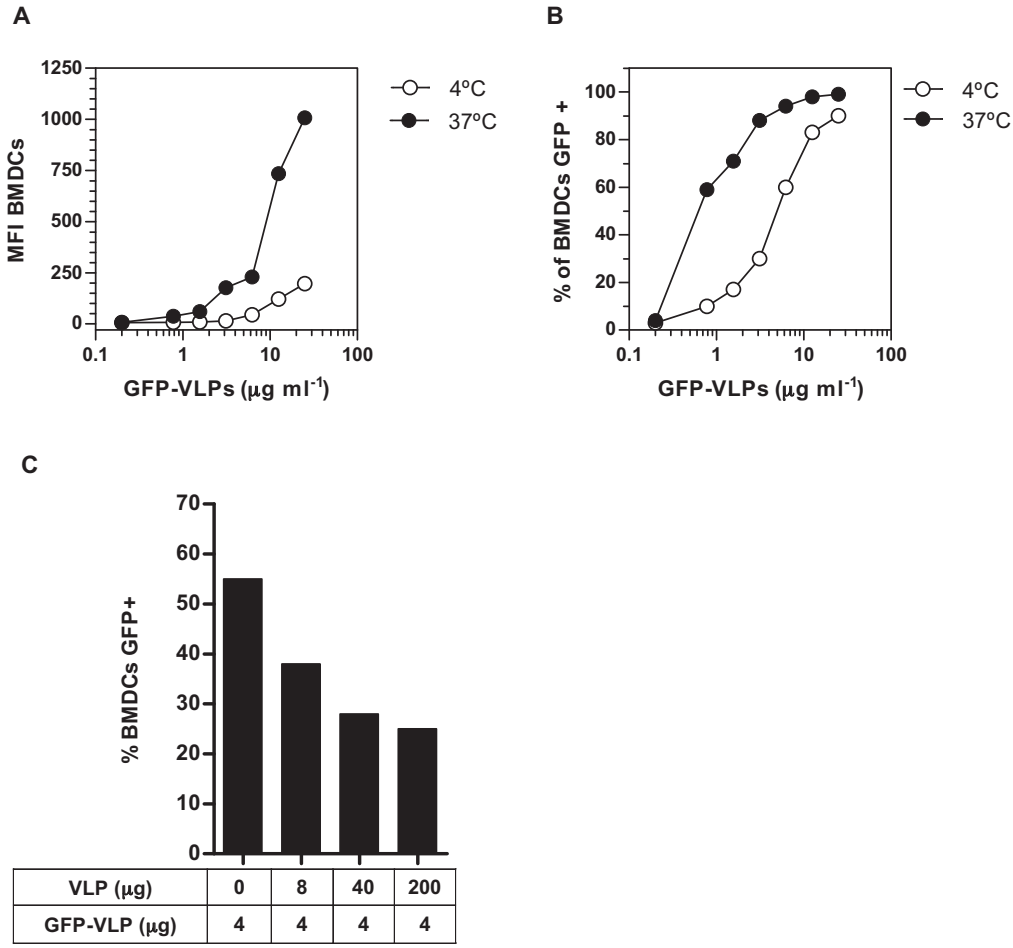

Fig. 3. *In vitro* uptake of chimeric VLPs by dendritic cells. (A and B) BMDCs were incubated with GFP-VLPs at 37 °C and 4 °C and analyzed by flow cytometry. The geometric Mean fluorescence Intensity (MFI) of total BMDCs (A) and the percentage of BMDCs positive in FL1 channel (B) were determined. (C) Competition assay between GFP-VLPs and wt VLPs: BMDCs were co-incubated with a fixed amount of GFP-VLPs (4 μg ml^{-1}) and increasing amounts of wt VLPs (0–200 μg ml^{-1}) for 2 h at 37 °C. Then, the percentage of BMDCs positive in FL1 channel was determined.
The results are representative of two independent experiments.

OVA-VLPs. For this purpose, mice were vaccinated i.v. with 10 μg of chimeric VLPs and antibody titers to VP6, and OVA were subsequently measured by direct ELISA. Titers as high as 1:10,000 of anti-VP6 antibodies were observed in mice inoculated with OVA-VLPs, but we failed to detect anti-OVA antibodies (data not shown), the OVA sequence was not accessible to B lymphocytes. This suggests that a high proportion of chimeric VLPs are circulating as entire particles in the blood of the injected mice.

