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Chimeric calicivirus-like particles elicit protective anti-viral cytotoxic responses without adjuvant

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ABSTRACT

We have analyzed the potential of virus-like particles (VLPs) from rabbit hemorrhagic disease virus (RHDV) as a delivery system for foreign T cell epitopes. To accomplish this goal, we generated chimeric RHDV-VLPs incorporating a $CD8^+$ T cell epitope (SIINFEKL) derived from chicken ovalbumin (OVA). The OVA epitope was inserted in the capsid protein (VP60) of RHDV at two different locations: 1) the N-terminus, predicted to be facing to the inner core of the VLPs, and 2) a novel insertion site predicted to be located within an exposed loop. Both constructions correctly assembled into VLPs. *In vitro*, the chimeric VLPs activated dendritic cells for TNF-α secretion and they were processed and presented to specific T cells. *In vivo*, mice immunized with the chimeric VLPs without adjuvant were able to induce specific cellular responses mediated by cytotoxic and memory T cells. More importantly, immunization with chimeric VLPs was able to resolve an infection by a recombinant vaccinia virus expressing OVA protein.

Introduction

Caliciviruses cause a variety of diseases in humans and animals (Green, 2006). The family *Caliciviridae* has been divided into four genera: *Norovirus*, *Sapovirus*, *Vesivirus* and *Lagovirus*. Rabbit hemorrhagic disease virus (RHDV), the prototype strain of the genus *Lagovirus*, is the causative agent of a highly infectious disease of rabbits (Angulo and Barcena, 2007; Cooke, 2002; Fenner and Fantini, 1999). Infected rabbits usually die within 48 to 72 h of necrotizing hepatitis. The virions (~40 nm in diameter) are non-enveloped and icosahedral and have a 7.4-kb single-stranded positive-sense RNA genome. The genomic RNA is organized into two open reading frames (ORFs). The first ORF encodes a polyprotein that is processed giving rise to several mature nonstructural proteins and the capsid protein subunit of 60 kDa (VP60) (Meyers et al., 2000; Thumfart and Meyers, 2002). The second ORF encodes a small minor structural protein, VP2. The RHDV-VP60 protein, expressed in several heterologous systems, has been shown to induce full protection of rabbits against a lethal challenge with RHDV (Barcena et al., 2000; Boga et al., 1994; Boga et al., 1997; Castanon et al., 1999; Fernandez-Fernandez et al., 2001; Laurent et al., 1994; Perez-Filgueira et al., 2007).

A major breakthrough in calicivirus research was the finding that the capsid protein of Norwalk virus (NV, the prototypic strain of the genus *Norovirus*), expressed in insect cells self-assembled into virus-like particles (VLPs) that are morphologically and antigenically identical to the infectious particles (Green et al., 1993; Jiang et al., 1992). Subsequently, recombinant calicivirus VLPs from the four genera have been reported (Chen et al., 2004; Di Martino, Marsilio, and Roy, 2007; Jiang et al., 1999; Laurent et al., 1994). The three-dimensional structure of several calicivirus recombinant VLPs as well as authentic virions has been determined to low resolution by cryo-electron microscopy and three-dimensional reconstruction techniques (Barcena et al., 2004; Chen et al., 2004; Prasad et al., 1994a, 1994b). These studies showed that caliciviruses are 35–40 nm in diameter with a $T=3$ icosahedral capsid formed by 90 dimers of the capsid protein, which surround 32 large hollows or cup-shaped depressions. X-ray crystallographic structures are available for NV VLPs and San Miguel sea lion virus (SMSV, the prototypic strain of the genus *Vesivirus*) (Chen et al., 2006; Prasad et al., 1999). Each capsid monomer has two major domains, the S (shell) and P (protruding) domains, linked by a hinge region. The N-terminal S domain is responsible for the formation of the continuous shell of the capsid, while the C-terminal P domain forms the arch-like structures extending from the shell. The P domain can be further divided into P1 and P2 subdomains, with P2 subdomain located on the surface of the capsid.

Generally speaking, VLPs are appealing as vaccine candidates because their inherent properties (ie, multimeric antigens and

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particulate structure) are optimal for the induction of an efficient immune response. The most important advantages of VLP-based vaccines as a specific type of subunit vaccines are: 1) the absence of infectivity/reversion to virulent phenotype since they contain no genetic material, 2) the robust immunogenicity, due to their repetitive, high density display of epitopes, and 3) their ability to elicit both protective neutralizing antibodies and cellular-mediated immunity. Some examples of VLPs that have been successfully used for vaccine development and have been licensed commercially include VLPs derived from hepatitis B virus (HBV) and human papilloma virus (HPV) (recently reviewed in Chackerian, 2007; Jennings and Bachmann, 2007; Ramqvist, Andreasson, and Dalianis, 2007; Spohn and Bachmann, 2008). In addition to being used to induce immune responses against the particle itself, VLPs have been successfully used as platforms for inducing immune responses against inserted foreign immunogenic epitopes (chimeric VLPs) (Dalsgaard et al., 1997; Langeveld et al., 2001; Neirynck et al., 1999; Netter et al., 2001; Sedlik et al., 1997; Varsani et al., 2003; Woo et al., 2006).

We have previously performed an exhaustive structural analysis of the RHDV capsid protein and obtained a pseudo-atomic model of VP60 protein (Barcena et al., 2004). Our group and others have shown that VP60 protein can accommodate insertions of foreign amino acid sequences at both, the N- and C-terminal regions, without disrupting VLP formation (Barcena et al., 2004; El Mehdaoui et al., 2000; Nagesha, Wang, and Hyatt, 1999), raising the possibility of using RHDV-VLPs as foreign epitope carriers for vaccine development. In addition, this structural focus allowed us to infer specific sites at the outermost region of VP60 as potential insertion sites for foreign epitopes.

Here we report the generation of recombinant chimeric RHDV-VLPs incorporating a well defined $CD8^+$ T cell epitope corresponding to aa 257–264 (SIINFEKL) from chicken ovalbumin (OVA). This epitope is restricted for MHC class I H-2Kb presentation (Rotzschke et al., 1991). The foreign epitope was inserted at two different locations: 1) at the N-terminus of VP60 protein, which is predicted to be buried in the internal face of the VLPs, and 2) at a novel insertion site between amino acid positions 306 and 307 of VP60 protein, which is predicted to be located within an exposed loop at the P2 subdomain of VP60 protein. We analyzed the immunogenic potential of both chimeric VLPs (RHDV-VLPs-OVA) *in vitro* and *in vivo*. Results of *in vitro* assays showed that RHDV-VLPs activated dendritic cells (DCs), as determined by analysis of TNF-α secretion. Furthermore, DCs were able to process and present SIINFEKL peptide from RHDV-VLPs-OVA for $CD8^+$ specific recognition. Both chimeric RHDV-VLPs were also analyzed *in vivo* as vaccine vectors in the total absence of adjuvant. Interestingly, RHDV-VLPs-OVA were able to stimulate specific IFNγ-producing cell priming and to generate a powerful and specific cytotoxic response *in vivo*. Moreover, mice inoculated with RHDV-VLPs-OVA were able to control an infection by a recombinant vaccinia virus expressing OVA (VV-OVA) in target organs.

Results

Generation of RHDV recombinant particles

In order to analyze the potential of RHDV-VLPs as a delivery system for foreign T cell epitopes we produced recombinant baculoviruses expressing different VP60 constructs (Fig. 1). The foreign amino acid sequence inserted: GSQLESIINFEKLTEGS (17 aa) contained the T cell epitope SIINFEKL, flanked by its natural sequences in the OVA protein (three and two amino acids flanking the N and C terminus of the OVA T cell epitope, respectively), to promote the correct processing of the immunogenic epitope by antigen-presenting cells (Rueda et al., 2004). In addition, the OVA derived sequence was flanked by amino acids glycine and serine (GS). This two-residue sequence, encoded by the DNA sequence of *BamHI* restriction site, might constitute a flexible linker that facilitates capsid assembly. The foreign sequence was generated by annealing two complementary oligonucleotides, which were inserted at unique *BamHI* restriction sites engineered by site-directed mutagenesis at defined locations in VP60 gene, as described in Materials and methods.

