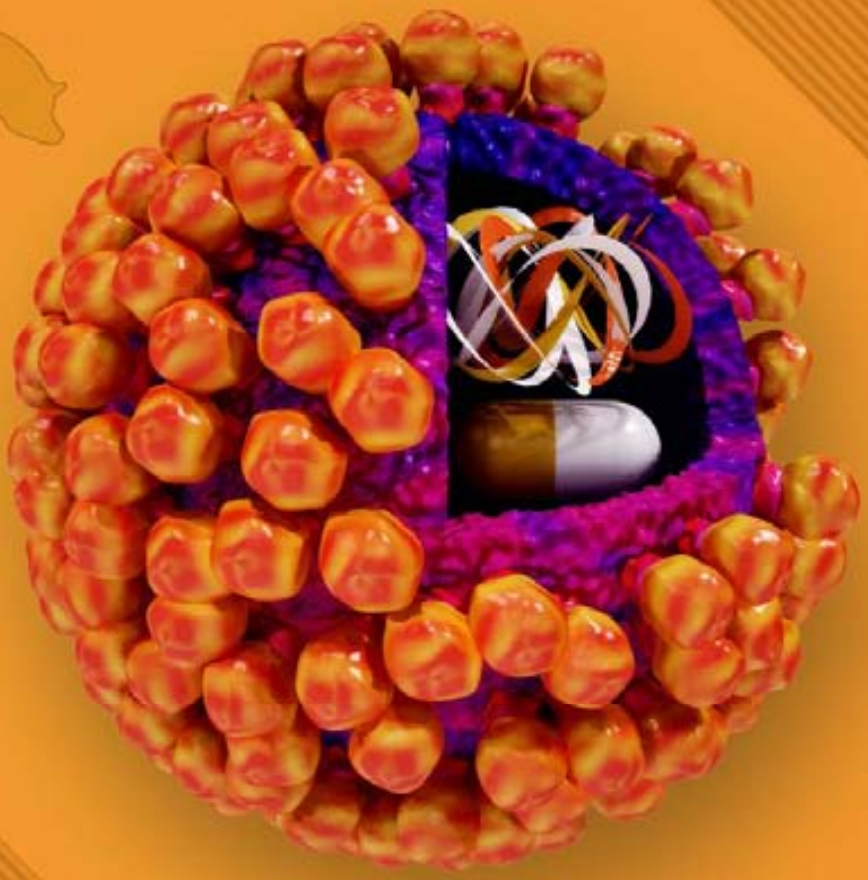
Immunogenic Properties of Calicivirus-Like Particles as Vaccine Vectors



Elisa Crisci

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IMMUNOGENIC PROPERTIES OF CALICIVIRUS-LIKE PARTICLES AS VACCINE VECTORS

Elisa Crisci

Directora: Maria Montoya González
Tutor: Joaquim Segalés i Coma

Dept. de Sanitat i Anatomia Animals
Facultat de Medicina Veterinària
Universitat Autònoma de Barcelona

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La Dra. Maria Montoya González, investigadora del Centre de Recerca en Sanitat Animal (CReSA) y el Dr. Joaquim Segalés i Coma, professor titular del Dept. de Sanitat i Anatomia Animals de la Universitat Autónoma de Barcelona,

CERTIFICAN:

Que la tesis titulada: "IMMUNOGENIC PROPERTIES OF CALICIVIRUS-LIKE PARTICLES AS VACCINE VECTORS" ha sido realizada por Elisa Crisci bajo su dirección, en el Centre de Recerca en Sanitat Animal (CReSA), y que es apta para ser presentada y optar al grado de Doctor en Medicina y Sanidad Animal por la Universitat Autònoma de Barcelona.

Y para que quede constancia firman la presente certificación a Bellaterra, 30 de Septiembre 2011.

Directora
Dra. Maria Montoya González

Tutor
Dr. Joaquim Segalés i Coma

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CONTENTS

ABBREVIATIONS

A

Ab: Antibody

Ag: Antigen

APCs: Antigen Presenting Cells

B

BV: Baculovirus

BM: Bone Marrow

BTV: Bluetongue Virus

C

CAV: Chicken Anaemia Virus

cDCs: Conventional Dendritic Cells

CD: Cluster of Differentiation

CPMV: Cowpea Mosaic Virus

CPV: Canine Parvovirus

CTL: Cytotoxic T Lymphocyte

CRP: C-Reactive Protein

D

DAPI: 4', 6-Diamidino-2-Phenylindole

DCs: Dendritic Cells

DIVA: Differentiating Infected from Vaccinated Animals

DNA: Deoxyribonucleic Acid

DOX: Doxorubicin

DPV: Muscovy Duck Parvovirus

E

ELISA: Enzyme-Linked ImmunoSorbent Assay

ELISPOT: Enzime-Linked InmunoSorbent SPOT

EMCV: Encephalomyocarditis Virus

ER: Endoplasmic Reticulum

ERAV: Equine Rhinitis A Virus

F

FCV: Feline Calicivirus

FITC: Fluorescein Isothiocyanate

FMDV: Foot and Mouth Disease Virus

FLU: Influenza Virus

G

GPV: Goose Parvovirus

H

HBcAg: Hepatitis B Virus core Antigen

HBV: Hepatitis B Virus

HP: Haptoglobin

HPV: Human Papillomavirus

I

IBDV: Infectious Bursal Disease Virus

ICCS: Intracellular Cytokine Staining

IFN-α: Interferon alpha

IFN-γ: Interferon gamma

IHC: Immunohistochemistry

IL: Interleukin

L

LCMV: Lymphocytic Choriomeningitis Virus

M

mAb: Monoclonal Antibody

MDCK cells: Madin-Darby Canine Kidney cells

MEV: Mink Enteritis Virus

MHC: Major Histocompatibility Complex

N

NDV: Newcastle Disease Virus

NNV: Nervous Necrosis Virus

NP: Nucleoprotein

NV: Norwalk Virus

O

OVA: Ovalbumin

P

PAMPs: Pathogen-Associated Molecular Patterns

PBMCs: Peripheral Blood Mononuclear Cells

PCV2: Porcine Circovirus type 2

pDCs: Plasmacytoid Dendritic Cells

PE: Phycoerythrin

PoBMDCs: Porcine Bone Marrow derived Dendritic Cells

PPV: Porcine Parvovirus

PRM: Pattern Recognition Molecules

PRRs: Pattern Recognition Receptors

PRRSV: Porcine Reproductive and Respiratory Syndrome Virus

PTX3: Long Pentraxin 3

R

RNA: Ribonucleic Acid

RHDV: Rabbit Haemorrhagic Disease Virus

RSV: Respiratory Syncytial Virus

S

SLA: Swine Leucocyte Antigens

T

TAP: Transporter associated with Antigen Presentation

Th1: T cell helper response type 1

TMV: Tobacco Mosaic Virus

TNF: Tumour Necrosis Factor

TRITC: Tetramethyl Rhodamine Isothiocyanate

V

VLPs: Virus-like Particles

VV: Vaccinia Virus

ABSTRACT

New subunit vaccines are getting a foothold in veterinary vaccinology and virus-like particles (VLPs) are one of the most appealing approaches opening up frontiers in animal vaccines. VLPs are robust protein cages in the nanometer range exhibiting well-defined geometry and remarkable uniformity that mimic the overall structure of the native virions. VLPs have an important advantage in terms of safety; indeed, lacking the genome of the virus avoid any of the risks associated with virus replication, reversion, recombination or re-assortment. Rabbit haemorrhagic disease virus (RHDV) capsid protein is able to form RHDV-VLPs and these particles showed a strong immunogenicity and protected the natural host after a lethal challenge. Additionally, previous studies described the possibility to use RHDV-VLPs as platform for the insertion of foreign epitopes or for DNA packaging. Nowadays, one study has shown the possibility to use RHDV-VLPs as carrier for improving cancer immunotherapies but no studies have investigated the possibility to use RHDV-VLPs as vaccine vectors carrying epitopes corresponding to viral animal diseases.

This thesis is aimed to study the potential immunogenicity of RHDV-VLPs as epitope carriers for viral disease in different animal models. In the first two studies, the immunogenicity of chimeric RHDV-VLPs was investigated in a murine system *in vitro* and *in vivo*. Results from these studies demonstrated that the inserted epitope was processed and presented in an MHC-I context by dendritic cells (DCs) and that the different sites of insertion of the epitope influenced the immunogenicity of the VLPs. Chimeric RHDV-VLPs were able to protect mice from a viral challenge. Also, the route of antigen delivery influenced the immunogenicity of the particles. The third study confirmed the initial results but this time in *in vitro* experiments using porcine cells. Lastly, chimeric RHDV-VLPs were studied as immunogens in pigs. The results showed that the delivery route and adjuvant influenced immune responses after chimeric RHDV-VLP immunization and more importantly that pigs exhibited very good cellular and humoral immune responses against not only RHDV-VLPs but also against the antigenic epitope. Further studies have to be performed to prove protection in pigs. In conclusion, in this thesis we demonstrated the potential of RHDV-VLPs as immunogens in two different animal systems.

RESUMEN

Las nuevas vacunas de subunidades están abriéndose paso dentro de la vacunología veterinaria y entre ellas, las pseudopartículas virales o VLPs (por su nombre en inglés "*virus-like particles*") son una de las estrategias más atractivas que están abriendo nuevas fronteras en la vacunación de animales. Las VLPs son estructuras proteicas rígidas con un tamaño dentro del rango de los nanómetros, que presentan una geometría muy bien definida y una espectacular uniformidad que mimetiza la estructura de los virus nativos de los que proceden. Las VLPs tienen importantes ventajas en relación a la seguridad por el hecho de carecer de genoma viral, que elimina cualquier riesgo asociado a la replicación viral, reversión, recombinación o reorganizamiento genómico. La proteína de la cápside del virus de la fiebre hemorrágica del conejo (por su nombre en inglés "*Rabbit haemorrhagic disease virus*" o RHDV) es capaz de formar RHDV-VLPs y estas partículas han demostrado poseer una fuerte inmunogenicidad, protegiendo al hospedador natural tras un desafío mortal. Además, estudios anteriores apuntaron la posibilidad de usar RHDV-VLPs como vector para mejorar la inmunoterapia contra el cáncer. Sin embargo, no hay estudios que hayan investigado la posibilidad de usar las RHDV-VLPs como vector vacunal con epitopos de enfermedades virales de animales.

El objetivo de esta tesis es estudiar el potencial inmunogénico de las RHDV-VLPs como vectores vacunales de enfermedades virales en diferentes animales. En los dos primeros estudios, se investigó la inmunogenicidad de las RHDV-VLPs en el modelo murino, tanto *in vitro* como *in vivo*. Los resultados de estos estudios demostraron que el epítopo insertado era procesado y presentado mediante MHC-I por células dendríticas y que la inmunogenicidad dependía de los diferentes sitios de inserción. Las RHDV-VLPs quiméricas fueron capaces de proteger a los ratones frente a un desafío viral. También, la respuesta se vio alterada según la ruta de administración del antígeno. El tercer estudio confirmó los resultados en ratón, pero esta vez en experimentos *in vitro* con células de cerdo. Por último, se estudió inmunogenicidad de las RHDV-VLPs quiméricas en cerdo. Los resultados mostraron que la ruta de administración y el adjuvante determinaron la respuesta inmune después de la inmunización con las RHDV-VLPs quiméricas y que los animales presentaban muy buena respuesta inmune celular y humoral, no solo frente a RHDV-VLPs sino frente al epítopo antigénico. Estudios

posteriores se tendrán que abordar para demostrar la protección de los cerdos. En conclusión, en esta tesis se demuestra el potencial de las RHDV-VLPs como inmunógenos en dos sistemas animales diferentes.

INTRODUCTION

Virus-like particles

Vaccination is considered one of the most effective ways to control pathogens and prevent diseases in human and in the veterinary field. Several efforts to develop a protective and lasting immunity, allowing the formation of memory cells have been done and different types of vaccines were set up in the last decades. Traditional vaccines against viral diseases are based on inactivated or attenuated viruses, but new subunit vaccines are getting a foothold in vaccinology. Among these subunit vaccines, virus-like particles (VLPs) are one of the most appealing approaches, as far as Jennings and Bachmann defined the last era as "the coming age of virus-like particle vaccines" (1). The statement underlines the recent interest in these new vaccines, due to their intrinsic properties and safety, remarked also by several reviews appeared in the last ten years (1-11). Although different reviews on VLPs were published, none of these have fully described the VLP-based vaccines in veterinary. Thus, the first part of this introduction will provide an outline of VLPs development as vaccine candidates and the immunological implications in the veterinary field. The second part will focus on rabbit haemorrhagic disease virus and its ability to form VLPs.

Why VLPs?

In comparison with conventional vaccines, VLPs incorporate key features that underlay their immunogenicity, safety and vaccine potential (**Fig. 1**): 1) well-defined geometry and remarkable uniformity with repetitive and ordered surface structures, 2) particulate and multivalent nature, 3) preservation of native antigenic conformation, 4) safety for being non-infectious and non replicating candidates, 5) higher stability than soluble antigens in extreme environmental conditions, 6) applicability as carriers of foreign epitopes or drugs and 7) possibility to follow the DIVA (Differentiating Infected from Vaccinated Animals)-compliance concerns. These general features will be discussed in more detail in the following sections.