Finally, we evaluated the ability of OVA-VLP to elicit OVA-specific cellular immune responses in mice. Seven days post i.v. inoculation of 10 μg of chimeric VLPs, a vigorous CTL response to the $OVA_{257–264}$ CD8 epitope was evidenced, as determined by an *in vivo* killing assay with CFSE-labeled target cells (Fig. 4A–C). This response was accompanied by a high production of IFNγ *in vitro* upon re-stimulation of splenocytes with OVA protein and peptide (Fig. 4C), in particular by CD8 cells (Fig. 4B and D). In fact, 10 μg of OVA-VLPs contain about 4 ng of OVA protein. When we inoculated the same quantity of OVA protein alone, no CTL responses were be detected (data not shown); which indicates that VLPs are excellent carriers to deliver the transported antigen to MHC-I presentation pathway.

4. Discussion

In this study, we demonstrated that the addition of OVA protein replacing the first 92 amino acids of VP2 did not interfere with normal VLP morphogenesis. Indeed, high quantities of VLPs in supernatants of infected insect cells were rendered, and these VLPs were indistinguishable of VLPs made of ΔVP2 and wtVP6. Furthermore, these chimeric VLPs were able to elicit strong cellular immune responses against the heterologous sequence when i.v. injected in mice. This finding demonstrates the ability of chimeric VLPs to reach the MHC-I route of presentation. The fact that a chimeric VLP can access the major histocompatibility complex class I was also recently demonstrated by Moffat et al., 2013 by using a HbsAgS HBV-OVA chimeric VLP [25].

The infection of insect cells with individual recombinant baculovirus coding for VP2 or ΔVP2 renders core-like VP2 particles, which aggregate inside cells. When both VP2 and VP6 recombinant proteins are simultaneously expressed, they render DLPs that are assembled inside the insect cell and can be easily recovered from the supernatants of co-infected cultures. Only the co-expression of VP2 and VP6 rendered high levels of chimeric VLPs in the supernatants of infected cells. It is interesting to point out that we were able to obtain chimeric core-like particles by expressing only chimeric VP2 proteins and that these particles were indistinguishable of ΔVP2 (data not shown). However, VP6 seems to be essential for a proper localization and self-assemble.

Usually, the immunogenicity of soluble, purified proteins is very low and the use of strong adjuvants is needed to stimulate immune responses. In particular, it is extremely difficult for soluble exogenous proteins to reach the cytosol of dendritic cells,