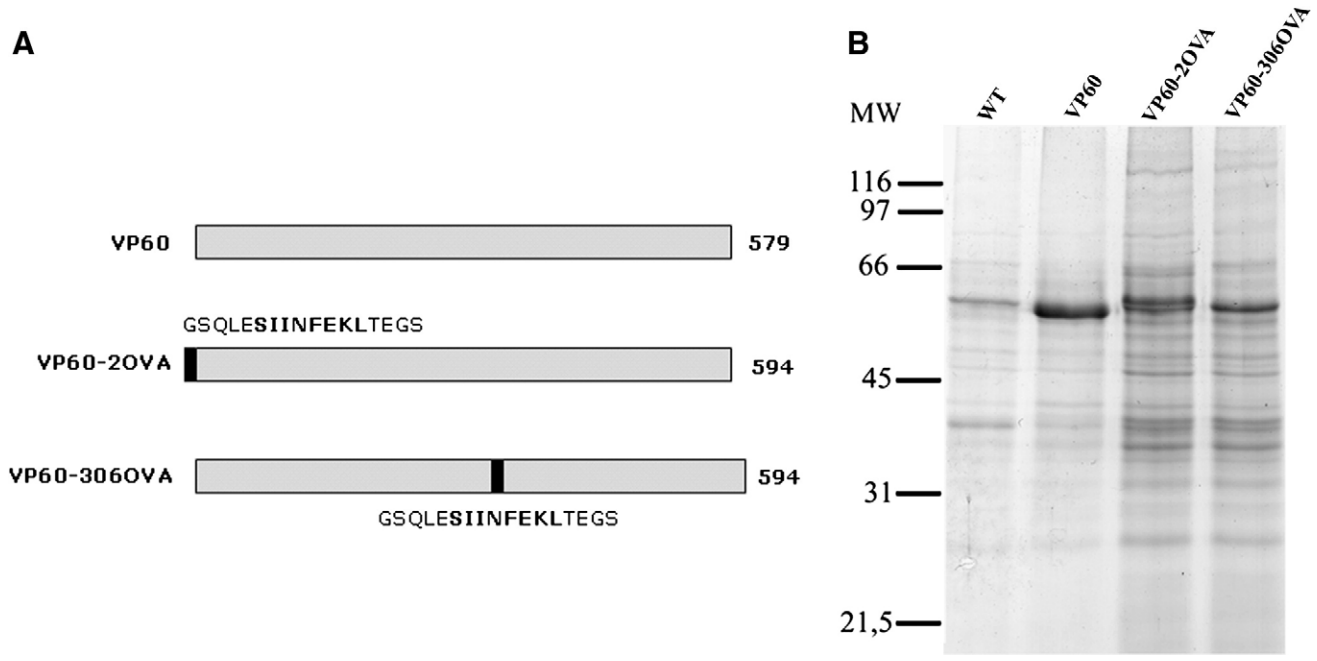

Fig. 1. Schematic representation and expression of the recombinant VP60 constructs used in this study. (A) Scheme of each construct showing names and numbers of amino acid residues. The chimeric proteins VP60-2OVA and VP60-306OVA harbour the depicted foreign peptide sequence containing de OVA derived T cell epitope at the indicated positions. (B) H5 cells were infected by wild-type baculovirus (WT) or the indicated recombinant baculoviruses. The infected-cell lysates were analyzed by SDS-10% PAGE and Coomassie brilliant blue staining. Molecular weight markers (MW; $\times 10^3$ Da) are given on the left.

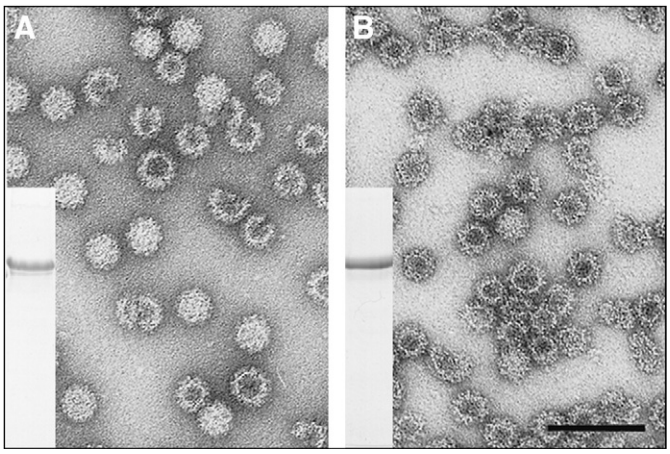

Fig. 2. Analysis of chimeric VP60 particles by negative staining and SDS-PAGE. Electron microscopy of negatively stained purified chimeric VP60 particles (A) VP60-2OVA and (B) VP60-306OVA. Purified particles were analyzed for protein content by SDS-10% PAGE and stained with Coomassie brilliant blue (insets). Scale bar, 100 nm.

The foreign peptide sequence was inserted at two different locations within the VP60 protein (Fig. 1A) on the basis of structural considerations. A chimeric mutant was generated by inserting the foreign sequence between amino acid positions 2 and 3 of VP60 protein sequence (VP60-2OVA). According to the structural model of RHDV capsid (Barcena et al., 2004), the N-terminus of VP60 protein is facing to the inner core of the viral capsid. Another chimeric mutant was produced by inserting the foreign peptide sequence between amino acid positions 306 and 307 of VP60 protein (VP60-306OVA). This novel insertion site was predicted based on the structural model of VP60 protein (Barcena et al., 2004), where this site would be part of an exposed loop at the P2 subdomain of VP60 protein, and thus might be a suitable location for inserting heterologous amino acid sequences without altering the ability of the protein to form VLPs.

Expression of the VP60 constructs in H5 insect cell cultures infected with the corresponding recombinant baculoviruses was verified by SDS-10% PAGE. As shown in Fig. 1B, extracts from insect cells infected with recombinant baculoviruses harbouring VP60 constructs exhibited a major protein band with the expected size of ~60 kDa, which was not present in wild-type baculovirus-infected cells. As expected, the chimeric VP60 constructs containing the OVA derived epitope displayed a slightly slower electrophoretic mobility than the VP60 protein, reflecting the presence of the inserted heterologous peptide sequence. Monoclonal antibodies directed against RHDV-VP60 protein specifically detected baculovirus-expressed VP60 protein as well as the chimeric mutants by ELISA and Western blotting (data not shown).

To determine whether the chimeric VP60 constructs self-assembled into VLPs, supernatants from infected H5 cell cultures were subjected to CsCl-gradient centrifugation and characterized by electron microscopy (Fig. 2). Negatively stained fractions enriched in the recombinant VP60 constructs (Fig. 2, insets), revealed VLPs of approximately 40 nm in diameter, which were morphologically identical to the VLPs formed by the native VP60 protein (data not shown). Thus, three different RHDV-derived VLPs were generated for our analysis: the native VLPs (RHDV-VLP), the chimeric VLPs harbouring the immunogenic epitope at the N-terminus of VP60 protein (RHDV-VLP-2) and the chimeric VLPs with the immunogenic epitope inserted between residues 306 and 307 of the capsid protein (RHDV-VLP-306). The yield of the purified chimeric VLPs was estimated to be around 5 mg/10^9 cells, which is within the range of that previously reported for other calicivirus VLPs (Ball et al., 1998; Guo et al., 2001; Jiang et al., 1995; Laurent et al., 1994).

Antigen presentation of VLP exogenous antigenic peptides by dendritic cells in vitro

Once both chimeric and control RHDV-VLPs were generated, we firstly investigated whether they were able to activate murine bone marrow derived dendritic cells (BM-DCs) *in vitro*. When DCs internalize proteins to stimulate T cell responses, the DCs must undergo maturation and migrate from the periphery to regional lymph nodes. It is also well established that DCs are a significant source of a wide range of cytokines that are secreted in response to various stimuli, e.g., viruses or bacteria and their products. To investigate whether RHDV-VLPs were capable of activating DCs and therefore to induce pro-inflammatory cytokine production, TNF-α levels were determined in supernatants of BM-DCs cultured either for 6 h (data not shown) or overnight in the presence or absence of different concentrations of RHDV-VLPs. In fact, all RHDV-VLPs induced TNF-α secretion in a dose dependent manner (Fig. 3), indicating a certain degree of BM-DCs activation after incubation with the VLPs. Since it has been demonstrated that the presence of contaminating baculovirus in VLP preparations may induce potent immune responses that could be erroneously attributed to the VLPs (Hervas-Stubbs et al., 2007), we also analyzed as a control, material prepared from insect cells infected with wild-type baculovirus subjected to the same purification procedure as the VLPs (mock VLPs). The average of TNF-α production induced by mock VLPs was 116.6 pg/ml ± 12 (mean ± SD), which was close to background levels (untreated DCs 44.29 pg/ml ± 10).

DCs have been recognized as being the most potent antigen-presenting cells (APCs) capable of stimulating naïve T cells. Therefore, we analyzed whether DCs could process RHDV-VLPs-OVA and present the OVA257-264-H2-Kb MHC-I complex to a specific $CD8^+$ T cell (B3Z) hybridoma, as the first step of cytotoxic T cells (CTL) induction, using an antigen presentation assay. B3Z hybridoma specifically recognizes SIINFEKL peptide presented in combination with H2-Kb MHC-I (Karttunen, Sanderson, and Shastri, 1992) thereby releasing IL-2 in the culture supernatants. Serial dilutions of SIINFEKL peptide were added to BM-DCs and IL-2 levels in the supernatants were recorded as a measure of antigen presentation in the assay. As shown in Fig. 4A, SIINFEKL peptide was specifically recognized when exogenously added to BM-DCs for antigenic presentation to B3Z hybridome, in a dose dependent manner. Under our experimental conditions,

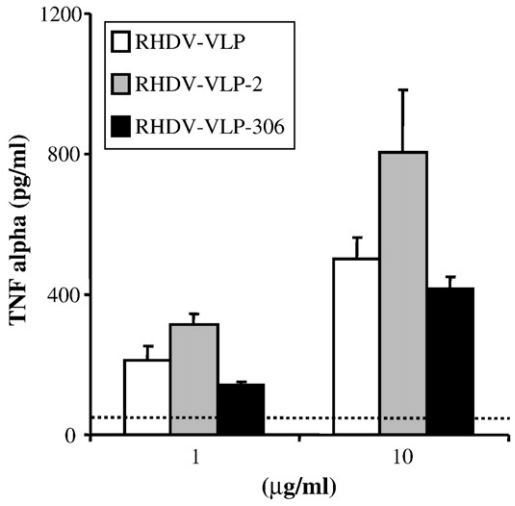

Fig. 3. Bone marrow derived DCs secreted TNF-α after incubation with control and chimeric RHDV-VLPs at different concentrations. The column colours indicate the different RHDV-VLPs: RHDV-VLP (white), RHDV-VLP-2 (grey) and RHDV-VLP-306 (black). Dotted line indicates background level of TNF-α secretion by untreated cells and error bars representing one standard deviation (SD) above the mean. Data are representative of two independent experiments.