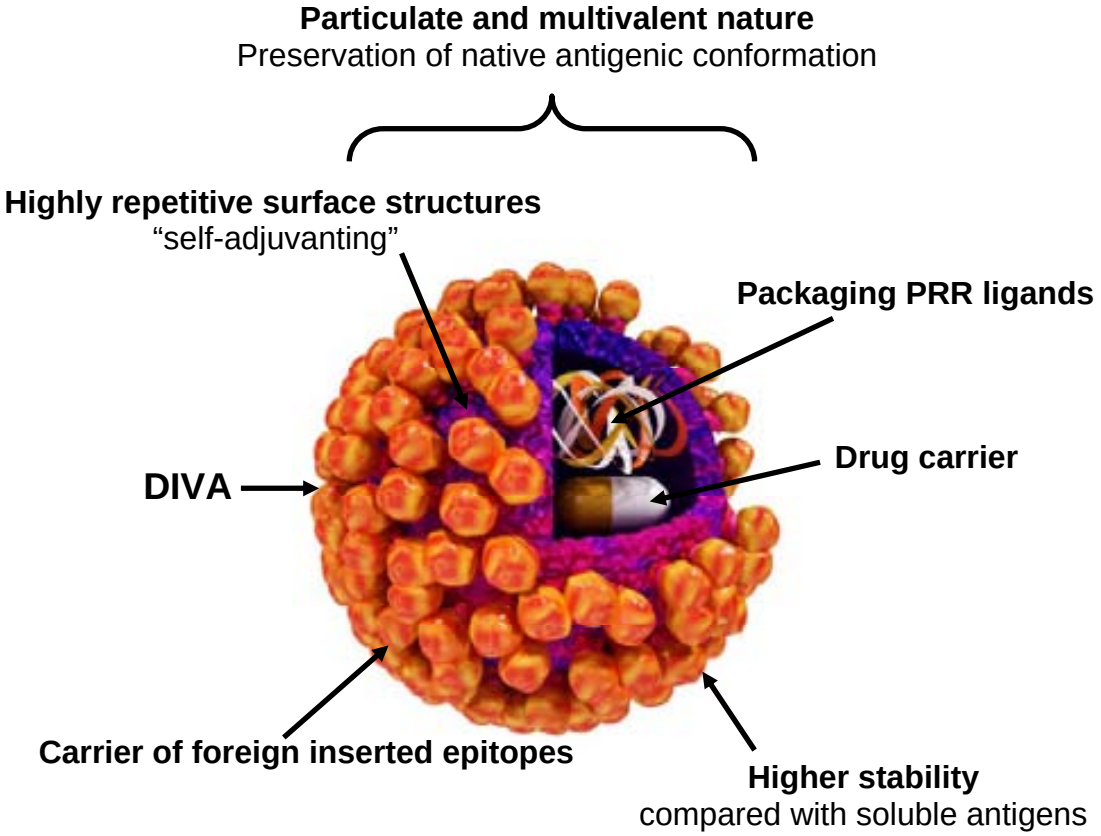


Fig. 1. Immunogenic features of a VLP presenting foreign antigens. VLPs incorporate key features that underlay their immunogenicity, safety and vaccine potential: 1) well-defined geometry and remarkable uniformity with repetitive and ordered surface structures; the multivalent display and highly ordered structure of VLPs constitute PAMPs motifs common to many pathogens but not to the host, that trigger innate immune sensing mechanism. PAMPs can be recognized by TLRs and other PRRs on the surface of the host cells; 2) particulate and multivalent nature; this feature means that VLPs are efficiently taken up by APCs. Their tendency to be a suitable size for uptake by DCs for processing and presentation by MHC-II and MHC-I (cross-presentation) pathways led to describe VLPs as "self-adjuvanting"; 3) preservation of native antigenic conformation; 4) safety for being non-infectious and non replicating candidates; VLPs lack the DNA or RNA genome of the virus altogether eliminate any of the risks associated with virus replication, reversion, recombination or re-assortment; 5) higher stability than soluble antigens in extreme environmental conditions; 6) applicability as carriers of foreign epitopes or drugs; 7) possibility to follow the DIVA (Differentiating Infected from Vaccinated Animals)-compliance concerns. *(Figure created by Carla Martínez Castro and Elisa Crisci).*

Firstly, subunit vaccines based on recombinant proteins can suffer from poor immunogenicity owing to incorrect folding of the target protein or poor presentation to the immune system; moreover, they are less effective when expressed and purified in the absence of the other viral components. Also, to promote efficient induction of immune responses the addition of adjuvants may be required (11). However, virus-like

particles are supra-molecular assemblages with well-defined geometry, usually icosahedrons or rod-like structures with diameters in the range of 25-100 nm (12) that mimic the overall structure of the native virions. These protein cages are based on the natural intrinsic ability of many types of viral coat subunits, often the major protein in the capsid or envelop, that spontaneously self assemble into VLPs (13). They are composed of multiple copies of one or more viral proteins and are often antigenically indistinguishable from the virus from which they were derived (1).

Secondly, the multivalent display and highly ordered structure of VLPs constitute pathogen-associated molecular patterns (PAMPs) motifs common to many pathogens but not to the host, that trigger innate immune sensing mechanisms (3). PAMPs can be recognized by Toll-like receptors (TLRs) and other pattern-recognition receptors (PRRs) on the surface of the host cells. Moreover, PAMPs stimulate antigen uptake by antigen presenting cells (APCs) and the subsequent presentation of antigens to cells of the adaptive immune response. Beyond this property, the particulate nature and dimensions of VLPs means that they are efficiently taken up by APCs, in particular dendritic cells (DCs). Uptake of antigens by DCs depends upon different properties, such a size, shape, surface charge etc., but the antigen size is a key factor. APCs of the immune system are able to uptake antigens with pathogen-like dimensions (20 nm-3 μm) and VLPs have dimensions that fit within this range. Particulate antigens have large surfaces that have charged, hydrophobic or receptor-interacting properties and this leads to a better interaction of APCs with particles than with soluble proteins (5). Besides, it has been demonstrated that DCs optimally uptake antigens with diameters of approximately 40 nm (14). Their tendency to be a suitable size for uptake by DCs for processing and presentation by MHC-II and MHC-I (cross-presentation) pathways led to describe VLPs as “self-adjuvanting” (10). Thanks to this ability, VLPs stimulate strong B-cell-mediated immune responses and are highly effective at stimulating $CD4^+$ T cell proliferative responses and cytotoxic T lymphocyte (CTL) responses (15-17).

Besides, VLPs combine the properties of whole virus vaccines and recombinant subunit vaccine into one system and have advantages in terms of safety. Indeed, the fact that VLPs lack the DNA or RNA genome of the virus altogether eliminate any of the risks associated with virus replication, reversion, recombination or re-assortment. They can be prepared independent to the culture of replicating virus, hence, the safety issues of

virus vaccines relating to reversion mutants and immunocompromised individuals are obviated (1).

VLPs contain many authentic antigenic sites and since no inactivation of VLPs is required, these empty shells are still capable of entering target cells; important epitopes are maintained and new ones are probably not generated (3). Importantly, in contrast to many virus vaccines, VLPs may be formed without viral proteins that down-regulate host immune responses (18).

Additionally, VLPs have been produced for a wide range of taxonomically and structurally distinct viruses, more than thirty from non-enveloped and enveloped viruses that infect humans and other animals are reviewed in (3, 8, 11). Clearly, not all of the viruses were used as vaccine targets and some VLPs have also been generated as a means of understanding the assembly or architecture of viruses (11).

In addition to being effective vaccines against the corresponding virus from which they are derived, VLPs can also be used as carrier molecules to present foreign chosen epitopes, DNA, small molecules and drugs to the immune system. Different strategies for nucleic acid inclusion, gene or drug delivery were developed, but in general, the typical approaches are genetic fusion or chemical conjugation. In particular, target epitopes may either be genetically fused into proteins of VLPs to form chimeras or attached to the surface of the VLPs by covalent or non-covalent binding.

This new technology has expanded their use in vaccination, from immunizing against pathogens to immunotherapy for chronic diseases. Indeed, VLPs have been used to induce auto-antibodies to disease-associated self-molecules involved in chronic diseases, such as hypertension and Alzheimer's disease (1).

The ability of VLPs to spontaneously assemble allows them to be disassembled and reassembled *in vitro*, a process which enables incorporating of a different range of molecules, including proteins, polypeptides, capsular polysaccharides and haptens. For example, stimulators of innate immunity, such as Toll-like receptor (TLR) ligands were packaged within VLPs. In this way the co-delivery of antigens and activators of innate immunity to DCs induces efficient T-cell responses (19), shaping the subsequent

adaptive immune response to be of appropriate magnitude, quality and specificity. Besides, VLPs may be combined with depot forming adjuvants to better simulate the immune responses.

Finally, one of the most interesting features of VLPs is the possibility to create DIVA vaccines to differentiate naturally infected animals from vaccinated animals. Even though this potential ability is well known in human research and has an important potential utility, mainly in the obligatory declared animal disease, the majority of the VLP-base vaccine candidates are not still commercialized. At present, the formal proof that DIVA vaccines are possible for all the VLP-base candidates remained unanswered under field conditions in the veterinary field.

VLPs as candidate vaccines for animals

The two most successful VLP-based vaccines that have been licensed and approved for use in humans are hepatitis B and human papillomavirus vaccines and progresses have been made in developing VLPs for hepatitis C virus, Ebola virus, Marburg virus, SARS coronavirus and Chikungunya virus (3).

Although various candidates are in course of study in the veterinary field (**Table 1**), only porcine circovirus type 2 (PCV2) VLP-based vaccine, Ingelvac CircoFLEX® developed by Boehringer Ingelheim (Germany) is licensed and commercially available.

PCV2, a member of the family *Circoviridae*, is associated with post-weaning multisystemic wasting syndrome, a swine disease characterized by wasting, weight loss, respiratory distress and diarrhoea that has a severe economic impact on the production (20).

The ORF 2 capsid protein was expressed in the baculovirus (BV) expression system and the protein was called purified circovirus antigen (PCA^{TM}). The PCV2-VLPs were adjuvanted with a well tolerated aqueous polymer (ImpranFLEX®) that has a slow release depot effect. This vaccine effectively induces a cellular immune response following a single injection and has good results in pig farms (21). For the same virus, another similar BV expressed sub-unit commercial vaccine, Porcilis PCV® (Intervet International, The Netherlands) with α-tocopherol+liquid paraffin-based adjuvant, is

licensed. It has shown to induce humoral and cell-mediated immunity and protection against porcine circovirus-associated disease under field conditions following one intramuscular dose (22); it is not described whether this vaccine forms VLPs.

Table 1. Virus like particles as candidate vaccines in the veterinary field.

See "Abbreviations". + indicate VLPs that protected the natural target host.

***Family*/Virus**	**Composition**	**Stage of development**	**Expression system**	**Reference**
Birnaviridae				
IBDV	VP2,VPX,PP	+ Animal studies	BV	(33)
Caliciviridae				
FCV	VP1	Animal studies	BV	(96)
RHDV	VP60	+ Animal studies	BV	(99)
Circoviridae				
CAV	VPs	+ Animal studies	BV	(30,31)
PCV2	ORF2 protein	Licensed	BV	Boehringer Ingelheim; Intervet International
Nodaviridae				
NNV	Coat protein	+ Animal studies	*E. Coli* BV	(55,56)
Orthomyxoviridae				
FLU	HA, NA, M1, M2	Clinical trials	Plants BV	Reviewed in (18,4,6,7) and (35)
Paramyxoviridae				
NDV	NP, M, F, HN	Animal studies	Avian and mammalian cells	(18,34)
Parvoviridae				
CPV	VP2	+ Animal studies	BV	(27,28)
MEV	VP2	+ Animal studies	BV	(26)
DPV	VPs	+ Animal studies	BV	(25)
GPV	VPs	Animal studies	BV	(24)
PPV	VP2	+ Animal studies	BV	(23)
Picornaviridae				
EMCV	P1, 2A, 3C	+ Animal studies	BV	(52 ,196)
ERAV	P12A, 3C	Animal studies	mammalian	(49)
FMDV	P12A, 3C	Animal studies	BV	(53)
Reoviridae				
BTV	VPs	+ Animal studies	BV	(46,47)
Rotavirus	VPs	Animal studies	BV	(43, 197-201)

Other swine viruses have been investigated as candidates for VLP-based vaccines and the first one studied was porcine parvovirus (PPV), a highly infectious virus causing reproductive failure in pigs. PPV-VLPs were tested in different animal models with a single intramuscular immunization coupled with different adjuvants. A microgram dose was highly immunogenic, very efficient in preventing trans-placental virus transmission and gilts were protected against PPV-induced reproductive failure (23). Besides, canine parvovirus (CPV), muscovy duck parvovirus (DPV), goose parvovirus (GPV) and mink enteritis virus (MEV) VLPs were also studied as vaccine candidates. In a recent preliminary study with geese, GPV-VLPs injected once subcutaneously in 50% mineral oil have shown higher titres of neutralizing antibody compared with inactivated and attenuated virus *in vitro* (24). Likewise, the previous study in ducks has also shown the production of specific DPV-antibodies after DPV-VLP immunization and neutralizing antibodies levels were consistent with those observed in ducklings inoculated with a commercial inactivated vaccine (25). Also, MEV-VLPs have shown to elicit higher antibody response after revaccination compared with registered vaccine; interestingly, minks were protected against viral challenge and did not excrete MEV in faeces (26). In addition, two other studies used recombinant CPV-VLPs in a prime-boost strategy with adjuvant. Both VLPs were able to elicit neutralizing antibodies sufficient to render all the dogs vaccinated immune to viral challenge (27, 28). Thus, *Parvoriridae* has been shown to be a suitable virus family for the production of VLPs probably due to its non enveloped icosahedral structure and appropriate dimension.

Poultry industry is also another veterinary area searching for safe, immunogenic, protective and less expensive vaccines; hence, economically important avian viruses were considered as potential subunit vaccines. Chicken anemia virus (CAV) belongs to the *Circoviridae* family and causes anaemia and immunodeficiency in newly hatched chickens, with important economic losses (29). CAV proteins, that Roy and Noab included in the baculovirus derived CAV-VLPs (8) were used for immunization in chickens. The formation of CAV-VLP-structure was not studied, but VP1 and VP2, acting as scaffold (30) were able to elicit neutralizing antibodies and the progeny from immunized chickens was protected against challenge by CAV directly after hatching (31).

Another important disease affecting chickens is caused by infectious bursal disease virus (IBDV), a *Birnaviridae* virus that induces immunosuppression by the destruction of immature B-lymphocytes within the bursa of Fabricius (32). Various IBDV-particles, derived from a polyprotein differentially processed, were tested in chicken using one dose. The results established that all the IBDV-VLPs were effective at inducing humoral responses, but not all have shown the same virus-neutralising capacity. They conferred protection to all the vaccinated chickens, as did the inactivated commercial vaccine, but no clear dose-effect was observed (33).

The most interesting poultry VLPs vaccine candidate was described by Morrison in a recently published manuscript (18). VLPs formed with structural proteins of Newcastle disease virus (NDV), an avian enveloped paramyxovirus causing respiratory and/or nervous disease, have demonstrated their effectiveness as an immunogen in a murine model, compared with responses stimulated by immunization with comparable amounts of an UV-inactivated vaccine strain. Levels of soluble antibodies, characterized by ELISA and by neutralizing antibody titres, resulting from NDV-VLP immunization were as high or higher that those resulting from immunization with the inactivated virus vaccine. Furthermore, NDV-VLPs stimulated T-cell responses at levels slightly higher than those stimulated by the vaccine virus (34). Another important finding was that NDV-VLPs can be used also to express peptide sequences from other target pathogens but this topic will be commented in the next section.