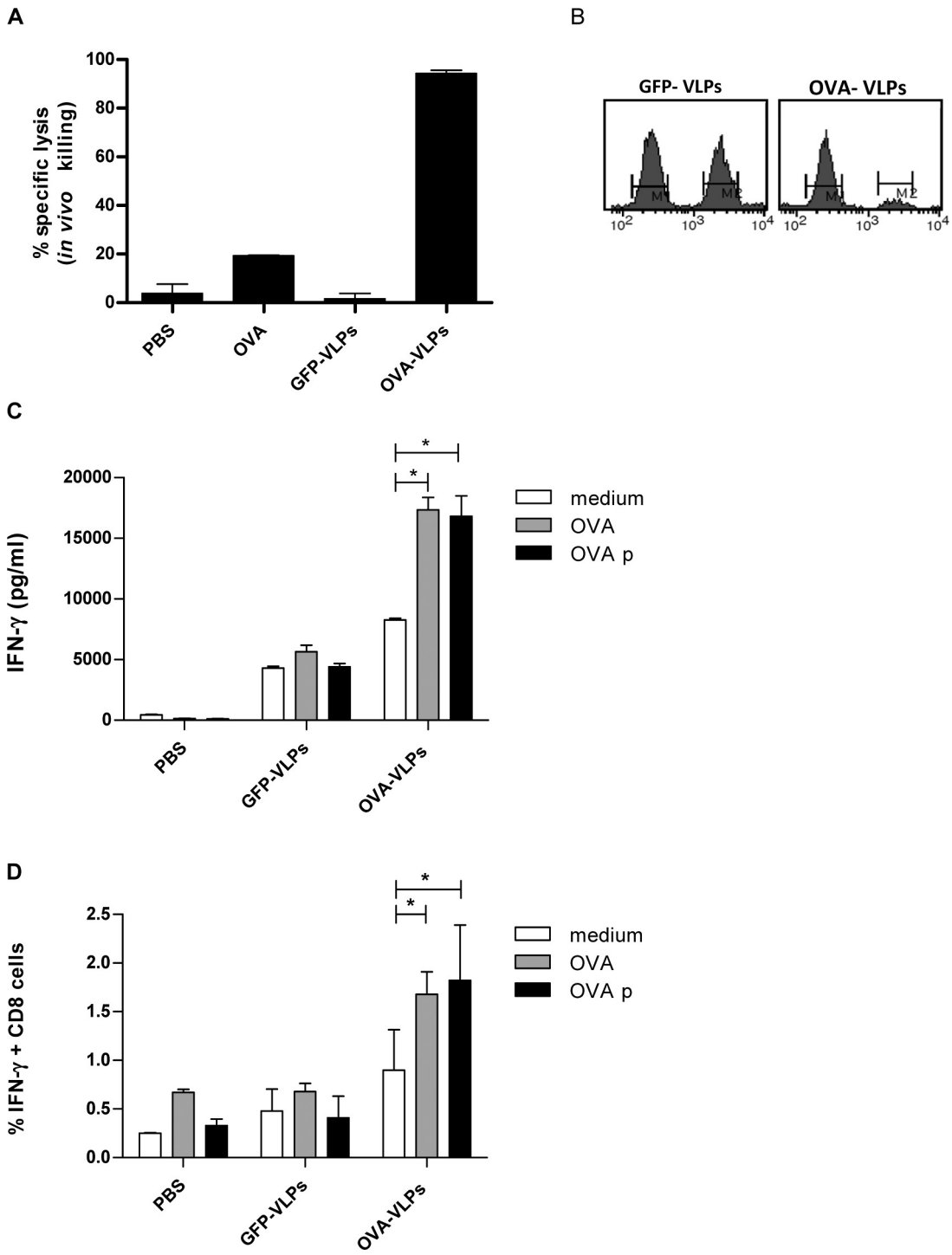

Fig. 4. Anti-OVA CTL response in mice injected with OVA-VLPs. C57BL/6 mice were immunized by a single i.v. injection of GFP-VLPs, OVA-VLP, OVA alone (1 mg) or PBS. Seven days later, immunized mice received an injection i.v. of a mixture (1:1) of $OVA_{256-264}$ peptide-loaded $CFSE_{high}$ and unloaded $CFSE_{low}$ splenocytes as target cells. (A) The percentage of specific *in vivo* killing of one representative experiment. (B) A representative histogram of target cells from vaccinated mice. (C) IFNγ content in supernatants of spleen cells from immunized mice determined by ELISA. Spleen cells were recovered and cultured for 48 h in the presence of OVA protein (1 mg ml^{-1}) or $OVA_{256-264}$ peptide (0.1 μg). As control, spleen cells without stimulus were also cultured. $^{*}p = 0.05$; $^{**}p = 0.01$. (D) The percentage of IFNγ+ CD8 T cells. Spleen cells were recovered and cultured for 12 h in the presence of OVA protein or $OVA_{256-264}$, and incubated in the presence of Brefeldin A for 6 additional hours. Then, the cells were labeled for CD8 markers and intracellular IFNγ. The results are representative of at least two independent experiments and are expressed as mean ± 2 SEM ($n = 4$).

unless unphysiologically large amounts of proteins are provided or an adjuvant is added. Although the safety of subunit vaccines is one of the most valuable advantages, the failure to elicit cytotoxic responses restricts their use and limits their efficacy with respect to life vaccines. Significantly, this study demonstrates that the chimeric rotavirus VLPs herein constructed displayed high immunogenicity eliciting strong CTL responses to internal sequences without adjuvants. This agrees with results recently obtained with other chimeric VLPs [10,26].