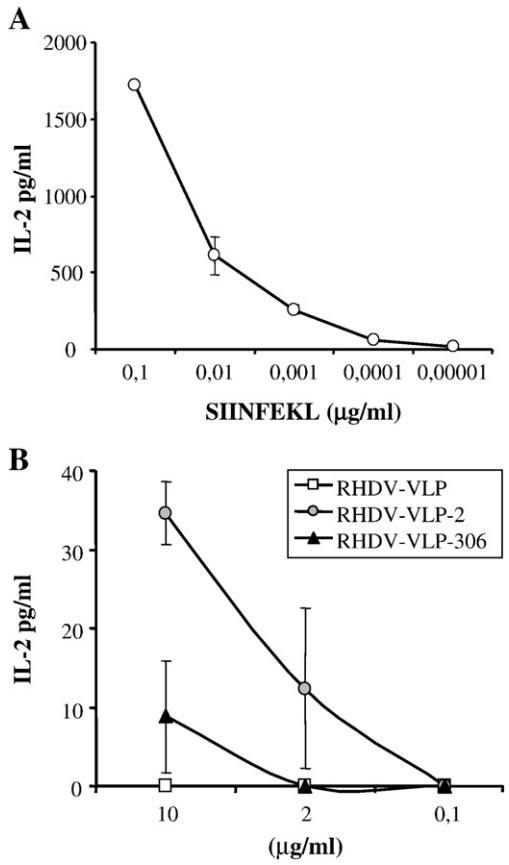

Fig. 4. Mouse DCs are able to process and present SIINFEKL peptide for $CD8^+$ specific recognition *in vitro* in a dose dependent manner. (A) C57Bl/6 BM-DCs were incubated for 6–7 h in the presence of the indicated concentration of synthetic SIINFEKL peptide, and IL-2 released was measured by ELISA. (B) IL-2 released after VLP incubation with C57Bl/6 BM-DCs at different concentrations. The colours indicate the different VLPs: RHDV-VLP (white), RHDV-VLP-2 (grey) and RHDV-VLP-306 (black). The data shown are the means of results obtained in triplicate wells and error bars representing one standard deviation (SD) above the mean. Data are representative of three independent experiments.

antigenic presentation was detected in a range from 100 to 0.1 ng/ml (Fig. 4A).

When BM-DCs were incubated with chimeric VLPs containing the SIINFEKL antigenic peptide (RHDV-VLP-2 and RHDV-VLP-306), IL-2 production was detected, reflecting specific antigen presentation from both chimeric RHDV-VLPs. No specific recognition was detected from BM-DCs incubated with the native control VLPs (RHDV-VLP) (Fig. 4B). Antigen presentation showed a dose dependent pattern. Whereas antigenic peptide presentation was detected when BM-DCs were incubated with RHDV-VLP-2 at the two highest concentrations used (10 and 2 μg/ml), presentation of SIINFEKL peptide from RHDV-VLP-306 was only detected at 10 μg/ml (Fig. 4B). Thus, insertion of the SIINFEKL peptide in the amino terminal position (RHDV-VLP-2) seems to favour processing and presentation by DCs in comparison with SIINFEKL insertion at the exposed loop (RHDV-VLP-306).

All in all, our data indicate that BM-DCs were able to efficiently process and present SIINFEKL peptide from recombinant RHDV-VLPs-OVA for $CD8^+$ specific recognition in a dose- and insert position-dependent manner.

Induction of cellular responses by recombinant VLPs in mice

The results of SIINFEKL presentation by BM-DCs *in vitro* led us to investigate whether the chimeric RHDV-VLPs expressing SIINFEKL peptide in two different positions induced any specific immunity in mice. Groups of three C57BL/6 mice were immunized twice by intraperitoneal injections of either 8 or 40 μg of each chimeric RHDV-VLPs-OVA or control RHDV-VLPs in PBS without adjuvant. A group of mice infected with VV-OVA was used as a positive control. Taking into account that SIINFEKL sequence is an immunodominant T cell epitope in C57Bl/6 mice (possessing H2-Kb MHC-I), it was conceivable to assume that a good vaccine vector carrying such epitope would induce specific IFN-γ-secreting cells and/or cytotoxic T cells (CTLs). Indeed, two weeks after the last inoculation of mice with RHDV-VLPs-OVA, specific IFN-γ-secreting cells were detected in spleens of mice by ELISPOT (Fig. 5). They exhibited a dose dependent pattern. At the highest dose of RHDV-VLPs-OVA used, both chimeric constructs induced similar numbers of IFN-γ-secreting cells. Noticeably, significant numbers of specific IFN-γ-secreting cells were detected at the lowest dose analyzed only when RHDV-VLP-2 was used. As expected, mice injected with control RHDV-VLPs did not show any significant response. Animals infected with VV-OVA without any previous treatment had 1200 ± 536 spots per 10^6 splenocytes of specific IFN-γ-secreting cells, six days after infection. This value is in a similar range than the ones from mice immunized with the chimeric RHDV-VLPs at the highest dose used. Therefore, although both chimeric RHDV-VLPs-OVA constructs were able to induce specific IFN-γ-secreting cells, insertion of the SIINFEKL peptide in the amino terminal position (RHDV-VLP-2) was more immunogenic than insertion in position 306 for induction of CTLs and anti-viral immunity.

Additionally, another mechanism for immune protection against a viral challenge is to induce enough specific memory CTLs. Therefore, cytotoxic activity was measured by an *in vivo* CTL assay (Le Bon et al., 2006) (Fig. 6A), where a low fluorescence peak of SIINFEKL-pulsed cells was used to calculate the percentage of specific killing compared with unpulsed high fluorescence cells injected in mice, as described in Materials and methods.

Without any viral infection, only the animals inoculated with RHDV-VLP-2 generated specific and functional CTL activity both at 40 μg (54.2% ± 15) and 8 μg (8.9% ± 3.2) dose of inoculation, whereas RHDV-VLP-306 was only able to generate detectable CTL activity at the higher dose (23.3% ± 10), and this was significantly lower than that induced by RHDV-VLP-2 (Fig. 6B). After infection with recombinant VV-OVA, all groups of mice exhibited a high level of cytotoxic activity (90–100%) (Fig. 6A).

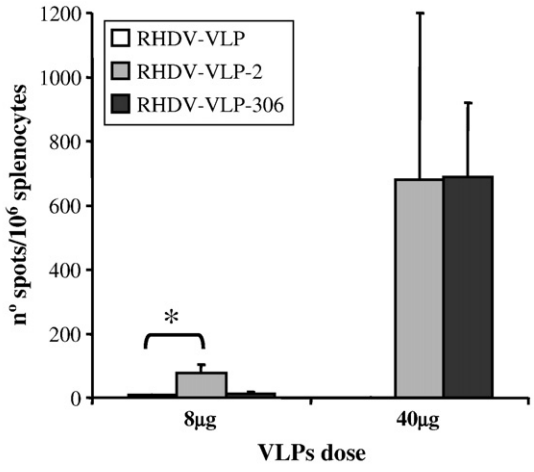

Fig. 5. Frequency of SIINFEKL-specific IFN-γ-producing cells in the spleen of treated mice. Groups of three mice were twice inoculated with 8 or 40 μg of the RHDV-VLPs and specific IFN-γ-producing cells were measured by ELISPOT assay. The column colours indicate the different RHDV-VLPs: RHDV-VLP (white), RHDV-VLP-2 (grey) and RHDV-VLP-306 (black). The data shown are the means of results obtained in groups of three mice, with the error bars representing one standard deviation above the mean. Data are representative of two independent experiments.

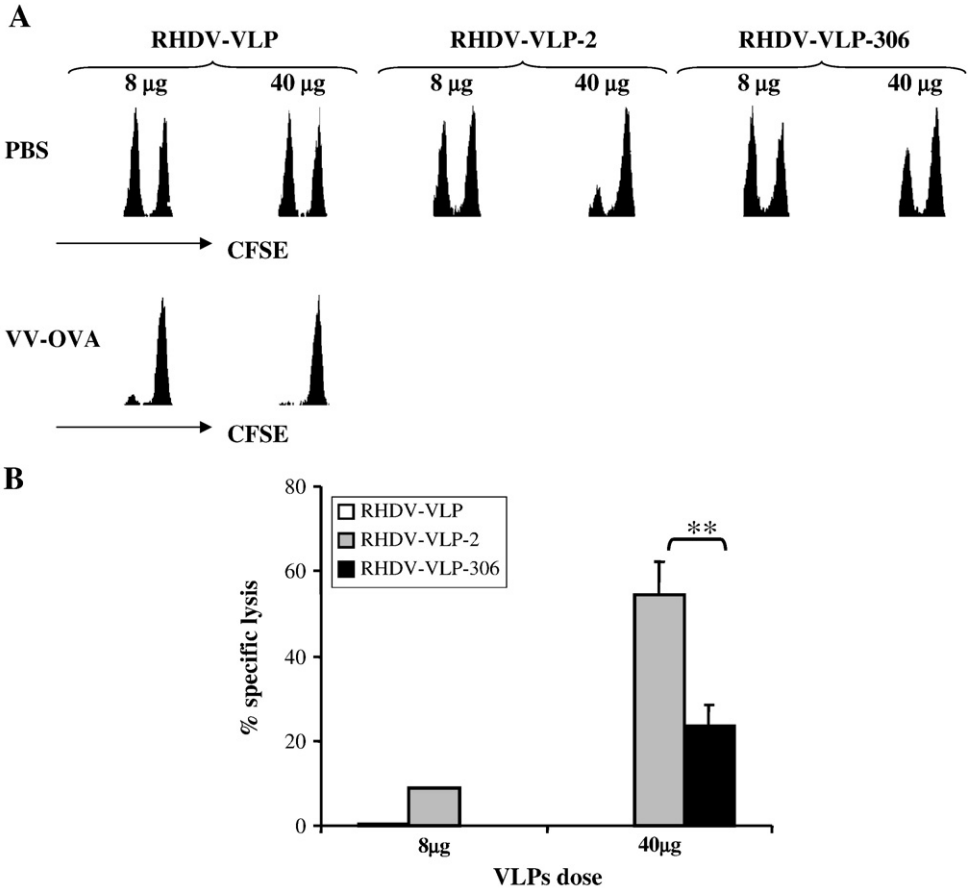

Fig. 6. OVA-specific CTL cell responses in VLP immunized mice by measuring SIINFEKL-specific cytotoxic activity using *in vivo* CTL assay as described in Materials and methods. Groups of three mice were twice inoculated with 8 or 40 µg of the different RHDV-VLPS: RHDV-VLP, RHDV-VLP-2 and RHDV-VLP-306. A control group for 100% of lysis, was set with a group of mice infected i.p. with VV-OVA. (A) Cytotoxic responses were assessed 7 days later measuring the percentage of specific lysis. Histograms represent target cells stained with high concentration of CFSE (control cells, right) and peptide-pulsed target cells stained with low concentration of CFSE (left). The data shown is from one representative mice per group. (B) Average of specific lysis per group of mice. The column colours indicate the different RHDV-VLP: RHDV-VLP (white), RHDV-VLP-2 (grey) and RHDV-VLP-306 (black). The data shown are the means of results obtained in groups of three mice, with the error bars representing one standard deviation above the mean. Data are representative of two independent experiments.