Within the enveloped viruses, influenza virus is in the foreground, since the disease is a zoonoses that remains one of the major threat to human health and involves a wide range of animal species, mainly avian and pigs. Influenza-VLPs (FLU-VLPs) are assembled in producer cells and released into the culture medium mimicking the viral budding process, which incorporates viral glycoproteins on their surface. These FLU-VLPs demonstrated protective immunity via either the intranasal or intramuscular route in the absence of adjuvants (6) and are exhaustively reviewed in (4, 6, 7). FLU-VLPs formed with the baculovirus system are now in clinical trials in humans (35) (NCT01072799, NCT01014806, NCT00903552 and NCT00519389) [September 2011. ClinicalTrials.gov. A service of the US NIH. http://clinicaltrials.gov/] [September 2011. Novavax. Research and development. Clinical trials. www.novavax.com/go.cfm?do=Page.View&pid=81] (18).

Other VLPs produced against important zoonoses are Rift valley fever (RVF) and rotavirus (RV) VLPs. RVF virus (RVFV) (*Bunyaviridae* family) is transmitted by several mosquito species and has a broad range of susceptible animal hosts (36). Interestingly, RVFV-VLPs produced in mammalian cells were able to elicit high titres of neutralizing antibodies and protect mice from a lethal challenge, suppressing virus replication (37). Also RV-VLPs expressing the main structural viral proteins has been assessed for their efficacy using different animal models such as mice (38), rabbits (39), gnotobiotic piglets (40) and cows (41). This virus is a *Reoviridae* virus widespread in the youngs of all mammalian species that cause severe dehydrating diarrhoea (42). By parenteral route RV-VLPs were proven to confer homologous protection in rabbits (39) and heterologous protection in mice (38). Moreover, homologous and heterologous RV-VLPs were immunogenic in mice and different levels of protective efficacy were achieved depending on the dose, route or co-administration with adjuvants (43).

Another VLP-based vaccine, also belonging to the same *Reoviridae* family, is the one generated with bluetongue virus-like particles (BTV-VLPs) derived from multiple baculovirus expression vectors and reviewed last year together with other BTV vaccines by (44). BT is a vector-borne disease of ruminants that causes haemorrhages and ulcers in the oral cavity and upper gastrointestinal tract (45). BTV-VLPs have been administered in the presence of various adjuvants to sheep, a vertebrate host susceptible to the virus. The results indicated that these multiprotein VLPs in conjunction with appropriate adjuvant elicited an immune response which protected against an infectious virus challenge (46). The combination of different outer capsid proteins elicited higher neutralizing-antibody titers as compared to VP2 alone (47) and since VLPs do not contain any non-structural protein it is possible to distinguish between vaccinated and infected animals (44).

Picornaviridae family shares a common replication strategy and self assembly of mature capsid proteins into VLPs. These properties have been shown for several picornaviruses, including equine rhinitis A virus (ERAV), foot and mouth disease virus (FMDV) and porcine encephalomyocarditis virus (EMCV). These VLPs were formed with similar proteins but using different expression systems: ERAV-VLPs were generated using a mammalian expression vector while the others were generated using the baculovirus system.

ERAV is a respiratory pathogen of horses that may present an acute febrile respiratory disease or subclinical infection (48). ERAV-VLPs were tested intramuscularly in mice with three injections of VLPs, following by boost with UV-inactivated virus. The VLP-immunized group showed higher titres of virus-neutralizing antibodies compared to the group without immunization and this enhanced response was consistent with the induction of a memory response to a neutralising epitope (49).

EMCV causes myocarditis in pre-weaned pigs and severe reproductive failure in sows (50, 51); EMCV-VLPs were used in the natural hosts once or twice with adjuvant from SEPPIC and the immunization elicited neutralizing antibody levels similar to those obtained with the commercial vaccine. In this study, a boost strategy was more effective than a single-dose immunization in inducing the production and maintenance of neutralizing antibodies (52).

Regarding FMDV-VLPs, derived from a virus causing an important animal disease affecting pigs, cattle and other cloven-hoofed livestock, guinea pigs were vaccinated twice with the particles and Montanide ISA 206 adjuvant (SEPPIC); both FMDV-specific antibodies and neutralizing antibodies were generated in VLP-vaccinated animals, but their levels were lower than those generated by the commercial vaccine. They exhibited good antigenicity and immunogenicity, however the conventional inactivated FMDV vaccine currently remains the most effective (53).

Viral fish diseases are also important in the veterinary context, since they are a serious problem in pisciculture and seafood market. Nervous necrosis virus (NNV), from *Nodaviridae* family, causes encephalopathy and retinopathy in many species of fishes (54). VLPs from this family, genus Betanovirus, were generated as vaccine candidates for different fish species. Two studies have shown that these VLPs were able to elicit neutralizing antibodies against NNV and the responses were dose dependent (55, 56). Additionally, Thiery *et al.* could demonstrate that vaccination with NNV-VLP was able to protect fish from a lethal challenge and to reduce virus spreading (55).

VLPs as vaccine vectors

As previously introduced, VLPs can also serve as a platform for multivalent heterologous epitope display to elicit an immune response against the protein or peptide that is attached through fusion or genetic insertion into the capsid proteins. They provide three surfaces (interior, exterior and subunit interface) for genetic and chemical modification with small molecules, peptides and nanoparticles at precise locations (57). Thus, VLPs are used both as a presentation scaffold for epitopes from another viral, bacterial or parasitic pathogen, and as an adjuvant to boost the immune response.

Exploration into attachment of self-peptides for the purpose of treating disease has also been attempted. The insertion often produces a heightened immune response due to the addition of a foreign protein and multivalent display that has the potential to break the tolerance. Various VLPs have been used to display self-antigens and are reviewed in (1, 3).

In recent years, there has been considerable interest in developing VLPs from animal viruses as effective drug delivery systems (57). Anticancer drug doxorubicin (DOX) was covalently conjugated to rotavirus-based VLPs (DVLPs) produced in *E. coli* protein expression system. DVLPs were further linked with lactobionic acid (LA), a cellular targeting ligand which contains galactose (DVLPLA), and intracellular uptake by different cells was examined. They demonstrated the release of DOX in the cells with different kinetics and the lower toxicity of this system compared with free DOX (57). Although good results have been obtained using this approach, VLPs-based systems for epitope presentation are limited by certain restrictions, mainly for the difficulty of adding larger epitopes and proteins to the scaffold that might impede VLP assembly or generate defective VLPs. In this section we will review different VLPs from viruses affecting animals used as vectors, but also epitopes from viruses affecting animals expressed in various scaffolds (summarized in **Table 2**).

Table 2. Virus like particles as vaccine vectors.

***Family*/virus**	**Composition**	**Target**	**Stage of development**	**Expression system**	**Reference**
Caliciviridae					
RHDV	OVA	tumour	Animal studies	BV	(113,114)
RHDV	HPV L1	gene transfer	*In vitro*	BV	(111)
Hepadnaviridae					
HBV	VP1 on HBcAg	FMDV	Animal studies	VV mammalian yeast	(58-60)
HBV	LCMVon HBcAg	LCMV	+ Animal studies	*E. Coli*	(61,62)
Paramyxoviridae					
NDV	NP, M, F, HN	Nipah virus G, FLU, RSV	Animal studies	Avian and mammalian cells	(18,70)
Parvoviridae					
PPV	NP	LCMV	+ Animal studies	BV	(68)
PPV	ORF2	PCV2	Animal studies	Adenovirus mammalian	(69)
Plant viruses					
CPMV	VP2	CPV	+ Animal studies	Plants	(66,67)
CPMV	VP2	MEV	+ Animal studies	Plants	(65)
TMV	VP1	FMDV	+ Animal studies	Plants	(63)
TMV	5B19	MHV	+ Animal studies	Plants	(64)
Reoviridae					
Rotavirus	DOX	Anticancer drug Δ	*In vitro*	*E. Coli*	(57)

See "Abbreviations". Δ indicate VLPs used for drug delivery. + indicate VLPs that protected the target host.

One of the first vectors used to express viral antigens was hepatitis B virus (HBV) and various epitopes from different viruses were displayed on the HBV platform. HBV belongs to *Hepadnaviridae* family and before the development of the vaccine it was an important threat for human health. VP1 FMDV epitope was attached to HBV core antigen (HBcAg). VP1-derived-VLPs elicited virus-neutralizing antibodies and provided immunogenicity stronger than the peptide alone. They were almost as immunogenic as inactivated virus; moreover, VLP-immunized guinea pigs were completely protected against virus challenge (58). Several other studies have reported the insertion of FMDV antigenic epitopes into HBcAg as an alternative vaccine approach. Beesley *et al.* selected a yeast expression system for HBcAg-FMDV-VLPs (59) while Jin *at al.* used HeLa cells (60) for more exhaustive studies. All these efforts demonstrate the utility of HBcAg as a VLP carrier for FMDV VP1 antigen that could be used to develop an effective FMDV vaccine.

HBcAg was also used to express different epitopes (MHC-I or MHC-II restricted peptides) of lymphocytic choriomeningitis virus (LCMV), a rodent-borne virus. This study was performed to investigate if pre-existing VLP-specific antibodies interfered with specific cytotoxic T-cell and Th-cell responses or with the induction of protective responses in mice. In this model, antigen presentation was not significantly affected *in vitro* and *in vivo* by the presence of anti-vector antibodies and protective immunity could be established in animals previously vaccinated with the vector (61). Also Storni *et al*. (62) used the HBcAg to express LCMV epitope to investigate the activation of APC for priming CTL responses after VLPs vaccination. In this model they demonstrated that VLPs alone were inefficient at inducing CTL responses and failed to mediate effective protection from viral challenge, but they became very powerful if applied together with other substance that activated APCs (e.g. anti-CD40 antibodies or CpG).

Plant viruses are another type of successful carriers and the most popular are cowpea mosaic virus (CPMV) and tobacco mosaic virus (TMV). They have been used as vectors for displaying animal virus epitopes. TMV particles expressing different peptides of FMDV VP1 were generated and tested in different animal models (mice, guinea pigs and pigs) (63). Guinea pigs parenterally injected with two VLP types were completely protected against the challenge with FMDV serotype O, while oral

administration gave partial protection. Also, suckling mice passively transferred with guinea pig antiserum were protected from viral challenge, showing that VLP vaccination was able to elicit neutralizing antibodies against FMDV. Finally, results in swine clearly demonstrated that VLPs protected pigs against a FMDV challenge (63). TMV was also used to express the 5B19 epitope of murine hepatitis virus (MHV) and different protocols were used to administer immunogens to mice. The particles protected mice against lethal challenge in a dose-dependent manner and the degree of protection was dependent of the route (parenteral or mucosal) of immunization. In this case, the regimen of vaccination positively correlated with the amount of antibody and the degree of protection; subcutaneous administration needed less amount of immunogen for effective protection (64). On the other side, CPMV particles have been genetically modified to include epitopes from mink enteritis virus (MEV) (65) and canine parvovirus (CPV) VP2 (66, 67). Also in these cases, both species immunized with the specific CPMV-VLPs were protected from lethal challenge.

Concerning epitopes of virus affecting animals that have been expressed in various scaffolds, it has been demonstrated that PPV VLPs were an effective vaccine in pigs; furthermore, PPV-VLPs were also the first animal-based vector used to carry viral epitopes. Sedlik *et al.* (68) generated recombinant PPV-VLPs expressing the $CD8^+$ CTL epitope from the LCMV nucleoprotein; this epitope was inserted in the N terminus of VP2 capsid protein of PPV and VLPs were tested *in vivo*. One intraperitoneal immunization with only 10 μg of PPV-LCMV-VLPs was able to induce complete protection of mice against a lethal viral infection through the induction of virus-specific MHC-I-restricted $CD8^+$ CTLs. The protection did not require $CD4^+$ T helper function or adjuvant and the strong *in vivo* CTL response induced by VLPs persisted for months after the immunization (68). PPV was also used to carry the ORF2 of PCV2, showing the induction of a strong antibody response in the absence of any adjuvant (69). Nowadays, ORF2 protein is the antigen of the PCV2 VLP-based vaccine commercially available.

As mentioned in previous sections, NDV VLPs can be also used to express peptide sequences from target pathogens. Morrison (18) elucidated the possibility to incorporate glycoprotein into NDV-VLPs; indeed, proteins from Nipah virus G, influenza virus and respiratory syncytial virus (RSV) were successfully inserted into these VLPs. An

interesting result was that immunization with NDV-RSV-VLPs provided complete protection from RSV replication in lungs after intranasal challenge with live virus in the murine system (70). According to Morrison's opinion, NDV-VLPs would be effective and safe human vaccines because there are no reports that NDV causes human disease in the general population and there are no reports of seropositive human populations. Moreover, there is no evidence for adverse effects in humans due to viral antigens, since live NDV has been injected intravenously in human clinical trials testing NDV as an oncolytic agent. These studies reported little to no toxicity and indicate that this was an ideal framework to base human vaccines (18). The same statements may comply with all the VLPs derived from specific animal viruses.

VLPs and immune responses

VLPs are commonly more immunogenic than subunit or recombinant protein immunogens (10) and are able to stimulate both humoral and cellular arms of the immune system (reviewed in (1, 10)).

Considering their key features previously described about mimicking the native virus and PAMPs and size properties, VLPs can enhance the production of neutralizing antibodies (reviewed also in (1, 10)). Different VLP candidates have been shown to be able to induce neutralizing antibodies and most of the times this ability resulted in protection in the animal model or in the natural host (24, 27, 28, 33, 34, 37, 49, 52, 55, 65, 67). These particles, with their size, efficiently drain or diffuse to lymph nodes from the site of injection, thus, VLPs can enter secondary lymphoid organ and interact directly with B-cells to trigger antibody responses (1).

An important unwanted aspect that may be considered about antibody responses is that it has been shown that pre-existing antibody responses against VLPs exert a detrimental effect on the efficacy of chimeric HPV-VLP-based vaccines (71). Even if this possibility can not be excluded, another study was performed more recently to investigate if pre-existing VLP-specific antibodies interfered with specific cytotoxic T-cell and Th-cell responses or with the induction of protective response in mice. In that model, antigen presentation was not significantly affected *in vitro* and *in vivo* by the presence of the vector antibodies and protective immunity could be established in carrier vaccinated animals. Ruedl *et al.* (HBcAg-LCMV-VLPs)(61) opened a new

perspective around VLP vectors and the classical concept that vaccine or maternal antibodies impair the induction of protective immune responses upon vaccination (72). Indeed, also in the veterinary field, the interference of colostral antibody has been described in animals vaccinated with inactivated virus (73). Interestingly, and contrary to previous vaccine results, the presence of VLP-specific antibodies did not affect induction of effector cells (61). Thus, suppression by VLP-specific antibodies may be considered low influential in VLP-based immunization also in young animals. However, further studies have to be performed to fully clarify this aspect.