VLPs have unique properties that make them promising tools for rationally designed vaccines. Firstly, the uptake of a single chimeric VLP incorporates hundreds of transported epitopes into

the processing and presentation machinery of antigen presenting cells (APCs). Besides, probably due to their size and structure, their uptake by APCs is highly efficient and could lead to the delivery to cell compartments normally reluctant to monomeric proteins. In particular, rotavirus VLPs bearing OVA or GFP proteins transport 120 copies of OVA or GFP per particle. These chimeric VLPs were efficiently uptaken by DCs, as revealed by flow cytometry experiments. In addition, the uptake of GFP-VLPs could be displaced by wt VLPs, suggesting that DCs interact with VLPs by binding of VP6 to a DC surface molecule. This assumption remains to be still determined.

The design of recombinant structures bearing heterologous sequences to elicit CTL responses should consider to include immunostimulatory motifs that allow the induction of an adequate effector adaptive immune response. Rotavirus VLPs of different compositions were able to elicit protective immune responses even without any adjuvants, suggesting that VLPs have intrinsic immunostimulatory properties. However, it has been recently demonstrated that live activated rotavirus particles but not complete VLPs are able to activate DCs despite they can be internalized [27].

Here, we used OVA-VLPs purified by ultracentrifugation in CsCl gradients as immunogens without the use of any external adjuvants. Although we did not detect baculovirus or double stranded RNA by Western blot or agarose gel electrophoresis followed by ethidium bromide staining in the VLP preparations, we cannot discard the presence of contaminants in the VLP bands. In fact, traces of budded baculovirus could be responsible for the DC activation as they are able to provide the signal necessary to mature BMDCs and to modulate humoral and CTL responses in mice against co-administered antigens [22,28]. However, the high CTL responses specific to OVA demonstrate that DLPs are excellent carriers to deliver heterologous epitopes to the MHC-I pathway and that they elicit OVA-specific cross priming through a replication-independent strategy. Although Charpilienne et al. detected antibodies against heterologous sequences transported inside chimeric rotavirus VLPs by intraperitoneal injection [18], we did not detect anti-OVA humoral responses (data not shown). This discrepancy could be explained by the different doses and routes used in each particular immunization protocol. Apart from this discordance, our findings revealed that in the i.v. route and at the assessed doses, these chimeric VLPs maintained its integrity.

Together, our results demonstrate that the localization of the heterologous sequence in rotavirus VLP influences the type of immune response. For instance, the internal localization of the transported heterologous sequences would make the antibody responses unlikely due to the difficult access to these sequences. This localization simultaneously could restrict the access of pre-existing antibodies. On the other hand, this localization would elicit cellular responses. Therefore, rotavirus VLPs could be excellent candidates for rational design of non-replicating vaccines.

References

[1] Acosta-Rivero N, Aguilar JC, Musacchio A, Falcon V, Vina A, de la Rosa MC, et al. Characterization of the HCV core virus-like particles produced in the methylotrophic yeast *Pichia pastoris*. Biochem Biophys Res Commun 2001;287:122–5.

[2] Emeny RT, Wheeler CM, Jansen KU, Hunt WC, Fu TM, Smith JF, et al. Priming of human papillomavirus type 11-specific humoral and cellular immune responses in college-aged women with a virus-like particle vaccine. J Virol 2002;76:7832–42.

[3] Valenzuela P, Medina A, Rutter WJ, Ammerer G, Hall BD. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. Nature 1982;298:347–50.

[4] Molinari P, Peralta A, Taboga O. Production of rotavirus-like particles in *Spodoptera frugiperda* larvae. J Virol Methods 2008;147:364–7.

[5] Palomares LA, Mena JA, Ramirez OT. Simultaneous expression of recombinant proteins in the insect cell-baculovirus system: production of virus-like particles. Methods 2012;56:389–95.

[6] Vieira HL, Estevao C, Roldao A, Peixoto CC, Sousa MF, Cruz PE, et al. Triple layered rotavirus VLP production: kinetics of vector replication, mRNA stability and recombinant protein production. J Biotechnol 2005;120:72–82.

[7] Yao L, Wang S, Su S, Yao N, He J, Peng L, et al. Construction of a baculovirus-silkworm multigene expression system and its application on producing virus-like particles. PLoS ONE 2012;7:e32510.

[8] Wang KC, Wu JC, Chung YC, Ho YC, Chang MD, Hu YC. Baculovirus as a highly efficient gene delivery vector for the expression of hepatitis delta virus antigens in mammalian cells. Biotechnol Bioeng 2005;89:464–73.