Viral titers in mice immunized with recombinant VLPs after VV-OVA challenge

Finally, to determine whether or not the immune response induced by the chimeric RHDV-VLPs was effective against a viral challenge, viral titers in ovaries were measured 6 days after infection with VV-OVA, in mice untreated or mice previously inoculated twice with the recombinant RHDV-VLPs in the absence of adjuvant. Viral titers in mice previously inoculated with either 8 or 40 µg of control RHDV-VLPs (Fig. 7) were in the same range to the ones induced in untreated mice infected with VV-OVA ($6 \pm 0.3 \times 10^7$ pfu/g). When mice were twice inoculated with 40 µg of RHDV-VLP-306, there was a two-logarithm reduction in virus titers, as compared with those from mice inoculated with control RHDV-VLP, indicating that some extent of protective immunity had been generated. Surprisingly, VV-OVA titers decreased to undetectable levels (limit of detection in our assay was 4 pfu/g) in ovaries from mice immunized with RHDV-VLP-2 (Fig. 7). Viral titers from mice inoculated twice with 8 µg of the chimeric VLPs exhibited a non significant reduction as compared with those from mice inoculated with control RHDV-VLPs.

In conclusion, immunization of mice with the chimeric VLPs at the highest dose tested elicited great viral titer reductions upon a VV-OVA challenge, suggesting that the immune response induced by the chimeric VLPs was able to cope with the viral infection. Again, the data obtained indicated that insertion of the foreign immunogenic peptide at the N-terminus of VP60 protein rendered better results than the insertion at the exposed loop.

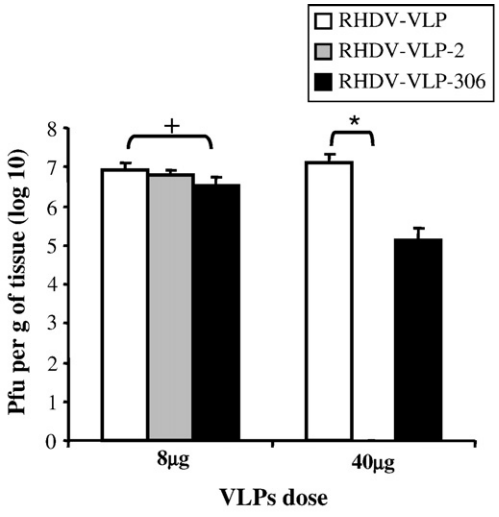

Fig. 7. Viral titers per gram of ovaries in mice immunized with the different RHDV-VLPs and subsequently challenged with VV-OVA. Mice were infected with VV-OVA 6 days before analysis. Columns indicate viral titers after immunization with 2 doses of either 8 or 40 mg of RHDV-VLPs after VV-OVA challenge. White columns indicate immunization with RHDV-VLP, grey columns for RHDV-VLP-2 and black columns for RHDV-VLP-306. The data shown are the means of results obtained in duplicates for groups of three mice, with the error bars representing one standard deviation above the mean. Data is representative of two independent experiments (* indicate $p<0.05$ and + indicate $p=0.1$). Detection limit in the assay was 4 pfu/g.

Discussion

VLPs have clearly demonstrated their potential as vectors for vaccination (reviewed in Dyer, Renner, and Bachmann, 2006; Jennings and Bachmann, 2008; Ludwig and Wagner, 2007) and have proven to be a potent CTL inducer when compared with other vectors (Allsopp et al., 1996). Recombinant VLPs derived from NV and other human noroviruses have been used to induce systemic and mucosal immune responses in mice (Ball et al., 1998; Guerrero et al., 2001; Nicollier-Jamot et al., 2004; Periwal et al., 2003; Xia, Farkas, and Jiang, 2007), and they are currently being evaluated in human clinical trials (Ball et al., 1999; Tacket et al., 2003). Norovirus-derived VLPs have also been used to immunize calves and pigs (Han et al., 2006; Souza et al., 2007). In addition, VLPs derived from other calicivirus genera like RHDV (*Lagovirus*) and feline calicivirus (*Vesivirus*) have been shown to be highly immunogenic (Boga et al., 1997; Di Martino, Marsilio, and Roy, 2007; Laurent et al., 1994; Nagesha, Wang, and Hyatt, 1999; Plana-Duran et al., 1996). Taken together, these results indicate that calicivirus VLPs could be excellent candidates to induce a potent immune response to foreign antigens inserted in their particulate structure and therefore to be a vaccine vector. However, chimeric calicivirus VLPs for vaccine purpose have not been reported.

We have developed a system for the generation of chimeric VLPs derived from RHDV that has shown a powerful capacity to elicit a potent anti-viral response. We have also compared the immunogenicity induced by a foreign T cell epitope inserted at two different sites within the capsid structure. *In vitro*, both chimeric RHDV-VLPs not only activated DCs for TNF-α secretion but they also were processed and presented to specific T cells. Additional experiments *in vivo* revealed that mice immunized with chimeric RHDV-VLPs-OVA without adjuvant were able to induce specific cellular responses. More importantly, immunization with chimeric RHDV-VLPs without adjuvant was able to resolve or to reduce an infection by a recombinant vaccinia virus expressing OVA protein.

Nowadays, the attention is focused on the immunological pattern of VLPs and studies have shown their immunomodulation potential when interacting with DCs (Moron et al., 2003). DCs are essential for activating the innate and adaptive immunity, and the particulate nature of the RHDV-VLPs, in the size range of around 40 nm, appears to be optimal for uptake by DCs for processing and presentation by MHC and for promoting DC maturation and migration (Gamvrellis et al., 2004). Several mechanisms responsible for the processing of exogenous antigens in the MHC class I pathway have been described (Rock, 1996), which are generally called cross-presentation. Cross-presentation is the process whereby APCs acquire, process and present exogenous antigen as peptides bound to MHC class I molecules to $CD8^+$ T cells (reviewed in Heath and Carbone, 2001). In the present study, we demonstrated that although chimeric RHDV-VLPs are exogenous antigens, they are presented by BM-DCs in the context of MHC class I as evidenced by stimulation of hybridoma cells B3Z *in vitro*. Cross-presentation of VLPs has been reported to occur via a TAP-independent, endosomal pathway, or by a TAP-dependent, endosome to cytosol pathway (Ruedl et al., 2002). To initiate cross-presentation, APCs must both capture extracellular antigens and receive specific activation signals, a process known as cross-priming where type I IFN has been described to play an important role (Le Bon et al., 2003). To gain insight into type I IFN contribution to the cross-presentation of our chimeric RHDV-VLPs, type I receptor knock-out BM-DCs were tested for antigen presentation *in vitro*. Our results show that cross-presentation of the OVA epitope was independent of type I IFN in the conditions tested (data not shown). Thus, cross-presentation of chimeric RHDV-VLPs would follow a type I IFN independent pathway that will require further investigation. Stimulation of DCs by papillomavirus-like particles has been shown to involve IFN-α secretion and it is mediated by MyD88 (Yang et al., 2004). Our results suggest that type I IFNs do not play a mayor role, but MyD88 implication remains to be elucidated for RHDV-VLPs.

$CD8^+$ effector T cells are central mediators of anti-viral immunity. These cells have been found to exert their anti-viral functions by at least two distinct mechanisms. First, $CD8^+$ effector T cells can recognize and kill virus-infected cells either via perforin-dependent lysis or through Fas–Fas ligand interaction, leading to apoptosis of the target cell (Kagi et al., 1994; Topham, Tripp, and Doherty, 1997). Second, virus-specific $CD8^+$ T cells are potent producers of anti-viral cytokines, in particular IFN-γ, which may attenuate viral replication (Ramshaw et al., 1997). The relative importance of these two different effector mechanisms (cell lysis versus anti-viral cytokines) in the elimination of a viral infection is hypothesized to be heavily influenced by the virus and its life cycle. Thus, resolution of cytopathic viruses is thought to be mediated mainly by soluble mediators, whereas cytotoxicity should be crucial for the clearance of a non-cytopathic virus (Kagi and Hengartner, 1996). VLPs from porcine parvovirus have been previously reported to be processed by MHC class I pathway by cross-presentation (Moron et al., 2003) for activation of $CD8^+$ T cells which are essential for the clearance of intracellular pathogens such a virus (Sedlik et al., 1999; Sedlik et al., 1997). The stimulation of this effective response is based on the induction of high frequency, efficiency and avidity CTLs (Sedlik et al., 2000). In our system, the fact that both chimeric RHDV-VLPs not only induce specific CTLs but also IFN-γ secreting cells indicated that chimeric RHDV-VLPs might constitute excellent vaccine platforms against both, cytopathic or non-cytopathic viruses, as they stimulate both effector mechanisms.