Ludwig and Wagner (9) divided VLPs in type-1 VLPs, which are capable of eliciting strong CTL responses towards the internally inserted epitopes, and type-2 VLPs exposing incorporated envelope proteins on their surface that have been more successful in inducing humoral antibody responses. In general, VLPs do not appear to require the use of adjuvants to achieve potent immune stimulation. Indeed, the self-adjuvanting effects of VLPs are inherent to their properties: uptake by DCs for processing and presentation by MHC-II and for directly promoting DC maturation and migration, essential for stimulation of innate immune response (14, 74). Several types of VLPs have been reported to directly induce phenotypic and functional maturation or activation of DC (75, 76).

Exogenous VLPs can also be taken up and processed via the MHC-I pathway (cross-presentation) for activation of $CD8^+$ T cells, which are essential for the clearance of intracellular pathogens such as viruses. The ability of VLPs to target DCs is an important advantage of VLP-vaccines, as targeting of this cell types is now understood to be essential for activating innate and adaptive immune responses. Some VLPs that resemble infectious viruses and retain their receptor binding regions are able to target and enter cells via their normal receptor and are taken up by APC as exogenous Ag for class I presentation (9, 10).

Ludwig and Wagner (9) showed a putative mechanism of VLP-mediated stimulation of innate and cognate immune response, summarized in the **figure 2** (9). We added some modifications regarding VLP internalization by clathrin-dependent macropinocytosis described by Win *et al.* (77).

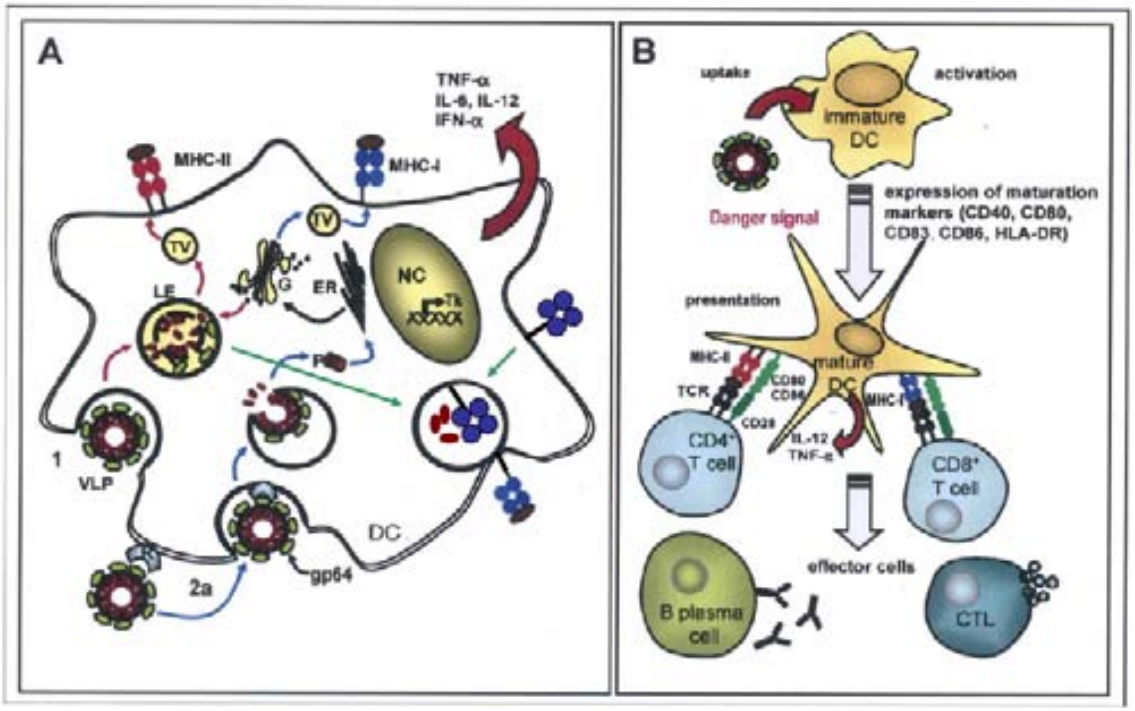


Fig. 2. Putative mechanism of VLP-mediated stimulation of innate and cognate immune responses. (**A**) **Model for the activation of DCs by BV-derived VLP preparation**. VLPs are taken up by DCs via endocytosis (1) directing antigen processing in late endosomes (LE) and presentation via the MHC-II pathway (red path), or via receptor (R)-mediated fusion triggered by gp64 (BV envelope fusion protein) (2a) resulting in proteasomal Ag processing in the cytoplasm and subsequent presentation on MHC-I (Blue path). VLPs are taken up by DCs via clathrin-dependent macropinocytosis (1) and VLP-derived peptides are loaded onto MHC-I that have been recycled from the cell surface (Green path). Danger signals are recognised by endosomal (E) TLR and transmitted by signalling pathway resulting in activation of transcription (Tk) and production of inflammatory cytokines and type I IFN. (**B**) **VLP-mediated maturation of DCs**. Uptake of VLP activates DCs via danger signals resulting in upregulation of DC maturation markers. Mature DCs present VLP-derived Ags to naïve $CD4^+$ and $CD8^+$ T cells via MHC-II and MHC-I. Secretion of cytokines by DCs stimulates differentiation into B and T effector cells resulting in antibody release and CTL responses. *(Modification of figure 2 from Ludwig and Wagner 2007, Current Opinion in Biotechnology, 18:537-545).*

VLPs production: immunological implications

Different production systems have been exploited for VLP generation for human and veterinary vaccines. Genes encoding VLPs have been acquired from many different sources, including animal viruses, plant viruses, bacteriophages and yeasts (3). These genes were recombinantly expressed in a variety of hosts and VLPs have been efficiently released from yeast cells (59) or using vaccinia virus expression system (58). Also plants were used to produce VLPs of various origins and with various structural characteristics (64, 65); plant derived VLPs, when administered properly, induced a potent immune response in animal and human systems. Plant systems have some advantages such as the absence of risk of contamination with animal pathogens, potentially low production costs and offer the option of producing edible vaccines (78). Various agroinfiltration methodologies have evolved and several whole plants can be infiltrated simultaneously for large-scale production; moreover, massive expression of recombinant proteins has been reported using agroinfiltration-based system, mainly influenza VLPs, reviewed in (4). Other systems, as transfection of DNA into mammalian cell (34, 49) or gut bacteria (e.g. *E. coli* (57)) have been developed.

All these technologies are considered useful because these methods can produce large amount of proteins *in vitro*, but among all, BV expression vector system has been used extensively for production of VLPs for human and veterinary vaccine candidates. The strong immunogenicity of the expressed products, the high productivity of the system and its ability to achieve a rapid implementation at production scale, together with the fact that the production is cost-effective, have turned towards this system (23).

The majority of veterinary VLPs listed in **Table 1** have been produced using the BV expression system. The BV system is based on the ability of this virus to produce "polyhedra" or "occlusion bodies" during productive viral infection in insect cells (79). The BV system has demonstrated to be a valuable tool for producing a variety of complex eukaryotic proteins and it has been used for investigating capsid formation of many viruses (80). The BV system has various advantages in the production of recombinant glycoprotein: a) the eukaryotic nature of the insect cells, b) BV host range

is limited to insect cells and c) BV is easily inactivated by chemical treatment (81), but some other issues regarding this system are involved.

First of all, VLPs derived from this technology possess insect-cell glycosylation patterns, with altered carbohydrate modification of the glycoprotein synthesized in this cells (7, 10, 18) compared with mammalian cells. These marked differences in glycosylation raise important questions regarding potential differences in immunogenicity between mammalian cell- and insect cell-produced VLPs (discussed in (7)).

A second issue is related to BV-derived enveloped VLPs, mainly influenza-VLPs, which probably may contain a finite quantity of insect cell-derived lipid raft-associated proteins and cellular proteins that can be incorporated into VLPs as they bud from the surface of the cell. These proteins would probably be immunogenic and may lead to the presence of pre-existing immune response to common surface proteins of VLPs. However, even when a previous study in mice demonstrated no measurable effect of pre-existing immunity to a common VLP antigen (61), successive immunizations with different VLP subtypes may present unforeseen hurdles (7).

Moreover, the encapsidation of nucleic acids during the production seems be a controversial issue; on one side, it may give unknown and unwanted effects during VLP-vaccination, but on the other side, these nucleic acids may stimulate particular TLRs (discussed in (3)) and potentiate the immune response.

One of the most relevant immunological aspect related to BV production is the presence of contaminating BV in the VLP preparations. The hypothesis is that immunogenicity of BV-derived VLP vaccines may be enhanced by contaminating baculovirus particles, since it has been demonstrated that live BV particles can stimulated short-term innate immunity and can act as adjuvant when mixed with other antigens (commented in (7, 9)). Indeed, BV has abundant CpG motifs in the viral genome (82) and has been reported to induce strong innate immunity upon intranasal inoculation capable of confering protection from a lethal influenza virus challenge in mice, indicating that inoculation with BV imparts unspecific antiviral activity (83). Besides, BV promotes humoral and CTL responses against co-administered antigens, DCs maturation and

production of inflammatory cytokines (84). This activity is essentially mediated by MyD88/TLR9-dependent signalling pathway, but signalling molecules other than MyD88 may also participate in the IFN-α production in response to BV (**Fig. 3**)(82, 84). Additionally, unwanted inflammatory reactions or a theoretical risk of integration of BV DNA into the cellular genome of the host have to be considered. Purification of VLPs from mammalian cells would eliminate any issue with immunogenicity associated with the altered post-translational modifications of proteins produced in insect cells and would eliminate concerns about the presence of BV in the vaccine preparations (18) but this is less controllable and more costly for production (10). Recently, recombinant baculovirus were shown to be capable of entering into various mammalian cells without any replication and of expressing foreign genes under the control of mammalian promoters (85); also, improved insect cell lines, "humanized" cells, that perform mammalian-like post-translation modification, are becoming available (86, 87). Thus, this option would be an interesting system for future protein expression.

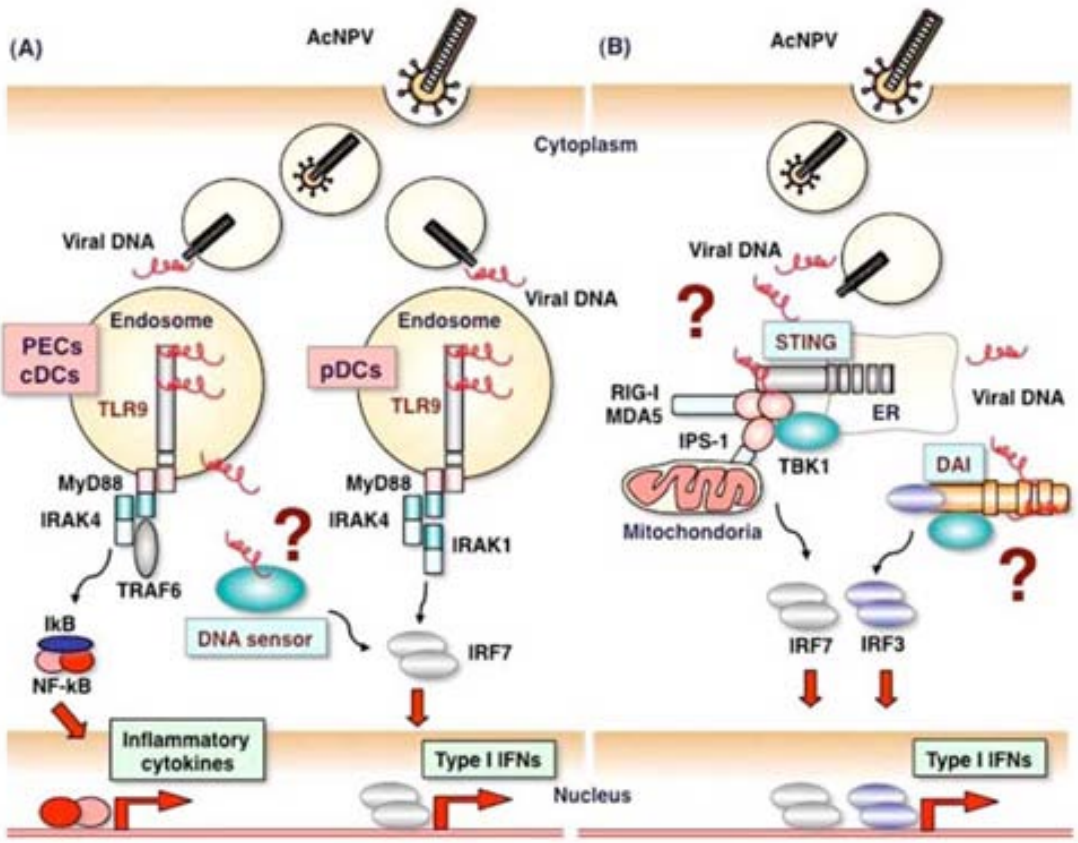


Fig. 3. Induction of a host innate immune response by baculovirus. (**A**) The current model of TLR9 activation by the baculovirus genome. Baculovirus particles (AcNPV) are internalized into the immune competent cells through an endocytotic or phagocytotic pathway, and the viral genome in the compartments activates TLR9 and subsequently produces pro-inflammatory cytokines and type I IFNs through the NF-kB- and IRF-dependent pathway, respectively. However, production of type I IFNs in immune competent cells other than pDCs upon infection with baculovirus is not mediated by the TLR9/MyD88-dependent pathway. (**B**) The cytoplasmic dsRNA sensors, including RIG-I, MDA5, and IPS-1, are not involved in the production of type I IFNs by baculovirus in immune cells and MEFs. However, it is possible that putative DNA sensors such as DAI and STING participate in the production of type I IFNs upon infection with baculovirus. *(Figure 1 from Takayuki Abe and Yoshiharu Matsuura 2010 Current Gene Therapy, 10, 226-231; permission of reproduction by Bentham Science Publishers Ltd).*

Rabbit haemorrhagic disease calicivirus

One suitable virus family for generation of VLPs is the *Caliciviridae* family. This family has been divided into four genera: Norovirus, Sapovirus, Vesivirus and Lagovirus. Caliciviruses cause a variety of diseases in humans and animals (88) and rabbit hemorrhagic disease virus (RHDV), the prototype strain of the genus Lagovirus, is the causative agent of a highly infectious disease of rabbits (89-91). Infected rabbits usually die within 48 to 72 h due to 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 into several mature non-structural proteins and the capsid protein subunit of 60 kDa (VP60) (92, 93). The second ORF encodes a small minor structural protein, VP2.
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 VLPs that were morphologically and antigenically identical to the infectious particles (94, 95). Subsequently, recombinant calicivirus VLPs from the four genera have been reported (96-99).