[9] Zhang S, Liang M, Gu W, Li C, Miao F, Wang X, et al. Vaccination with dengue virus-like particles induces humoral and cellular immune responses in mice. Virol J 2011;8:333.

[10] Kawano M, Morikawa K, Suda T, Ohno N, Matsushita S, Akatsuka T, et al. Chimeric SV40 virus-like particles induce specific cytotoxicity and protective immunity against influenza A virus without the need of adjuvants. Virology 2014;448:159–67.

[11] Peralta A, Molinari P, Taboga O. Chimeric recombinant rotavirus-like particles as a vehicle for the display of heterologous epitopes. Virol J 2009;6:192.

[12] Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, et al. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J Immunol 2004;173:3148–54.

[13] Schodel F, Peterson D, Milich D. Hepatitis B virus core and e antigen: immune recognition and use as a vaccine carrier moiety. Intervirology 1996;39:104–10.

[14] Sedlik C, Saron M, Sarraseca J, Casal I, Leclerc C. Recombinant parvovirus-like particles as an antigen carrier: a novel nonreplicative exogenous antigen to elicit protective antiviral cytotoxic T cells. Proc Natl Acad Sci U S A 1997;94:7503–8.

[15] Sedlik C, Deriaud E, Leclerc C. Lack of Th1 or Th2 polarization of CD4+ T cell response induced by particulate antigen targeted to phagocytic cells. Int Immunol 1997;9:91–103.

[16] Natilla A, Nemchinov LG. Improvement of PVX/CMV CP expression tool for display of short foreign antigens. Protein Expr Purif 2008;59:117–21.

[17] Remond M, Da Costa B, Riffault S, Parida S, Breard E, Lebreton F, et al. Infectious bursal disease subviral particles displaying the foot-and-mouth disease virus major antigenic site. Vaccine 2009;27:93–8.

[18] Charpilienne A, Nejmeddine M, Berois M, Parez N, Neumann E, Hewat E, et al. Individual rotavirus-like particles containing 120 molecules of fluorescent protein are visible in living cells. J Biol Chem 2001;276:29361–7.

[19] O'Reilly DR, Miller LK, Luckow VA. Baculovirus expression vectors. A laboratory manual; 1994.

[20] Maletto BA, Ropolo AS, Liscovsky MV, Alignani DO, Glocker M, Pistoresi-Palencia MC. CpG oligodeoxinucleotides functions as an effective adjuvant in aged BALB/c mice. Clin Immunol 2005;117:251–61.

[21] Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 1992;176:1693–702.

[22] Molinari P, Crespo MI, Gravisaco MJ, Taboga O, Moron G. Baculovirus capsid display potentiates OVA cytotoxic and innate immune responses. PLoS ONE 2011;6:e24108.

[23] Durand V, Wong SY, Tough DF, Le Bon A. IFN-alpha/beta-dependent cross-priming induced by specific Toll-like receptor agonists. Vaccine 2006;24(Suppl. 2). S2-22-23.

[24] Roldao A, Mellado MC, Lima JC, Carrondo MJ, Alves PM, Oliveira R. On the effect of thermodynamic equilibrium on the assembly efficiency of complex multi-layered virus-like particles (VLP): the case of rotavirus VLP. PLoS Comput Biol 2012;8:e1002367.

[25] Moffat JM, Cheong WS, Villadangos JA, Mintern JD, Netter HJ. Hepatitis B virus-like particles access major histocompatibility class I and II antigen presentation pathways in primary dendritic cells. Vaccine 2013;31:2310–6.

[26] Mazeike E, Gedvilaite A, Blohm U. Induction of insert-specific immune response in mice by hamster polyomavirus VP1 derived virus-like particles carrying LCMV GP33 CTL epitope. Virus Res 2012;163:2–10.

[27] Istrate C, Douagi I, Charpilienne A, McInerney GM, Hidmark A, Johansen K, et al. Bone marrow dendritic cells internalize live RF-81 bovine rotavirus and rotavirus-like particles (RF 2/6-GFP-VLP and RF 8*2/6/7-VLP) but are only activated by live bovine rotavirus. Scand J Immunol 2007;65:494–502.

[28] Hervas-Stubbs S, Rueda P, Lopez L, Leclerc C. Insect baculoviruses strongly potentiate adaptive immune responses by inducing type I IFN. J Immunol 2007;178:2361–9.