Immunization of mice with chimeric VLPs induced a reduction in viral levels upon a VV-OVA challenge (Fig. 7). Moreover, immunization with chimeric RHDV-VLP-2 at the highest dose tested was able to resolve VV-OVA infection. Considering that chimeric RHDV-VLP-2 was the most efficient one at inducing specific CTLs and IFN-γ producing cells (Fig. 5 and 6), these two mechanisms might be the primary candidates to explain viral clearance. In addition, RHDV-VLP-306 was able to induce some detectable level of specific antibodies (data not shown) which did not correlate with protection (Fig. 7). Further experiments will determine the relative role of each mechanism in viral clearance.

Native RHDV-VLPs have been used to confer complete protection against RHD in immunized rabbits. Recently, Peacey et al. reported a procedure to chemically conjugate whole proteins and peptides to VLPs from RHDV (Peacey et al., 2007). They later showed that prophylactic immunization with the chemically coupled RHDV-VLPs administered with adjuvant has the capacity to elicit a potent cell-mediated and anti-tumor response (Peacey et al., 2008). Our results are in agreement and further extend those of Peacey et al. obtained with antigen conjugated to RHDV-VLPs. Firstly, the data presented in this work demonstrated induction of a different type of immunity, namely anti-viral immunity by induction of specific IFN-γ secreting cells and CTLs. Secondly, chimeric RHDV-VLPs used to immunize mice did not require the use of adjuvants, as the chemically engineered did (Peacey et al., 2008).

Finally, we have analyzed two potential sites to insert foreign epitopes into RHDV-VLPs. Both constructs were shown to be immunogenic but the one at the N-terminus, which is predicted to be buried in the internal face of the VLPs, exhibited a higher degree of immunogenicity for cell-mediated responses against VV-OVA infection. On the other hand, the novel insertion site located within a predicted exposed loop at the P2 subdomain of RHDV capsid protein, might constitute a suitable insertion site for B cell epitopes to induce specific antibodies. The P2 subdomain, located at the surface of the capsid, is an immunodominant region and contains the highest variability in the genome among caliciviruses. It contains the determinants of strain specificity, receptor binding (Chen et al., 2004; Tan, Meller, and Jiang, 2006), and potential neutralizing

antibody recognition sites (Chen et al., 2006; Lochridge et al., 2005), characteristics in principle appropriate for an efficient insertion site intended for foreign B cell epitopes. This is the case for porcine parvovirus derived VLPs, where T cell epitopes elicit efficient immune responses when inserted at the N-terminus of the VP2 protein, but neither the N nor the C termini of the protein can be used to insert foreign B cell epitopes (Sedlik et al., 1995), which must be inserted at exposed loops, in order to render an efficient specific immune response (Rueda et al., 1999). Work is in progress to analyze the ability of chimeric RHDV-VLPs to induce an immune response against foreign B cell epitopes inserted at different locations within the capsid protein.

It has been shown that pre-existing antibody responses against VLPs exert a detrimental effect on the efficacy of chimeric VLP-based vaccines (Da Silva et al., 2001). The use of different VLP scaffolds in prime-boost regimens to deliver the same antigen is a promising strategy to increase the efficacy and usefulness of this type of vaccines (Da Silva, Schiller, and Kast, 2003). In the case of RHDV-VLPs which are derived from a rabbit virus to which no pre-existing immunity is expected in humans or livestock species, they represent a suitable choice of delivery system and further extend the arsenal of VLPs to use for prime-boost regimens.

In conclusion our data demonstrated that the chimeric VLPs were able to protect mice from a viral challenge, suggesting the potential suitability of these constructions for new vaccine development against animal and human viral infections.

Materials and methods

Viruses, cells and mice

Derivatives of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) were used to obtain the recombinant baculoviruses expressing RHDV-VLPs. Baculoviruses were propagated in insect cell lines grown in suspension or monolayer cultures at 28°C in TNM-FH medium (Sigma) supplemented with 5% fetal calf serum (Gibco). *Spodoptera frugiperda* cells (SF9) were used for generation of recombinant baculoviruses, plaque assays, and the preparation of high titer viral stocks. *Trichoplusia ni* cells (H5) were used for high level expression of recombinant proteins.

Recombinant vaccinia virus (VV) expressing ovalbumin (VV-OVA) were originally obtained from J. Yewdell (National Institute of Health, Bethesda, Maryland, U.S.A.) (Restifo et al., 1995). Vaccinia virus was grown and titrated in Vero cells as previously described (Earl et al., 2001).

Mice, C57BL/6JOlaHsd (Harlan) of 7- to 8-wk-old age were used for immunization and for DCs primary cultures. Bone marrow from type I IFN-R KO mice in a C57BL/6 genetic background was kindly donated by Dr. N. Sevilla and was used for DCs primary cultures.

Construction of recombinant baculovirus transfer vectors

The primers used in this study are all shown in Table 1. The baculovirus transfer vector chosen to express the different VP60 constructs was plasmid pBacPAK8HA. This plasmid is a derivative of pBacPAK8 (Clontech), in which several restriction sites were eliminated from the multiple cloning site. To generate pBacPAK8HA, first, pBacPAK8 was digested with *BamH*I and *Xba*I, blunt ended, and religated. The resulting plasmid, pBacPAK8XB, was used as template for a PCR reaction using the primer pair BacHAF/Bac1R. After gel purification (FlexiPrep Kit, Amersham Pharmacia) of the PCR product, the DNA fragment was digested with *Bgl*II and *Hind*III, and inserted into the plasmid pBacPAK8XB, previously digested with the same restriction enzymes, generating pBacPAK8HA.

A DNA fragment containing the coding sequences of proteins VP60 and VP2, and the 3′ untranslated region of RHDV (strain AST/89), was obtained by PCR using as template plasmid pUC2.4-1, which contained the full-length RHDV subgenomic RNA (Barcena et al., 2004), and the primer pair PolihedF/KpnISgRHDR. The PCR product obtained was digested with *Bgl*II and *Kpn*I and inserted into unique restriction sites of pBacPAK8HA, creating pHAPhSubG. This plasmid was subsequently modified to eliminate a natural *Bam*HI site present in the VP60 gene, without changing the encoded amino acid sequence of the protein. For this purpose, two separate PCR reactions were performed using the primer pairs Bac1F/T93R and T93F/KpnISgRHDR, and plasmid pHAPhSubG as template. The PCR products obtained were gel purified, denatured and annealed together in a secondary PCR in which the extended template was amplified using the external primers PolihedF/KpnISgRHDR. The PCR product obtained was digested with *Bgl*II and *Kpn*I and inserted into unique restriction sites of pBacPAK8HA, generating pHAPhSubGB.

The next step was the engineering by site-directed mutagenesis of unique BamHI sites at defined locations within VP60 gene: at the region corresponding to amino acid positions 2 and 3 (plasmid pHAPh2GS), and at the region corresponding to amino acid positions 306 and 307 (plasmid pHAPh306GS). To generate pHAPh2GS, a PCR was performed using the primer pair 2GSF/KpnISgRHDR, and plasmid pHAPhSubGB as template. The PCR product obtained was digested with *Bgl*II and *Bst*EII and inserted into unique restriction sites of pHAPhSubGB, creating pHAPh2GS. Plasmid pHAPh306GS was generated using two sequential PCRs. First, two separate PCR reactions were performed using the primer pairs PolihedF/306GSF and 306GSR/KpnISgRHDR, and plasmid pHAPhSubGB as template. The PCR products obtained were gel purified, denatured and annealed together in a secondary PCR in which the extended template was amplified using the external primers PolihedF/KpnISgRHDR. The PCR product obtained was digested with *Bgl*II and *Kpn*I and inserted into unique restriction sites of pBacPAK8HA, generating pHAPh306GS.

Finally, a DNA fragment containing the coding sequence of the immunogenic peptide SIINFEKL derived from OVA protein, plus 3 upstream (QLE) and 2 downstream (TE) flanking amino acids was generated by annealing synthetic oligonucleotides 3OVA2F and 3OVA2R, leaving *Bam*HI compatible ends. The annealed primers were subsequently ligated into plasmids pHAPh2GS and pHAPh306GS, previously linearized by *Bam*HI digestion and dephosphorilated, creating plasmids pHAVP60-2OVA and pHAVP60-306OVA. All the inserted sequences in the resulting recombinant plasmids were verified by sequence analyses.

Table 1
Oligonucleotide primers used for cloning.

Primer	Sequence (5′ to 3′)[a]
Bac1F	GACTCCAAGTGTGTGGGTGAAGTC
Bac1R	CACGCCCGATGTTAAATATGTCC
BacHAF	CTAGAAGATCTGGTACCGTTATTAGTACATTTATTAAGCGCTAGATTCTG
T93F	CGATGGCATGGACCCCGGCGTTGTG
T93R	CACAACGCCGGGGTCCATGCCATCG
PolihedF	TAAATAGATCTATAAAT**ATG**GAGGGCAAAGCCCGC
KpnISgRHDR	TCGACGGTACCATAGC**TTA**CTTTAAACTATAAACCCA
2GSF	TAAATAGATCTATAAAT**ATG**GGATCCAAAGCCCGC
306GSF	GCAAGTTACCCTGGATCCAACGCAACC
306GSR	GGTTGCGTTGGATCCAGGGTAACTTGC
3OVA2F	GATCTCAACTGGAGAGCATCATCAATTTCGAGAAACTTACCGAGG
3OVA2R	GATCCCTCGGTAAGTTTCTCGAAATTGATGATGCTCTCCAGTTGA

[a] Restriction site sequences are underlined. Start and stop codons are shown in boldface.