The RHDV-VP60 protein, expressed in several heterologous systems, has been shown to induce full protection of rabbits against a lethal challenge with RHDV (99-104). The first protection study was performed in 1994 by Boga *et al*. (104) expressing RHDV-VLPs with an *E. coli* system. Rabbits were immunized with two doses of RHDV-VLPs with complete Freund's adjuvant and challenged 7 days after the last immunization. The immunized rabbits survived developing anti-VP60 specific antibodies. In the same year, Laurent *et al.* (99) produced RHDV-VLPs with baculovirus system; these VLPs emulsified in Freund's complete adjuvant injected once intramuscularly to rabbits were able to protect rabbits against a virulent challenge (under the conditions used for commercial vaccine testing in France). Anti-VP60 antibodies could be detected as early as 5 days after the vaccination and the titres progressively increased over a 15-day period (99). Also the oral immunization of animals with BV-derived RHDV-VLPs conferred protection (105). Successively, other groups expressed the capsid protein using a plant-based production system. Castañon *et al*. (102) expressed VP60 in potato

plants and inoculated leaf extracts with adjuvant using subcutaneous route followed by intramuscular route. Similarly, Fernandez *et al.* (101) used a potyvirus-based vector in plants and was able to protect rabbits with two doses subcutaneously. Finally, Perez-Filgueira *et al.* (100) used BV insect larvae-derived VLPs to immunized rabbits once intramuscularly; these insectigens (recombinant subunit antigens produced with insect system) protected animals using a low-cost system (100).

The three dimensional structure of several calicivirus recombinant VLPs as well as authentic virions has been determined to low resolution by cryoelectron microscopy and three-dimensional reconstruction techniques (97, 106-108). 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 Norwalk Virus (NV) VLPs and San Miguel sea lion virus (SMSV, the prototypic strain of the genus Vesivirus) capisds (109, 110). Regarding NV, 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, whereas 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 (**Fig. 4**).

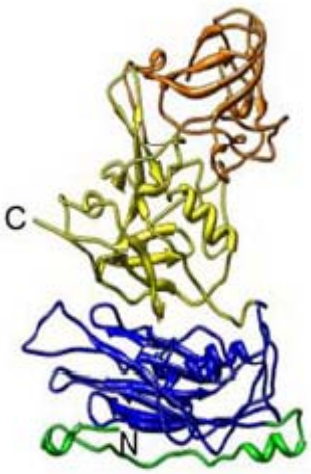


Fig. 4. Pseudo-atomic model of VP60. Ribbon representations of the structure of rNV capsid protein . The color coding for NV coat protein is as initially shown by (110): the N-terminal arm is in green, the S domain in blue, the P1 subdomain in yellow and P2 subdomain in orange. (*Modified figure from Iván Angulo Herrera's PhD Thesis 2007; reproduction permission from Bárcena J.*).

Barcena *et al.* (106) have previously performed an exhaustive structural analysis of the RHDV capsid protein and obtained a pseudo-atomic model of VP60 protein, also shown in Iván Angulo Herrera's PhD Thesis (**Fig. 5-6**). Different authors 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 (106, 111, 112), raising the possibility of using RHDV-VLPs as foreign epitope carriers for vaccine development. In addition, this structural focus allowed inferring specific sites at the outermost region of VP60 as potential insertion sites for foreign epitopes.

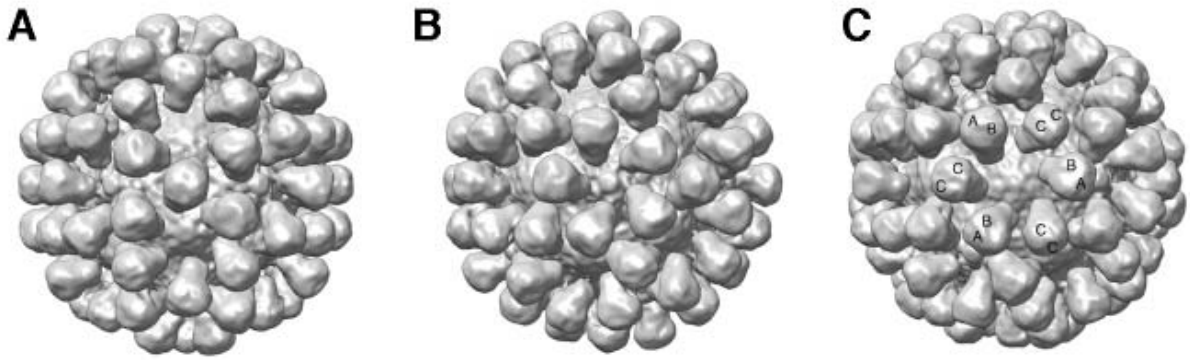


Fig. 5. Three-dimensional structure of capsid *T*=3 of VP60 at 10-Å resolution. (A-C) Surface-shaded representation of the outer surface of the VP60 capsid viewed along a 3-fold axis of icosahedral symmetry. The position of the three conformers of VP60 (A, B and C) are indicated. *(Figure from Iván Angulo Herrera's PhD Thesis 2007; reproduction permission from Bárcena J.).*

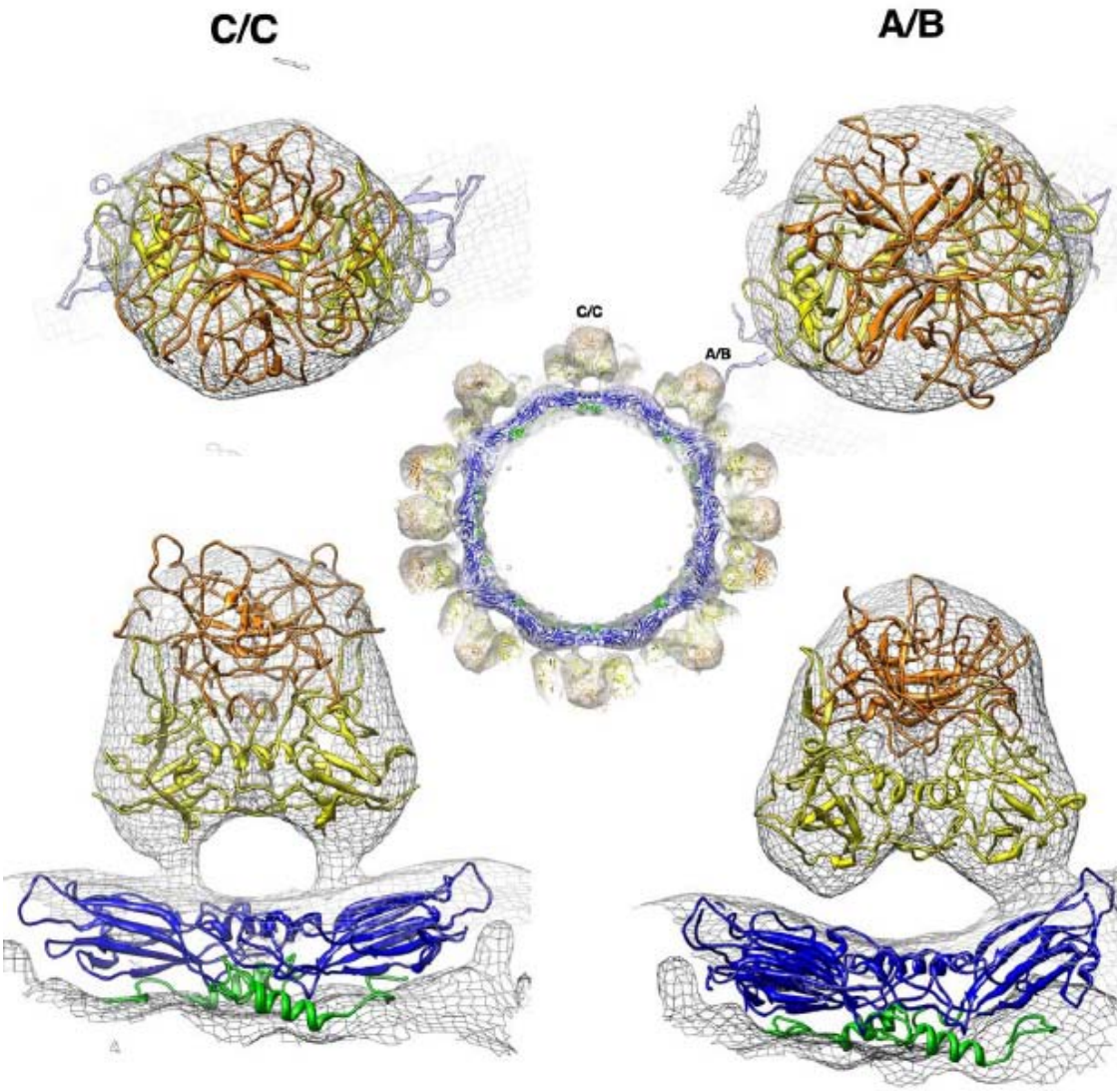


Fig. 6. Docking of the VP60 model in the cryo-electron microscopy reconstruction of the RHDV particles. The envelope of the three-dimensional map obtained from cryo-electron microscopy images is depicted in grey and the structure of the dimers C/C (left) and A/B (right) is shown in top view (upper line) or lateral view (lower line). In the middle is shown the transverse section of T=3 capsid (~ 50 Å). *(Figure from Iván Angulo Herrera's PhD Thesis 2007; reproduction permission from Bárcena J.).*

Taking into account the ability of RHDV-VLPs to be an epitope vector, Peacey *et al.* (113) described for the first time the chemical covalent conjugation of the model antigen OVA in the N-terminus of VP60 protein. In this study, they investigated the immuno-stimulatory properties of the underlying viral shell to the conjugated antigen, showing that RHDV-VLPs enabled the initiation of both antigen specific humoral and cell-mediated immune responses (113). In the second study, this research group used two well-defined CD4 and CD8 peptide from OVA in RHDV-VLPs and compared their ability to impair tumour growth with these VLPs. These RHDV-OVA-VLPs used with adjuvant (CpG) were able to impair tumour growth of B16.OVA melanoma (114). Another work used RHDV-VLPs as gene transfer vector; indeed, chimeric RHDV-VLPs were able to package plasmid DNA and to transfer genes into animal cells (Cos-7), opening the way for a new alternative method for gene transfer (111).

The well-documented immunogenicity of VLPs is probably due to their interaction with DCs (13). Peacey *et al.* (114) demonstrated that RHDV-VLPs are cross-presented by DC but the mechanism by which this occurs was not fully understood. Thus, a recent study used RHDV-VLPs chemically conjugated with antigen with both murine and human DCs to elucidate these pathways. Win *et al.* (77) demonstrated that RHDV-VLPs are taken up by clathrin-dependent macropinocytosis and phagocytosis before being degraded in acidic lysosomal compartments (summarised in **Fig. 2** in VLP and immune responses paragraph). Neither proteosomal nor transporter associated with antigen presentation (TAP) inhibitors prevented the effective presentation of VLP-derived antigen to peptide-specific $CD8^+$ T Cells, so they concluded that these peptides did not escape into the cytosol nor did they enter the endoplasmic reticulum. Their evidence suggests that VLP-derived peptides are loaded onto MHC-I that have been recycled from the cell surface as seen with inhibition of cross-presentation by primaquine and not from nascent MHC-I molecules in the endoplasmic reticulum (ER) as is the case for endogenously derived antigens. Different antigens were used to demonstrate cross-presentation via this alternative, receptor recycling pathway, which operated independently of the proteasome and the transporter-associated with antigen presentation. Finally, they found that cross-presentation of RHDV-VLPs *in vivo* was not confined to $CD8\alpha^+$ DC subsets. These data define the cross-presentation pathway for RHDV-VLPs and the authors could not find evidence for receptor-mediated acquisition

by DCs in the species under study. RHDV does not naturally infect mice or humans and it is possible that the lack of receptor enabling efficient entry into host cells contribute to this (77).

HYPOTHESIS AND OBJECTIVES

RHDV-VLPs have been shown to be an effective vaccine against the natural disease and also a suitable vector for foreign epitopes.
Previous studies have shown that the chemical conjugation of an antigen enabled these RHDV-VLPs to be an interesting tool for improving cancer immunotherapies and also they may be a new alternative method for gene transfer.

The main working hypothesis of this thesis was that chimeric RHDV-VLPs could be a potent vaccine vector against animal viral diseases.
Therefore, the specific objectives were the following:

1. To generate chimeric RHDV-VLPs with a model epitope (OVA) (RHDV-VLPs-OVA) in different positions using baculovirus expression system.

2. To study the immunogenicity of chimeric RHDV-VLPs-OVA in a murine system *in vitro* and *in vivo*.

3. To study the immunogenicity of other chimeric RHDV-VLPs (RHDV-NP-VLPs) in mice using different immunization routes.

4. To study the immunogenicity of RHDV-VLPs in porcine cells *in vitro*.

5. To study the immunogenicity of chimeric RHDV-3A-VLPs in pigs *in vivo*.

“The woman and the mouse”

Martin Drolling

France, XVIII

CHAPTER 1

CHIMERIC CALICIVIRUS-LIKE PARTICLES ELICIT PROTECTIVE ANTI-VIRAL CYTOTOXIC RESPONSES WITHOUT ADJUVANT

INTRODUCTION

In this study the generation of recombinant chimeric RHDV-VLPs incorporating a well defined $CD8^+$ T cell epitope corresponding to amino acid (aa) 257–264 (SIINFEKL peptide) from chicken ovalbumin (OVA) is reported. This epitope is restricted for MHC-I H-2Kb presentation (115). 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 aa positions 306 and 307 of VP60 protein, which is predicted to be located within an exposed loop at the P2 subdomain of VP60 protein.

The aim of this study was to analyze the immunogenic potential of both chimeric VLPs (RHDV-VLPs-OVA) in a murine system *in vitro* and *in vivo*.