Generation of recombinant baculoviruses

All recombinant baculoviruses were produced using the BacPAK baculovirus expression system (Clontech) as described previously (Barcena et al., 2004). Briefly, monolayers of SF9 insect cells were co-transfected with recombinant transfer vectors and *Bsu*36I triple-cut

AcMNPV DNA (Kitts and Possee, 1993) using lipofectamine (Invitrogen). Recombinant baculoviruses were selected on the basis of their LacZ-negative phenotypes, plaque purified, and propagated as described elsewhere (King and Possee, 1992).

Expression and purification of the recombinant RHDV-VLPs

The recombinant VP60 and the chimeric VP60 constructs were expressed and the self-assembled VLPs were purified by previously described methods (Almanza et al., 2008). Briefly, H5 insect cell monolayers were infected with recombinant baculoviruses at a multiplicity of infection of 10. After incubation (6–7 days, 28 °C) infected cells were scraped into the medium. The culture medium was then clarified by centrifugation (at 10,000 rpm for 10 min with a GSA rotor), and the supernatant was centrifuged at 26,000 rpm for 2 h with a Beckman SW28 rotor. The pelleted material was resuspended in 0.2 M phosphate-buffered saline for VLPs (PBS-V; 0.2 M sodium phosphate, 0.1 M NaCl, pH 6.0), extracted twice with Vertrel® XF, and subjected to centrifugation (at 35,000 rpm for 2 h with a Beckman SW55 rotor) through a 20% sucrose cushion of 1.5 ml made with PBS-V. Subsequently, the pellet was suspended in a solution of CsCl (0.42 g/ml) and subjected to isopycnic gradient centrifugation at 35,000 rpm for 18 h in a Beckman SW55 rotor. The visible opalescent band in the CsCl gradient was collected by micropipetting, diluted in PBS-V, and pelleted by centrifugation at 26,000 rpm for 2 h in a Sorvall TH-641 rotor to remove CsCl. The pellet was finally resuspended in PBS-V containing protease inhibitors (Complete, Roche) and stored at 4°C. The protein concentrations of the VLP preparations were determined with a bicinchoninic acid protein assay kit (BCA protein assay kit, Pierce).

Electron microscopy

Samples (approximately 5 μl) were applied to glowdischarged carbon-coated grids for 2 min. and negatively stained with 2% (wt/vol) aqueous uranyl acetate. Micrographs were recorded with a Jeol 1200 EXII electron microscope operating at 100 kV at a nominal magnification of ×40,000.

Mouse bone marrow derived dendritic cells (BM-DCs) generation

BM-DCs were generated from cultures of bone marrow cells of C57Bl/6 and type I IFN-R KO mice. They were prepared as described previously (Montoya et al., 2002). Briefly, bone marrow was extracted from the tibia and femur, and cell suspensions were cultured in RPMI 1640 complete medium (Gibco) containing 10% heat-inactivated fetal calf serum (FCS), 50 μM 2-ME, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 U/mL polymyxin B (Sigma), and 20 ng/mL recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF) (R and D Systems, Abingdon, Oxon, United Kingdom). Fresh medium was given every other day. $CD11c^+$ ranged between 95% and 98% without any further sorting or treatment. The BM progenitors were incubated at 37°C in 5% CO_2 in Petri dishes for 8 days as previously described (Lutz et al., 1999) and then immature BM-DCs were used for the antigen presentation assay.

DC activation and antigen presentation assay

Different concentrations of our different RHDV-VLPs (RHDV-VLP-2, RHDV-VLP-306 and negative control RHDV-VLP) were plated in triplicates with murine immature BM-DCs (10^5 cells/well) and they were incubated for 6–7 h in 96-well culture microplates in a final volume of 100 μl of RPMI 1640 complete medium. Activation of DCs was analyzed by TNF-α release in the supernatant using a specific ELISA (R&D Systems, Abingdon, Oxon, United Kingdom). Detection limit in ELISA was 9 pg/ml. Then, BM-DCs were thoroughly washed and 10^5 cell/well of specific $CD8^+$ hybridoma (B3Z) recognizing SIINFEKL peptide (Karttunen, Sanderson, and Shastri, 1992) were added and incubated overnight at 37°C in 5% CO_2. Antigen presentation to B3Z presented in combination with H2-Kb MHC-I was analyzed by IL-2 release in the supernatant using an specific ELISA (R&D Systems, Abingdon, Oxon, United Kingdom). Detection limit in ELISA was 6 pg/ml. BM-DCs cells stimulated only with SIINFEKL peptide (ProImmune, Abingdon, UK) were used as control of presentation efficiency range in our *in vitro* experiment (Moron et al., 2003).

Immunization protocol

The following immunization scheme was used in two independent experiments: female C57BL/6JOlaHsd (Harlan) of 7- to 8-wk-old age, kept under specific-pathogen-free-conditions, were randomly divided in groups of 6 animals and intraperitoneally inoculated twice in 2 week intervals with 40 μg or 8 μg of VLPs resuspended in 200 μl of sterile PBS (a control group was inoculated with PBS alone). No adjuvant was used in the immunisations. Two weeks after the second VLP inoculation, three animals in each group were challenged intraperitoneally with 10^6 pfu/mice of VV-OVA.

Evaluation of cellular responses and CTL activity

Two weeks after the second immunization, spleen cells were collected and analyzed for specific IFN-γ production by ELISPOT Set following manufactures instructions (Becton Dickinson UK). Spleen cells were added to triplicate wells at concentrations of 10^4, 10^5 and 10^6 cells/well with SIINFEKL peptide (ProImmune, Abingdon, UK) at a concentration of 10^{-6} M per well. Triplicate wells with 10^6 cells without peptide were used to estimate the non-specific activation. As positive control, triplicate wells with 10^6 cells were stimulated with phytohemagglutinin (PHA) (Sigma) at a concentration of 10 μg/ml per well.

For *in vivo* CTL assays, naïve spleen cells were pulsed *ex-vivo* for 1 h with 1 nM of SIINFEKL peptide. After extensive washing, cells were labelled with 0.1 μM CFSE (CFSElo) (Molecular Probes, The Netherlands). A control population, splenocytes unpulsed with peptide, was labelled with 1 μM CFSE (CFSEhi). CFSElo and CFSEhi cells were mixed in a 1:1 ratio and injected intravenously into naïve or immunized animals. After 18 h, spleens were removed and single-cell suspensions analyzed by flow cytometry to determine the ratio of CFSElo to CFSEhi cells. The percentage of specific lysis was calculated as follows: % of specific lysis = 100 − {100 × (% CFSElo immunised/% CFSEhi immunised) − (% CFSElo control/% CFSEhi control).

Evaluation of viral levels

Viral titers in ovaries of individual mice were determined at day 6 after infection by a plaque assay using Vero cells {Restifo, 1995 #100}. Detection limit was 4 pfu/g.

Statistical analysis

Experimental groups were compared through ANOVA followed by Tukey–Kramer post hoc test for multiple comparisons of unpaired observations. The significance level was established at $p<0.05$ and all the analyses were carried out with the NCSS 2004 and PASS 2005 software (Kavysville, Utah, USA).

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References

Allsopp, C.E., Plebanski, M., Gilbert, S., Sinden, R.E., Harris, S., Frankel, G., Dougan, G., Hioe, C., Nixon, D., Paoletti, E., Layton, G., Hill, A.V., 1996. Comparison of numerous delivery systems for the induction of cytotoxic T lymphocytes by immunization. Eur. J. Immunol. 26 (8), 1951–1959.

Almanza, H., Cubillos, C., Angulo, I., Mateos, F., Caston, J.R., van der Poel, W.H., Vinje, J., Barcena, J., Mena, I., 2008. Self-assembly of the recombinant capsid protein of a swine norovirus into virus-like particles and evaluation of monoclonal antibodies cross-reactive with a human strain from genogroup II. J. Clin. Microbiol. 46 (12), 3971–3979.

Angulo, E., Barcena, J., 2007. Towards a unique and transmissible vaccine against myxomatosis and rabbit haemorrhagic disease for rabbit populations. Wildlife Res. 34 (7), 567–577.

Ball, J.M., Hardy, M.E., Atmar, R.L., Conner, M.E., Estes, M.K., 1998. Oral immunization with recombinant Norwalk virus-like particles induces a systemic and mucosal immune response in mice. J. Virol. 72 (2), 1345–1353.

Ball, J.M., Graham, D.Y., Opekun, A.R., Gilger, M.A., Guerrero, R.A., Estes, M.K., 1999. Recombinant Norwalk virus-like particles given orally to volunteers: phase I study. Gastroenterology 117 (1), 40–48.

Barcena, J., Morales, M., Vazquez, B., Boga, J.A., Parra, F., Lucientes, J., Pages-Mante, A., Sanchez-Vizcaino, J.M., Blasco, R., Torres, J.M., 2000. Horizontal transmissible protection against myxomatosis and rabbit hemorrhagic disease by using a recombinant myxoma virus. J. Virol. 74 (3), 1114–1123.

Barcena, J., Verdaguer, N., Roca, R., Morales, M., Angulo, I., Risco, C., Carrascosa, J.L., Torres, J.M., Caston, J.R., 2004. The coat protein of rabbit hemorrhagic disease virus contains a molecular switch at the N-terminal region facing the inner surface of the capsid. Virology 322 (1), 118–134.

Boga, J.A., Casais, R., Marin, M.S., Martin-Alonso, J.M., Carmenes, R.S., Prieto, M., Parra, F., 1994. Molecular cloning, sequencing and expression in *Escherichia coli* of the capsid protein gene from rabbit haemorrhagic disease virus (Spanish isolate AST/89). J. Gen. Virol. 75 (Pt 9), 2409–2413.

Boga, J.A., Martin Alonso, J.M., Casais, R., Parra, F., 1997. A single dose immunization with rabbit haemorrhagic disease virus major capsid protein produced in *Saccharomyces cerevisiae* induces protection. J. Gen. Virol. 78 (Pt 9), 2315–2318.

Castanon, S., Marin, M.S., Martin-Alonso, J.M., Boga, J.A., Casais, R., Humara, J.M., Ordas, R.J., Parra, F., 1999. Immunization with potato plants expressing VP60 protein protects against rabbit hemorrhagic disease virus. J. Virol. 73 (5), 4452–4455.

Chackerian, B., 2007. Virus-like particles: flexible platforms for vaccine development. Expert. Rev. Vaccines 6 (3), 381–390.

Chen, R., Neill, J.D., Noel, J.S., Hutson, A.M., Glass, R.I., Estes, M.K., Prasad, B.V., 2004. Inter- and intragenus structural variations in caliciviruses and their functional implications. J. Virol. 78 (12), 6469–6479.

Chen, R., Neill, J.D., Estes, M.K., Prasad, B.V., 2006. X-ray structure of a native calicivirus: structural insights into antigenic diversity and host specificity. Proc. Natl. Acad. Sci. U. S. A. 103 (21), 8048–8053.

Cooke, B.D., 2002. Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations. Rev. Sci. Tech. 21 (2), 347–358.

Da Silva, D.M., Pastrana, D.V., Schiller, J.T., Kast, W.M., 2001. Effect of preexisting neutralizing antibodies on the anti-tumor immune response induced by chimeric human papillomavirus virus-like particle vaccines. Virology 290 (2), 350–360.

Da Silva, D.M., Schiller, J.T., Kast, W.M., 2003. Heterologous boosting increases immunogenicity of chimeric papillomavirus virus-like particle vaccines. Vaccine 21 (23), 3219–3227.

Dalsgaard, K., Uttenthal, A., Jones, T.D., Xu, F., Merryweather, A., Hamilton, W.D., Langeveld, J.P., Boshuizen, R.S., Kamstrup, S., Lomonossoff, G.P., Porta, C., Vela, C., Casal, J.I., Meloen, R.H., Rodgers, P.B., 1997. Plant-derived vaccine protects target animals against a viral disease. Nat. Biotechnol. 15 (3), 248–252.

Di Martino, B., Marsilio, F., Roy, P., 2007. Assembly of feline calicivirus-like particle and its immunogenicity. Vet. Microbiol. 120 (1–2), 173–178.

Dyer, M., Renner, W., Bachmann, M., 2006. A second vaccine revolution for the new epidemics of the 21st century. Drug Discov. Today 11 (21–22), 1028–1033.

Earl, P. L., Cooper, N., Wyatt, L. S., Moss, B., and Carroll, M. W. (2001). Preparation of cell cultures and vaccinia virus stocks. Curr Protoc Protein Sci Chapter 5, Unit5 12.

El Mehdaoui, S., Touze, A., Laurent, S., Sizaret, P.Y., Rasschaert, D., Coursaget, P., 2000. Gene transfer using recombinant rabbit hemorrhagic disease virus capsids with genetically modified DNA encapsidation capacity by addition of packaging sequences from the L1 or L2 protein of human papillomavirus type 16. J. Virol. 74 (22), 10332–10340.

Fenner, F., Fantini, B., O. CABI Publishing, England., 1999. Biological control of vertebrate pests. The history of myxomatosis, an experiment in evolution. The Use of Rabbit Haemorrhagic Disease Virus for Rabbit Control. Oxford, England, pp. 236–272.

Fernandez-Fernandez, M.R., Mourino, M., Rivera, J., Rodriguez, F., Plana-Duran, J., Garcia, J.A., 2001. Protection of rabbits against rabbit hemorrhagic disease virus by immunization with the VP60 protein expressed in plants with a potyvirus-based vector. Virology 280 (2), 283–291.

Gamvrellis, A., Leong, D., Hanley, J.C., Xiang, S.D., Mottram, P., Plebanski, M., 2004. Vaccines that facilitate antigen entry into dendritic cells. Immunol. Cell. Biol. 82 (5), 506–516.

Green, K.Y., 2006. Fields virology, In: Knipe, A.P.M.H.D.M. (Ed.), 5th ed. *Caliciviridae*: The Noroviruses, Vol. 1. Lippincott and Wilkins, Philadelphia, pp. 49–979.

Green, K.Y., Lew, J.F., Jiang, X., Kapikian, A.Z., Estes, M.K., 1993. Comparison of the reactivities of baculovirus-expressed recombinant Norwalk virus capsid antigen with those of the native Norwalk virus antigen in serologic assays and some epidemiologic observations. J. Clin. Microbiol. 31 (8), 2185–2191.

Guerrero, R.A., Ball, J.M., Krater, S.S., Pacheco, S.E., Clements, J.D., Estes, M.K., 2001. Recombinant Norwalk virus-like particles administered intranasally to mice induce systemic and mucosal (fecal and vaginal) immune responses. J. Virol. 75 (20), 9713–9722.

Guo, M., Qian, Y., Chang, K.O., Saif, L.J., 2001. Expression and self-assembly in baculovirus of porcine enteric calicivirus capsids into virus-like particles and their use in an enzyme-linked immunosorbent assay for antibody detection in swine. J. Clin. Microbiol. 39 (4), 1487–1493.

Han, M.G., Cheetham, S., Azevedo, M., Thomas, C., Saif, L.J., 2006. Immune responses to bovine norovirus-like particles with various adjuvants and analysis of protection in gnotobiotic calves. Vaccine 24 (3), 317–326.

Heath, W.R., Carbone, F.R., 2001. Cross-presentation, dendritic cells, tolerance and immunity. Annu. Rev. Immunol. 19, 47–64.

Hervas-Stubbs, S., Rueda, P., Lopez, L., Leclerc, C., 2007. Insect baculoviruses strongly potentiate adaptive immune responses by inducing type I IFN. J. Immunol. 178 (4), 2361–2369.

Jennings, G.T., Bachmann, M.F., 2007. Designing recombinant vaccines with viral properties: a rational approach to more effective vaccines. Curr. Mol. Med. 7 (2), 143–155.

Jennings, G.T., Bachmann, M.F., 2008. The coming of age of virus-like particle vaccines. Biol. Chem. 389 (5), 521–536.

Jiang, X., Wang, M., Graham, D.Y., Estes, M.K., 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J. Virol. 66 (11), 6527–6532.

Jiang, X., Matson, D.O., Ruiz-Palacios, G.M., Hu, J., Treanor, J., Pickering, L.K., 1995. Expression, self-assembly, and antigenicity of a snow mountain agent-like calicivirus capsid protein. J. Clin. Microbiol. 33 (6), 1452–1455.

Jiang, X., Zhong, W., Kaplan, M., Pickering, L.K., Matson, D.O., 1999. Expression and characterization of Sapporo-like human calicivirus capsid proteins in baculovirus. J. Virol. Methods 78 (1–2), 81–91.

Kagi, D., Hengartner, H., 1996. Different roles for cytotoxic T cells in the control of infections with cytopathic versus noncytopathic viruses. Curr. Opin. Immunol. 8 (4), 472–477.

Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K.J., Podack, E.R., Zinkernagel, R.M., Hengartner, H., 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature 369 (6475), 31–37.

Karttunen, J., Sanderson, S., Shastri, N., 1992. Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. Proc. Natl. Acad. Sci. U. S. A. 89 (13), 6020–6024.

King, L.A., Possee, R.D., 1992. The Baculovirus Expression System. A Laboratory Manual. Chapman and Hall, London.

Kitts, P.A., Possee, R.D., 1993. A method for producing recombinant baculovirus expression vectors at high frequency. Biotechniques 14 (5), 810–817.

Langeveld, J.P., Brennan, F.R., Martinez-Torrecuadrada, J.L., Jones, T.D., Boshuizen, R.S., Vela, C., Casal, J.I., Kamstrup, S., Dalsgaard, K., Meloen, R.H., Bendig, M.M., Hamilton, W.D., 2001. Inactivated recombinant plant virus protects dogs from a lethal challenge with canine parvovirus. Vaccine 19 (27), 3661–3670.

Laurent, S., Vautherot, J.F., Madelaine, M.F., Le Gall, G., Rasschaert, D., 1994. Recombinant rabbit hemorrhagic disease virus capsid protein expressed in baculovirus self-assembles into viruslike particles and induces protection. J. Virol. 68 (10), 6794–6798.

Le Bon, A., Etchart, N., Rossmann, C., Ashton, M., Hou, S., Gewert, D., Borrow, P., Tough, D.F., 2003. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. Nat. Immunol. 4 (10), 1009–1015.

Le Bon, A., Montoya, M., Edwards, M.J., Thompson, C., Burke, S.A., Ashton, M., Lo, D., Tough, D.F., Borrow, P., 2006. A role for the transcription factor RelB in IFN-alpha production and in IFN-alpha-stimulated cross-priming. Eur. J. Immunol. 36 (8), 2085–2093.

Lochridge, V.P., Jutila, K.L., Graff, J.W., Hardy, M.E., 2005. Epitopes in the P2 domain of norovirus VP1 recognized by monoclonal antibodies that block cell interactions. J. Gen. Virol. 86 (Pt 10), 2799–2806.

Ludwig, C., Wagner, R., 2007. Virus-like particles-universal molecular toolboxes. Curr. Opin. Biotechnol. 2007 (6), 537–545.

Lutz, M.B., Kukutsch, N., Ogilvie, A.L., Rossner, S., Koch, F., Romani, N., Schuler, G., 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J. Immunol. Methods 223 (1), 77–92.