MATERIALS and METHODS

Viruses, cells and mice

Derivatives of *Autographa californica* nuclear polyhedrosis virus (AcNPV) were used to obtain the recombinant baculoviruses (BVs) expressing RHDV-VLPs. BVs were propagated in insect cell lines grown in suspension or monolayer cultures at 28°C in TNM-FH medium (Sigma) supplemented with 5% foetal calf serum (FCS) (Gibco).

Spodoptera frugiperda cells (SF9) were used for generation of recombinant BVs, 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 expressing OVA (VV-OVA) was originally obtained from J. Yewdell (National Institute of Health, Bethesda, Maryland, U.S.A.) (116). VV was grown and titrated in Vero cells as previously described (117).

Mice, C57BL/6JOlaHsd (Harlan) of 7- to 8-wk-old age, were used for immunization and for DCs primary cultures.

Construction of recombinant baculovirus transfer vectors

The primers used in this study are shown in **Table 3** (see **Annexes**).

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 BamHI and XbaI, 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 BglII and HindIII, 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 (106), and the primer pair PolihedF/KpnISgRHDR. The PCR product obtained was digested with BglII and KpnI and inserted into unique restriction sites of pBacPAK8HA, creating pHAPhSubG. This plasmid was subsequently modified to eliminate a natural BamHI 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 BglII and KpnI 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 BglII and BstEII 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 BglII and KpnI 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 BamHI compatible ends. The annealed primers were subsequently ligated into plasmids pHAPh2GS and pHAPh306GS, previously linearized by BamHI digestion and dephosphorilated, creating plasmids pHAVP60-2OVA and pHAVP60-306OVA. All the inserted sequences in the resulting recombinant plasmids were verified by sequence analyses.

Generation of recombinant baculoviruses

All recombinant BVs were produced using the BacPAK baculovirus expression system (Clontech) as described previously (106). Briefly, monolayers of SF9 insect cells were co-transfected with recombinant transfer vectors and Bsu36I triple-cut AcMNPV DNA (118) using lipofectamine (Invitrogen). Recombinant BVs were selected on the basis of their LacZ-negative phenotypes, plaque purified, and propagated as described elsewhere (119).

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 (120). Briefly, H5 insect cell monolayers were infected with recombinant BVs 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 (BMDCs) generation

BMDCs were generated from cultures of bone marrow cells of C57Bl/6 mice. They were prepared as described previously (121). 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 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&D Systems, Abingdon, Oxon, United Kingdom). Fresh medium was given every other day. The BM progenitors were incubated at 37°C in 5% CO_2 in Petri dishes for 8 days as previously described (122) and then immature BMDCs were used for the antigen presentation assay. $CD11c^+$ ranged between 95% and 98% without any further sorting or treatment.

DC activation and antigen presentation assay

Different concentrations of different RHDV-VLPs (RHDV-VLP-2, RHDV-VLP-306 and negative control RHDV-VLP) were plated in triplicates with murine immature BMDCs (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 of ELISA was 9 pg/ml. Then, BMDCs were thoroughly washed and 10^5 cell/well of specific $CD8^+$ hybridoma

(B3Z) recognizing SIINFEKL peptide (123) 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 a specific ELISA (R&D Systems, Abingdon, Oxon, United Kingdom). Detection limit of ELISA was 6 pg/ml. BMDCs cells cultured for 6-7 h only with SIINFEKL peptide (ProImmune, Abingdon, UK) were used as control of presentation efficiency range in the *in vitro* experiment (124).

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 (ip) 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 immunizations. 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 humoral responses

Sera of three animals of each group were collected two weeks after the second immunization and analyzed by ELISA assay. Briefly, polisorp plates (Nunc) were coated with 50 μg/well of OVA or 1 μg/well of VP60 diluted in coating buffer, incubated overnight at 4ºC and then washed extensively. Wells were blocked, and then serum in five-fold dilution (starting at 1:10) was added in duplicate to plates. Plates were incubated for 1 h at 37ºC, washed and HRP conjugated goat anti-mouse IgG antibody (Bio-Rad Laboratories) was added and incubated for 1 h at 37ºC. Plates were extensively washed, OPD substrate (Kem-En-Tec Diagnostic, Denmark) added, then the reaction stopped with 1N H_2SO_4 and OD determined at 492 nm.

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 manufacturer's 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 and incubated overnight. 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.

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 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 immunized/% CFSEhi immunized)−(% 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 (116). 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).

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 BVs expressing different VP60 constructs (**Fig. 7**). 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 (125). 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 the VP60 gene.

The foreign peptide sequence was inserted at two different locations within the VP60 protein (**Fig. 7A**) 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 (106), 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 (106), 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 BVs was verified by SDS-10% PAGE. As shown in **Fig. 7B**, extracts from insect cells infected with recombinant BVs harbouring VP60 constructs exhibited a major protein band with the expected size of ~ 60 kDa, which was not present in wild-type BV-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 BV expressed VP60 protein as well as the chimeric mutants by ELISA and Western blot (data not shown).

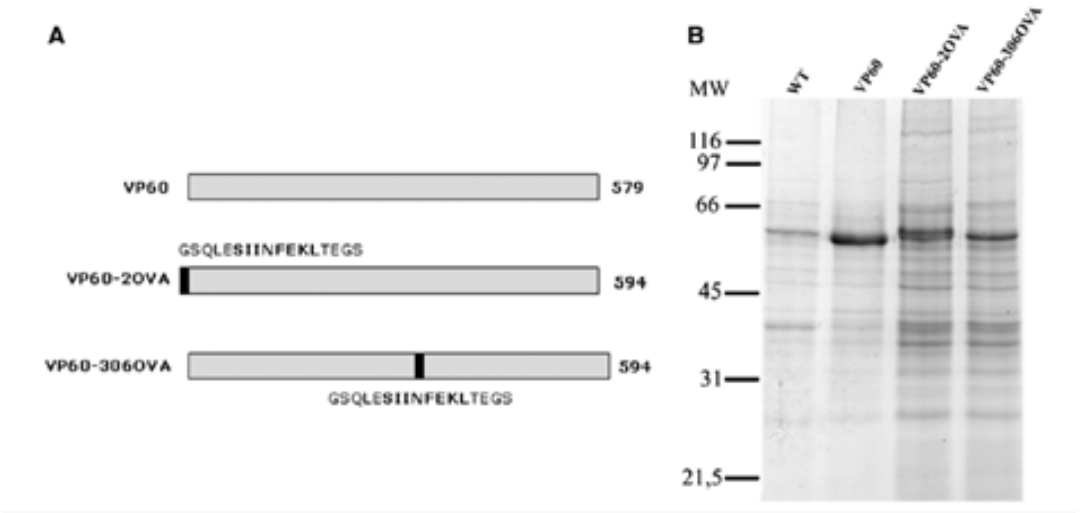


Fig. 7. Schematic representation and expression of the recombinant VP60 constructs used in this study. (A) Scheme of each construct showing names and numbers of amino acid residues. The chimeric proteins VP60-2OVA and VP60-306OVA harbour the depicted foreign peptide sequence containing de OVA derived 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; $x10^3$ Da) are given on the left.

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. 8**). Negatively stained fractions enriched in the recombinant VP60 constructs (**Fig. 8**, 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 (99, 126-128).

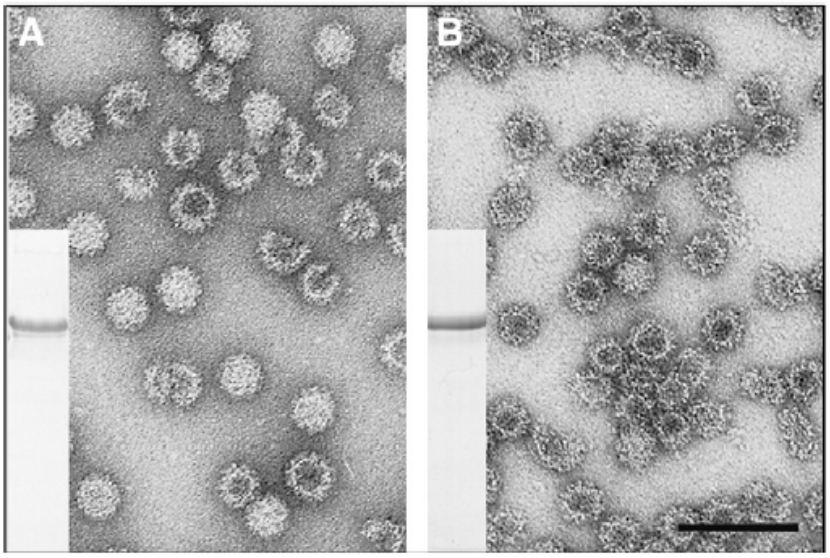


Fig. 8. Analysis of chimeric VP60 particles by negative staining and SDS–PAGE. Electron microscopy of negatively stained purified chimeric VP60 particles (A) VP60-2OVA and (B) VP60-306OVA. Purified particles were analyzed for protein content by SDS-10% PAGE and stained with Coomassie brilliant blue (insets). Scale bar, 100 nm.

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

Once both chimeric and control RHDV-VLPs were generated, we first investigated whether they were able to activate murine BMDCs *in vitro*. When DCs internalize proteins to stimulate T cell responses, the DCs 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 bacterias 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 BMDCs 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. 9**), indicating a certain degree of BMDCs activation after incubation with the VLPs.

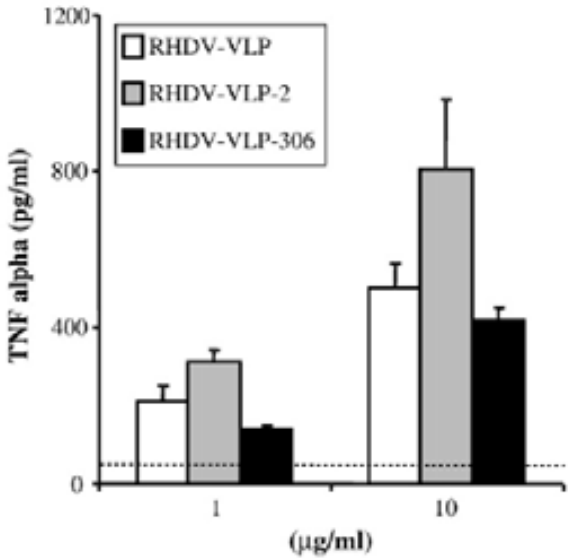


Fig. 9. Bone marrow derived DCs secreted TNF-α after overnight incubation with control and chimeric RHDV VLPs at different concentrations. The column colours indicate the different RHDV VLPs: RHDV-VLP (white), RHDV-VLP-2 (grey) and RHDV-VLP-306 (black). Dotted line indicates background level of TNF-α secretion by untreated cells. Data are representative of two independent experiments.

Since it has been demonstrated that the presence of contaminating BV in VLP preparations may induce potent immune responses that could be erroneously attributed to the VLPs (84), 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 VLP). The average of TNF-α production induced by mock VLPs was 116.6 ±12 pg/ml (mean±SD), which was close to background levels (untreated DCs 44.29 ±10 pg/ml) (**Fig. 10**). It is worth noticing that our VLP purification procedure, which includes extraction with Vertrel, centrifugation through a 20% sucrose cushion and centrifugation in a CsCl isopycnic gradient, is quite more stringent than that reported by Hervas-Stubbs *et al.* (84) (precipitation with 20% ammonium sulfate), and this may account for the difference in the BV content in the VLP samples between our system and theirs.

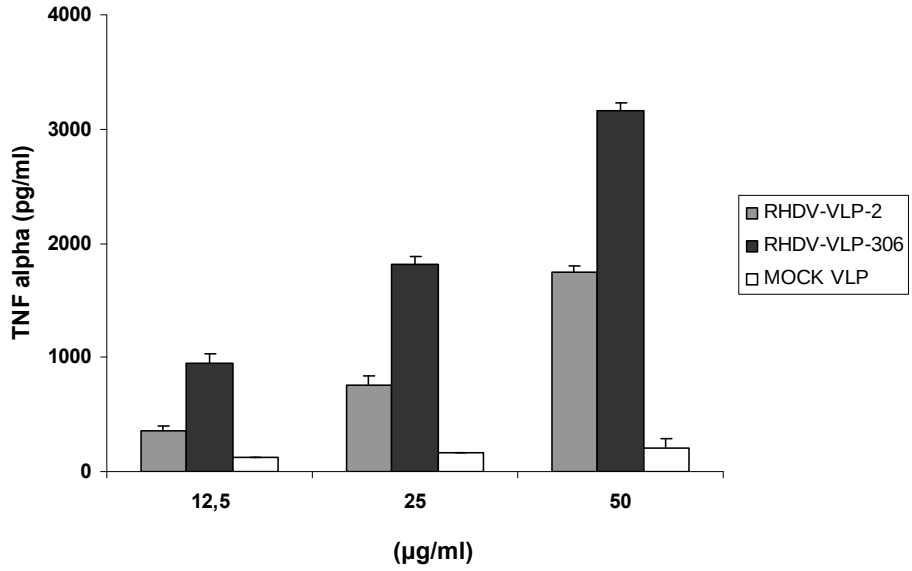


Fig. 10. Bone marrow derived DCs secreted TNF-α after overnight incubation with mock VLPs and chimeric RHDV VLPs at different concentrations. The column colours indicate the different RHDV VLPs: RHDV-VLP-2 (grey) and RHDV-VLP-306 (black). White columns indicate the negative control (mock VLP), material prepared from insect cells infected with wild-type BV that has undergone the same purification procedure as the VLPs. Background level was rested from all value. Error bars indicate standard deviation (SD) and data are representative of three independent experiments.

DCs have been recognized as being the most potent 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 (123) thereby releasing IL-2 in the culture supernatants. Serial dilutions of SIINFEKL peptide were added to BMDCs and IL-2 levels in the supernatants were recorded as a measure of antigen presentation in the assay. As shown in **Fig. 11A**, SIINFEKL peptide was specifically recognized when exogenously added to BMDCs for antigenic presentation to B3Z hybridoma, in a dose dependent manner. Under our experimental conditions, antigenic presentation was detected in a range from 100 to 0.1 ng/ml (**Fig. 11A**). When BMDCs 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 BMDCs incubated with the native control VLPs (RHDV-VLP) (**Fig. 11B**). Antigen presentation showed a dose dependent pattern. Whereas antigenic peptide presentation was detected when BMDCs 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. 11B**). 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 together, our data indicate that BMDCs 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.