Meyers, G., Wirblich, C., Thiel, H.J., Thumfart, J.O., 2000. Rabbit hemorrhagic disease virus: genome organization and polyprotein processing of a calicivirus studied after transient expression of cDNA constructs. Virology 276 (2), 349–363.

Montoya, M., Schiavoni, G., Mattei, F., Gresser, I., Belardelli, F., Borrow, P., Tough, D.F., 2002. Type I interferons produced by dendritic cells promote their phenotypic and functional activation. Blood 99 (9), 3263–3271.

Moron, V.G., Rueda, P., Sedlik, C., Leclerc, C., 2003. In vivo, dendritic cells can cross-present virus-like particles using an endosome-to-cytosol pathway. J. Immunol. 171 (5), 2242–2250.

Nagesha, H.S., Wang, L.F., Hyatt, A.D., 1999. Virus-like particles of calicivirus as epitope carriers. Arch. Virol. 144 (12), 2429–2439.

Neirynck, S., Deroo, T., Saelens, X., Vanlandschoot, P., Jou, W.M., Fiers, W., 1999. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat. Med. 5 (10), 1157–1163.

Netter, H.J., Macnaughton, T.B., Woo, W.P., Tindle, R., Gowans, E.J., 2001. Antigenicity and immunogenicity of novel chimeric hepatitis B surface antigen particles with exposed hepatitis C virus epitopes. J. Virol. 75 (5), 2130–2141.

Nicollier-Jamot, B., Ogier, A., Piroth, L., Pothier, P., Kohli, E., 2004. Recombinant virus-like particles of a norovirus (genogroup II strain) administered intranasally and orally with mucosal adjuvants LT and LT(R192G) in BALB/c mice induce specific humoral and cellular Th1/Th2-like immune responses. Vaccine 22 (9–10), 1079–1086.

Peacey, M., Wilson, S., Baird, M.A., Ward, V.K., 2007. Versatile RHDV virus-like particles: incorporation of antigens by genetic modification and chemical conjugation. Biotechnol. Bioeng. 98 (5), 968–977.

Peacey, M., Wilson, S., Perret, R., Ronchese, F., Ward, V.K., Young, V., Young, S.L., Baird, M.A., 2008. Virus-like particles from rabbit hemorrhagic disease virus can induce an anti-tumor response. Vaccine 26 (42), 5334–5337.

Perez-Filgueira, D.M., Resino-Talavan, P., Cubillos, C., Angulo, I., Barderas, M.G., Barcena, J., Escribano, J.M., 2007. Development of a low-cost, insect larvae-derived recombinant subunit vaccine against RHDV. Virology 364 (2), 422–430.

Periwal, S.B., Kourie, K.R., Ramachandaran, N., Blakeney, S.J., DeBruin, S., Zhu, D., Zamb, T.J., Smith, L., Udem, S., Eldridge, J.H., Shroff, K.E., Reilly, P.A., 2003. A modified cholera holotoxin CT-E29H enhances systemic and mucosal immune responses to recombinant Norwalk virus-virus like particle vaccine. Vaccine 21 (5–6), 376–385.

Plana-Duran, J., Bastons, M., Rodriguez, M.J., Climent, I., Cortes, E., Vela, C., Casal, I., 1996. Oral immunization of rabbits with VP60 particles confers protection against rabbit hemorrhagic disease. Arch. Virol. 141 (8), 1423–1436.

Prasad, B.V., Matson, D.O., Smith, A.W., 1994a. Three-dimensional structure of calicivirus. J. Mol. Biol. 240 (3), 256–264.

Prasad, B.V., Rothnagel, R., Jiang, X., Estes, M.K., 1994b. Three-dimensional structure of baculovirus-expressed Norwalk virus capsids. J. Virol. 68 (8), 5117–5125.

Prasad, B.V., Hardy, M.E., Dokland, T., Bella, J., Rossmann, M.G., Estes, M.K., 1999. X-ray crystallographic structure of the Norwalk virus capsid. Science 286 (5438), 287–290.

Ramqvist, T., Andreasson, K., Dalianis, T., 2007. Vaccination, immune and gene therapy based on virus-like particles against viral infections and cancer. Expert. Opin. Biol. Ther. 7 (7), 997–1007.

Ramshaw, I.A., Ramsay, A.J., Karupiah, G., Rolph, M.S., Mahalingam, S., Ruby, J.C., 1997. Cytokines and immunity to viral infections. Immunol. Rev. 159, 119–135.

Restifo, N.P., Bacik, I., Irvine, K.R., Yewdell, J.W., McCabe, B.J., Anderson, R.W., Eisenlohr, L.C., Rosenberg, S.A., Bennink, J.R., 1995. Antigen processing in vivo and the elicitation of primary CTL responses. J. Immunol. 154 (9), 4414–4422.

Rock, K.L., 1996. A new foreign policy: MHC class I molecules monitor the outside world. Immunol. Today 17 (3), 131–137.

Rotzschke, O., Falk, K., Stevanovic, S., Jung, G., Walden, P., Rammensee, H.G., 1991. Exact prediction of a natural T cell epitope. Eur. J. Immunol. 21 (11), 2891–2894.

Rueda, P., Martinez-Torrecuadrada, J.L., Sarraseca, J., Sedlik, C., del Barrio, M., Hurtado, A., Leclerc, C., Casal, J.I., 1999. Engineering parvovirus-like particles for the induction of B-cell, CD4(+) and CTL responses. Vaccine 18 (3–4), 325–332.

Rueda, P., Moron, G., Sarraseca, J., Leclerc, C., Casal, J.I., 2004. Influence of flanking sequences on presentation efficiency of a CD8+ cytotoxic T-cell epitope delivered by parvovirus-like particles. J. Gen. Virol. 85 (Pt 3), 563–572.

Ruedl, C., Storni, T., Lechner, F., Bachi, T., Bachmann, M.F., 2002. Cross-presentation of virus-like particles by skin-derived CD8(−) dendritic cells: a dispensable role for TAP. Eur. J. Immunol. 32 (3), 818–825.

Sedlik, C., Sarraseca, J., Rueda, P., Leclerc, C., Casal, I., 1995. Immunogenicity of poliovirus B and T cell epitopes presented by hybrid porcine parvovirus particles. J. Gen. Virol. 76 (Pt 9), 2361–2368.

Sedlik, C., Saron, M., Sarraseca, J., Casal, I., Leclerc, C., 1997. Recombinant parvovirus-like particles as an antigen carrier: a novel nonreplicative exogenous antigen to elicit protective antiviral cytotoxic T cells. Proc. Natl. Acad. Sci. U. S. A. 94 (14), 7503–7508.

Sedlik, C., Dridi, A., Deriaud, E., Saron, M.F., Rueda, P., Sarraseca, J., Casal, J.I., Leclerc, C., 1999. Intranasal delivery of recombinant parvovirus-like particles elicits cytotoxic T-cell and neutralizing antibody responses. J. Virol. 73 (4), 2739–2744.

Sedlik, C., Dadaglio, G., Saron, M.F., Deriaud, E., Rojas, M., Casal, S.I., Leclerc, C., 2000. In vivo induction of a high-avidity, high-frequency cytotoxic T-lymphocyte response is associated with antiviral protective immunity. J. Virol. 74 (13), 5769–5775.

Souza, M., Costantini, V., Azevedo, M.S., Saif, L.J., 2007. A human norovirus-like particle vaccine adjuvanted with ISCOM or mLT induces cytokine and antibody responses and protection to the homologous GII.4 human norovirus in a gnotobiotic pig disease model. Vaccine 25 (50), 8448–8459.

Spohn, G., Bachmann, M.F., 2008. Exploiting viral properties for the rational design of modern vaccines. Expert. Rev. Vaccines. 7 (1), 43–54.

Tacket, C.O., Sztein, M.B., Losonsky, G.A., Wasserman, S.S., Estes, M.K., 2003. Humoral, mucosal, and cellular immune responses to oral Norwalk virus-like particles in volunteers. Clin. Immunol. 108 (3), 241–247.

Tan, M., Meller, J., Jiang, X., 2006. C-terminal arginine cluster is essential for receptor binding of norovirus capsid protein. J. Virol. 80 (15), 7322–7331.

Thumfart, J.O., Meyers, G., 2002. Rabbit hemorrhagic disease virus: identification of a cleavage site in the viral polyprotein that is not processed by the known calicivirus protease. Virology 304 (2), 352–363.

Topham, D.J., Tripp, R.A., Doherty, P.C., 1997. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. J. Immunol. 159 (11), 5197–5200.

Varsani, A., Williamson, A.L., de Villiers, D., Becker, I., Christensen, N.D., Rybicki, E.P., 2003. Chimeric human papillomavirus type 16 (HPV-16) L1 particles presenting the common neutralizing epitope for the L2 minor capsid protein of HPV-6 and HPV-16. J. Virol. 77 (15), 8386–8393.

Woo, W.P., Doan, T., Herd, K.A., Netter, H.J., Tindle, R.W., 2006. Hepatitis B surface antigen vector delivers protective cytotoxic T-lymphocyte responses to disease-relevant foreign epitopes. J. Virol. 80 (8), 3975–3984.

Xia, M., Farkas, T., Jiang, X., 2007. Norovirus capsid protein expressed in yeast forms virus-like particles and stimulates systemic and mucosal immunity in mice following an oral administration of raw yeast extracts. J. Med. Virol. 79 (1), 74–83.

Yang, R., Murillo, F.M., Cui, H., Blosser, R., Uematsu, S., Takeda, K., Akira, S., Viscidi, R.P., Roden, R.B., 2004. Papillomavirus-like particles stimulate murine bone marrow-derived dendritic cells to produce alpha interferon and Th1 immune responses via MyD88. J. Virol. 78 (20), 11152–11160.