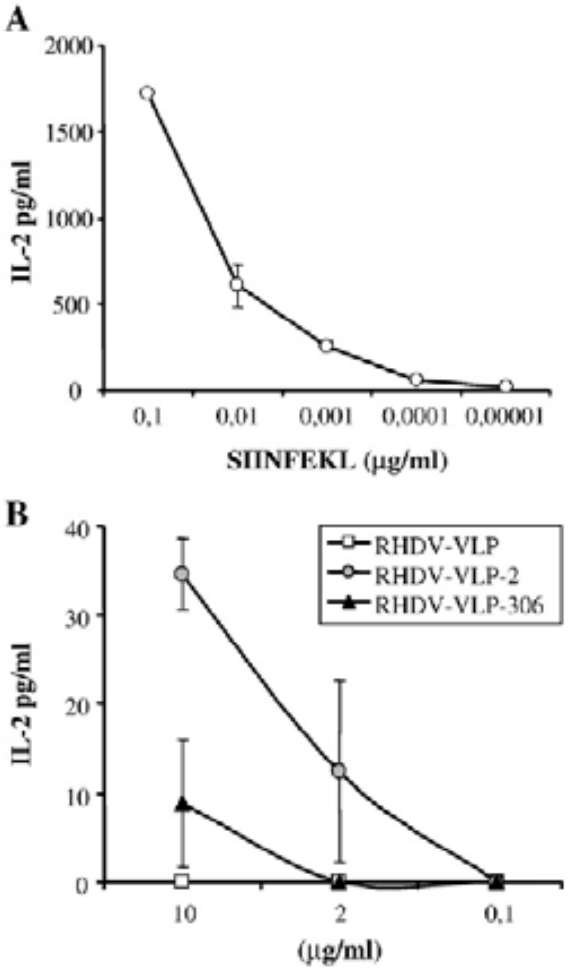


Fig. 11. Mouse DCs are able to process and present SIINFEKL peptide for $CD8^+$ specific recognition *in vitro* in a dose dependent manner. (A) C57Bl/6 BMDCs were incubated for 6-7 hours in the presence of the indicated concentration of synthetic SIINFEKL peptide, and IL-2 released was measured by ELISA. (B) IL-2 released after VLP incubation with C57Bl/6 BMDCs 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. Data are representative of three independent experiments.

RHDV-VLP and OVA specific antibody responses after two immunizations

The immunization with chimeric RHDV-VLPs was able to elicit specific OVA antibodies, mainly using the construct with the insertion in the exposed loop (RHDV-VLP-306) compared with VLP with the epitope in the N-terminus. The responses were dose dependent and interestingly RHDV-VLP-2 immunization was able to elicit specific OVA responses although the epitope insertion is predicted to be buried in the inner part of the shell. The RHDV-VLP and PBS control mice showed unspecific background levels (**Fig. 12**).

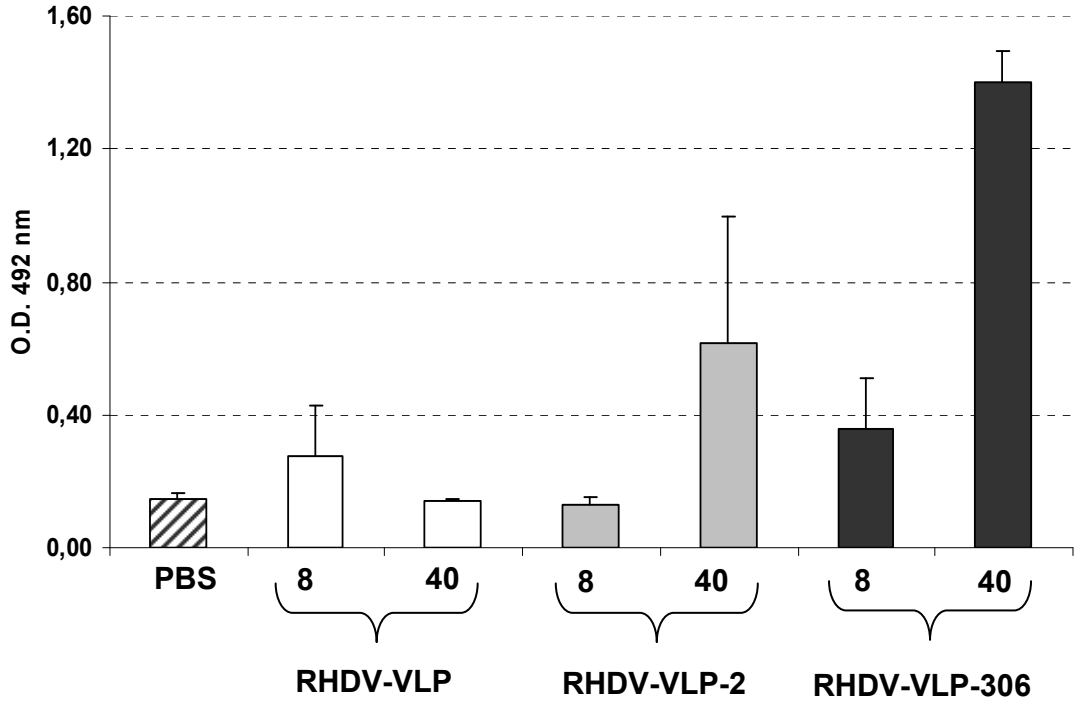


Fig. 12. Specific antibody responses against OVA after two immunizations analyzed by ELISA. Mice were inoculated with two different doses (8 μg and 40 μg) of chimeric RHDV-VLPs and sera were analyzed 14 days after the second immunization. PBS represents the negative control mice. PBS and RHDV-VLP mice show unspecific background levels. The data shown are the means of the results obtained in groups of three animals and the error bars represent one standard deviation above the mean. Data are representative of two independent experiments.

When we analysed the specific humoral responses against the vector, immunization with chimeric VLPs was able to elicit specific VP60 antibodies in all the groups and responses were not dose dependent. No differences were found between the different constructs for antibodies against VP60. As expected, PBS control mice did not show any specific VP60 humoral response (**Fig. 13**).

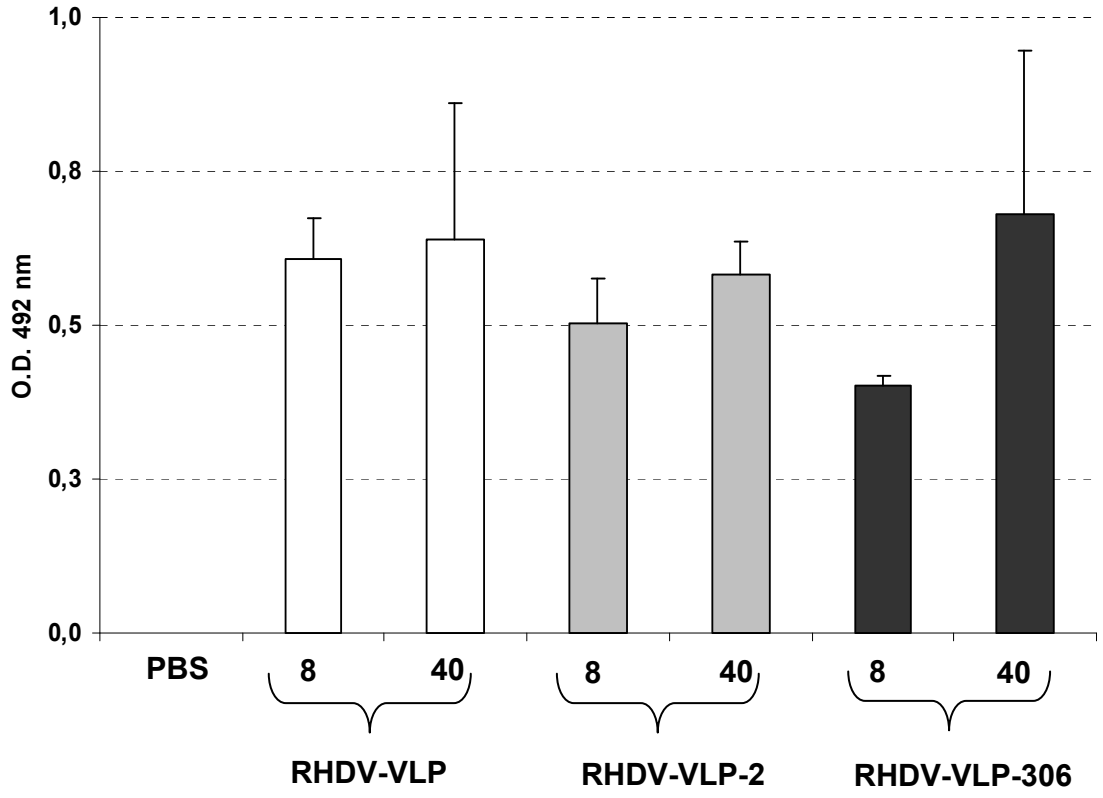


Fig. 13. Specific antibody responses against VP60 after two immunizations analyzed by ELISA. Mice were inoculated with two different doses (8 μg and 40 μg) of chimeric RHDV-VLPs and the sera were analyzed 14 days after the second immunization. PBS represents the negative control mice. No differences between the different VLPs and between doses were found. The data shown are the means of the results obtained in groups of three animals and the error bars represent one standard deviation above the mean. Data are representative of one experiment.

Induction of cellular responses by recombinant VLPs in mice

The results of SIINFEKL presentation by BMDCs *in vitro* led us to investigate whether the chimeric RHDV-VLPs expressing SIINFEKL peptide in two different positions induced any specific cell-mediated 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 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. 14**). 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 at the lower dose.

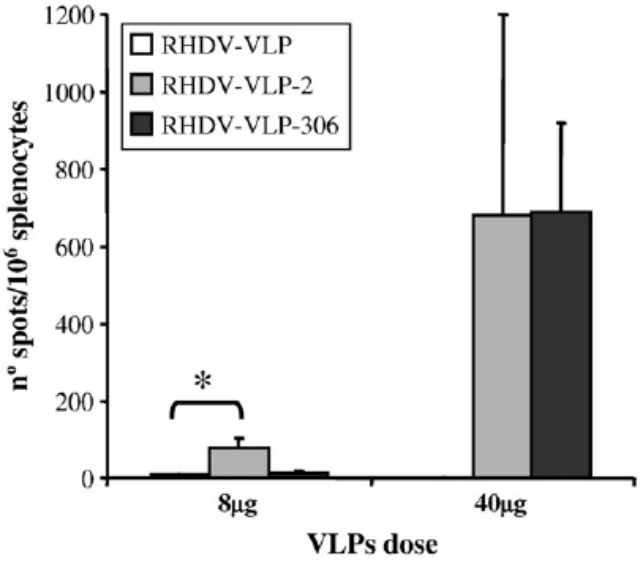


Fig. 14. Frequency of SIINFEKL-specific IFN-γ–producing cells in the spleen of treated mice. Groups of three mice were twice inoculated with 8 or 40 µg of the RHDV VLPs and specific IFN-γ–producing cells were measured by ELISPOT assay. The column colours indicate the different RHDV VLPs: RHDV-VLP (white), 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.

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 (129)(**Fig. 15A**), 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. 15B**). After infection with recombinant VV-OVA, all mice exhibited a high level of cytotoxic activity (90–100%) (**Fig. 15A**).

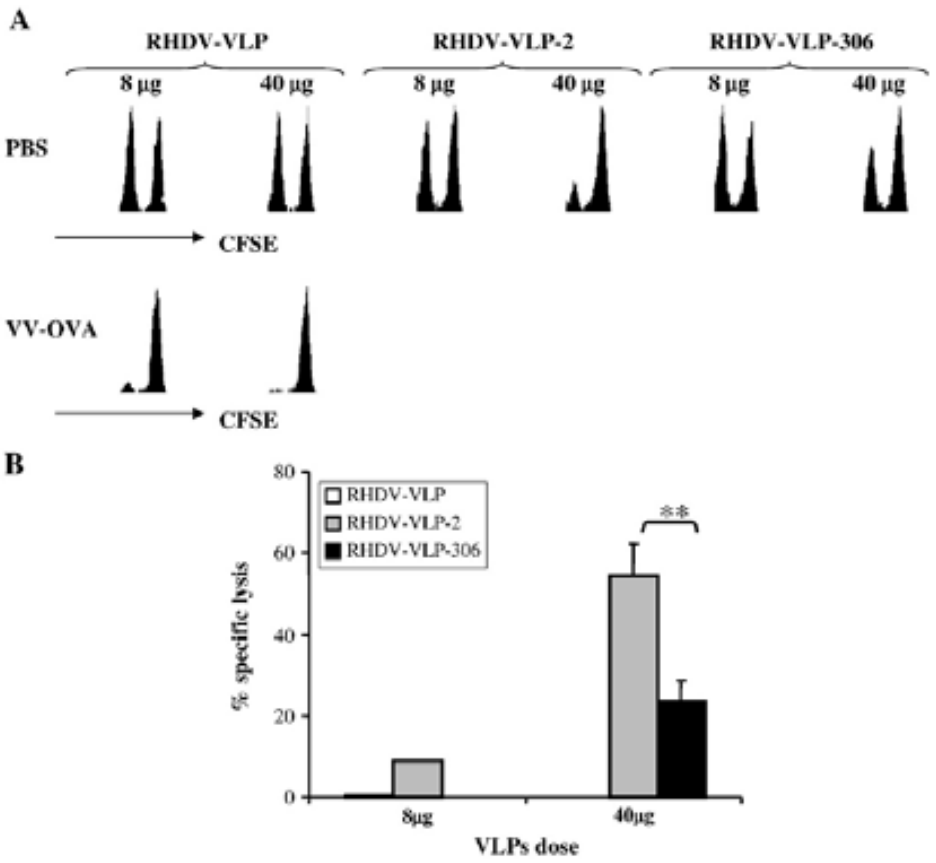


Fig. 15. OVA-specific CTL cell responses in VLP immunized mice by measuring SIINFEKL-specific cytotoxic activity using *in vivo* CTL assay. Groups of three mice were twice inoculated with 8 or 40 µg of the different RHDV VLPS: RHDV-VLP, RHDV-VLP-2 and RHDV-VLP-306. A control group for 100% of lysis, 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 untreated mice 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. 16**) were in the same range as the ones observed in untreated mice infected with VV-OVA ($6\pm0.3\times10^7$ 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 ($p<0.5$). Surprisingly, VV-OVA titers decreased to undetectable levels (limit of detection in our assay was 4 pfu/g) in ovaries from mice immunized with 40 μg of RHDV-VLP-2 (**Fig. 16**). 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 was more efficient than the insertion at the exposed loop.

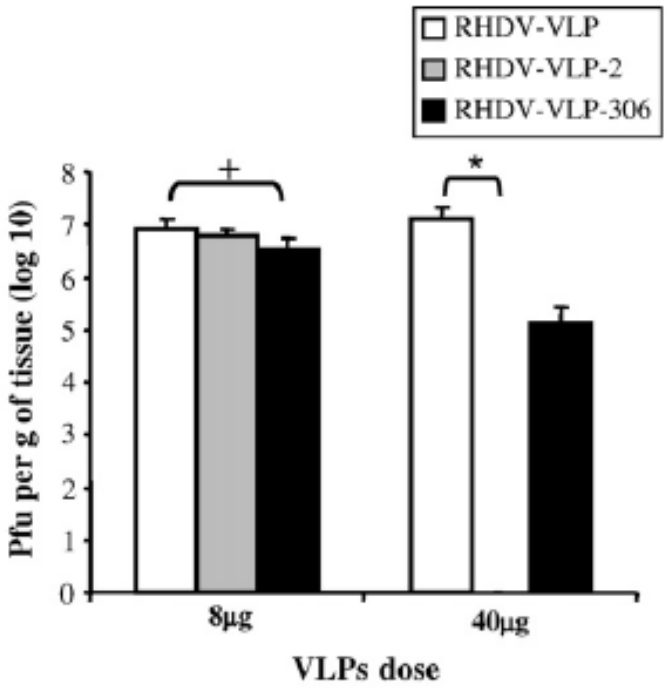


Fig. 16. Viral titres per gram of ovaries in mice immunized with the different RHDV VLPs and subsequently challenged with VV-OVA. Mice were infected with VV-OVA 6 days before analysis. Columns indicate viral titers after immunization with 2 doses of either 8 or 40 μg of RHDV VLPs after VV-OVA challenge. 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.

CHAPTER 2

DIFFERENTIAL CD8$^+$ T CELL RESPONSES GENERATED BY CHIMERIC CALICIVIRUS-LIKE PARTICLES DEPENDING ON THE ROUTE OF ADMINISTRATION

INTRODUCTION

Within the enveloped viruses, influenza virus is at the foreground, since the disease is a zoonose that remains one of the major threat to human health and is involving a wide range of animal species. Influenza virus is a negative-sense single stranded RNA virus belonging to the *Orthomyxoviridae* family. Influenza viruses A possess a segmented genome of eight segments and is surrounded by a lipid membrane containing two major glycoproteins, the hemagglutinin (HA) and neuraminidase (NA), and a minor but essential ion-channel protein M2 and a matrix protein M1, underneath the membrane (130). Additionally, within the virion, the nucleoprotein (NP) encapsidates the virus genome to form a ribonucleoprotein (RNP) particle for the purpose of transcription, replication and packaging. Phylogenetic analysis of virus strains isolated from different hosts revealed that NP gene is relatively well conserved (132). Moreover, it has been demonstrate that $NP_{366-374}$ (NP_{366}) peptide binds to class I H-$2D^b$ molecules and is a major cytotoxic T lymphocyte (CTL) epitope to protect influenza A infection in mice carring D^b (133). In human, the importance of this CTL response is not yet clearly defined, but studies suggest that it plays at least a co-operative role in virus clearance (134). In the absence of pre-existing strain-specific neutralizing antibodies, protection against influenza virus infection in humans mediated predominantly by CTLs has been inferred (135). The role of the CTL response is more clearly demonstrated by immunizing mice with influenza virus NP expressed by recombinant vaccinia virus (137-139), Semliki Forest virus (SFV) (140) or Sindbis virus (141), which have been shown to induce immune responses that, in some cases, confer at least partial protection (134). Additionally, immunization with naked DNA vectors encoding influenza virus NP has been shown to induce antibodies, cellular responses and protection against both homologous and heterologous challenge with influenza A virus variants (142-144). Another alternative strategy able to induce CTL responses against influenza A virus NP was by naked RNA immunization with replicons derived from poliovirus and SFV genomes. The intramuscular immunization of mice with these synthetic naked RNAs was able to induce antibodies against the NP. However, only mice immunized with rSFV-NP RNA also developed a CTL response against NP and reduced the virus load in the lungs after challenge with A/PR/8/34 virus (134). Finally, another study demonstrated that intranasal administration of synthetic NP_{366} peptide, encapsulated in

liposomes, was effective in inducing protective immunity against influenza virus in mice when anti-CD40 mAb was used as a mucosal adjuvant (145). We have to consider that no references are present in the bibliography about the possibility to use the CTL epitope NP inserted in VLPs.

In this study we report the generation of recombinant RHDV-VLPs, displaying a well defined $CD8^+$ T cell epitope of influenza A virus (FLU), strain A/PR/8/34. This foreign epitope corresponding to nucleoprotein (NP) aa 366-374 (ASNENMETM) was inserted at the N-terminus of RHDV capsid protein, which is predicted to be buried in the internal face of the VLPs. This N-terminal position to insert epitopes was described in the **chapter 1** of the present report to be appropriate for induction of cellular immune responses. Mice were immunized twice with RHDV-NP-VLPs without adjuvant, administered by intraperitoneal, subcutaneous or intranasal route.

The aim of this study was to analyze the immunogenic potential of chimeric VLPs (RHDV-NP-VLPs) in a murine system comparing different immunization routes using a FLU epitope.

MATERIALS and METHODS

Virus, cells and mice

The influenza virus strain used in the study was A/PR/8/34 that was obtained from Dr. Adolfo Garcia-Sastre (Mount Sinai School of Medicine, New York, USA). Viral stocks were prepared and titrated in MDCK cells.

Derivatives of *Autographa californica* nuclear polyhedrosis virus (AcNPV) were used to obtain the recombinant BVs expressing RHDV-VLPs. BVs were propagated in *Trichoplusia ni* cells (H5) grown in monolayer cultures at 28°C in TNM-FH medium (Sigma), supplemented with 5% FCS (Gibco). H5 cells were used for the generation of recombinant BVs, preparation of high titer viral stocks and for high level expression of the recombinant proteins.

Female C57BL/6JOlaHsd (Harlan) mice of 6- to 7-week-old age were used for immunization. All mice were kept under specific-pathogen-free-conditions and were maintained with free access to sterile food and water.

Construction of recombinant baculovirus transfer vectors

The primers used in this study are shown in **Table 4** (See **Annexes**). The same construction strategy described in **chapter 1** was used, changing the oligonucleotide primers used for cloning. DNA fragments were generated by annealing synthetic phosphorylated oligonucleotides NP366F/NP366R, leaving BamHI compatible ends as previously described in **chapter 1**. NP366F/NP366R encodes the amino acid sequence MGSVQIASNENMETMESGS, which contains the CTL epitope derived from influenza virus A/PR/8/34.

Generation of recombinant baculoviruses

The generation of recombinant BVs was performed as previously described in **chapter 1**.

Expression and purification of the recombinant RHDV-VLPs

For the purification of the different VLPs generated for this study we used a different protocol than in **chapter 1**.

Recombinant constructs VP60 and NP were expressed in H5 insect cell-cultures infected with the corrresponding recombinant BVs, as previously described (120). H5 cell monolayers were infected with recombinant baculoviruses at a multiplicity of infection of 10. After incubation (4 days, 28 °C), infected cells were gently dislodged into the growth medium and collected. The resulting suspensions were then washed three times with 0.2 M phosphate-buffered saline for VLPs (PBS-V; 0.2 M sodium phosphate, 0.1 M NaCl, pH 6.0) in order to separate intact cells from the culture medium. The pellets were then resuspended in distilled water, subjected to mild sonication and treated with DNAse I (Roche) for 1 h at RT. Next, samples were adjusted to 2% Sarkosyl (sodium N-lauroylsarcosine, Sigma) and 5mM EDTA in PBS-V, and incubated at 4ºC. Subsequently, cell lysates were clarified by low speed centrifugation and supernatants were centrifuged at 27,000 rpm for 2 h with a Beckman SW28 rotor. The pelleted material was resuspended in PBS-V, extracted twice with Vertrel XF (Fluka), and subjected to centrifugation (at 35,000 rpm for 2.5 h with a Beckman SW55 rotor) through a cushion of 1.5 ml of PBSv with 15% Opti-prep (a 60% solution of iodixanol in water, Gibco-BRL). The pellets were finally resuspended in PBS-V containing protease inhibitors (Complete, Roche) and stored at 4°C. Protein concentrations of VLP preparations were determined with a bicinchoninic acid protein assay kit (BCA protein assay kit, Pierce).

Immunization protocol

The following immunization scheme was used in two independent experiments: female mice were randomly divided in groups of six animals and inoculated twice in two week intervals with 100 µg of chimeric RHDV-NP-VLPs or RHDV-VLPs resuspended in 200 µl of sterile PBS (20 µl for intranasal inoculation). Mice were immunized without adjuvant using different routes: intranasal, subcutaneous and intraperitoneal. Finally, a group of mice were used as negative control. The experimental design is summarized in the **table 5**.

Table 5. Immunization protocol.

Group	Inoculum	Route
A	-	-
B	RHDV-VLPs	IP
C	RHDV-VLPs	SC
D	RHDV-VLPs	IN
E	RHDV-NP-VLPs	IP
F	RHDV-NP-VLPs	SC
G	RHDV-NP-VLPs	IN

IP: intraperitoneal. SC: subcutaneous. IN: intranasal

Evaluation of cellular responses

One week and two weeks after the second immunization, three mice were sacrificed and spleen cells were collected and analyzed for specific IFN-γ production by ELISPOT Set following manufacturer's instructions (Becton Dickinson UK). Spleen cells were added to triplicate wells at concentrations of $2x10^4$, 10^5 and $5x10^5$ cells/well with different stimuli: NP_{366-74} peptide (ASNENMETM, Sigma) at a concentration of 10 μM per well or VP60 at a concentration of 50 μg/ml per well or $5x10^6$ pfu/ml of PR8. *In vitro* culture was incubated overnight. Triplicate wells with $5x10^5$ cells without stimuli were used to estimate the non-specific activation. As positive control, triplicate wells with $5x10^5$ cells were stimulated with phytohemagglutinin (PHA) (Sigma) at a concentration of 10 μg/ml.

Intracellular cytokine staining assay

IFN-γ-secreting $CD8^+$ cells were detected using the protocol recommended by the manufacturer (Cytofix/Cytoperm Plus Kit, PharMingen, San Diego, CA, USA) at day 7 and day 14 after the second immunization. Briefly, splenocytes were isolated from the mouse, and a single cell suspension was incubated with 1 μM of influenza NP peptide for 6 h at 37ºC. At 3 h before the end of incubation, 2 μg/ml of Brefeldin A was added. The cells were washed and blocked with 4% normal mouse sera and stained with PerCP-conjugated anti-mouse CD8 mAb (BD). Cells were then resuspended in 250 μl of Cytofix/Cytoperm solution at 4ºC for 20 min, washed with Perm/Wash solution and

stained with anti-mouse IFN-γ mAb conjugated with FITC (BD) at 4ºC for 30 min followed by flow cytometric analysis.

Virus challenge

To test protection against FLU we performed another experiment using 9 mice/group with only intraperitoneal injection of VLPs. Two weeks after the last immunization, mice were anesthetized with avertin and infected intranasally with $4x10^3$ PFU/mice of influenza A/PR/8/34 (PR8) in 20 µl PBS.

These mice were kept under observation for 14 days and body weight and survival rates were calculated. Six mice of each group were sacrificed 3 days after challenge infection and lung homogenates were prepared and titred for virus on MDCK cell monolayer. As control, non-immunized, naïve mice were infected with the same amount of PR8 virus.

Statistical analysis

All statistical analysis was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). For all analyses, mouse was used as the experimental unit. The significance level (α) was set at 0.05 with statistical tendencies reported when $p<0.10$. The Shapiro Wilk´s and the Levene test were used to evaluate the normality of the distribution of the examined quantitative variables and the homogeneity of variances, respectively. A non-parametric test (Mann–Whitney) was chosen to compare the different values obtained for all the immunological parameters between groups all the sampling times.

RESULTS

Generation of RHDV recombinant VLPs

In this study chimeric RHDV-VLPs were generated with NP CTL peptide inserted in the N-terminus of VP60. Expression of VP60 construct in H5 insect cell cultures infected with the corresponding recombinant baculovirus was verified by SDS-10% PAGE, with similar results as shown in **figure 7** in **chapter 1**. In addition, self-assembling into VLPs was studied by electron microscopy, obtaining similar results as the one shown in **figure 8** in **chapter 1**.

Induction of cellular responses by chimeric RHDV-VLPs

Taking into account that NP_{366} sequence is an immunodominant CTL epitope in C57Bl/6 mice (possessing H2-D^b MHC-I), it was conceivable to assume that a good vaccine vector carrying such epitope would induce specific IFN-γ-secreting cells and/or CTLs. Indeed, one week and two weeks after the last immunization of mice with chimeric RHDV-VLPs, specific IFN-γ-secreting cells were detected in spleen by ELISPOT. On day 7 after the second immunization, groups inoculated IP and SC with chimeric RHDV-VLPs have shown higher responses against NP peptide compared with IN inoculated mice (**Fig. 17A**). IP and IN inoculations showed a statistical tendency with $p = 0.08$ when compared with control animals and vector-stimulated animals, whereas SC injection did not shown any statistical differences with the other groups. As expected, groups inoculated with native RHDV-VLP and control group did not show any specific NP response (**Fig. 17A**). Regarding the cellular immunity against the vector, group F showed the highest response compared with the other groups but it was not statistically significative; negative control group did not induce any specific response (**Fig. 17A**).

When the response was evaluated at day 14 after the second immunization, similar results were found against NP. Groups E and F elicited higher number of IFN-γ-secreting cells and responses were slightly higher than day 7 (**Fig. 17B**). In this case both routes showed statistical tendency ($p = 0.07$) when compared with control group. Groups inoculated with native VLPs and control group confirmed the same results of day 7, showing no responses against NP peptide. An interesting finding was that chimeric VLP immunization was able to induce similar specific IFN-γ-secreting cells

against the vector in all groups inoculated IP and SC on day 14 ($p > 0.1$). IN groups remained the lowest responsive ones and the negative control group did not show any specific response (**Fig. 17B**). Responses on day 14 against native VLPs showed the same pattern than the responses on day 7 except for group F (**Fig. 17**).

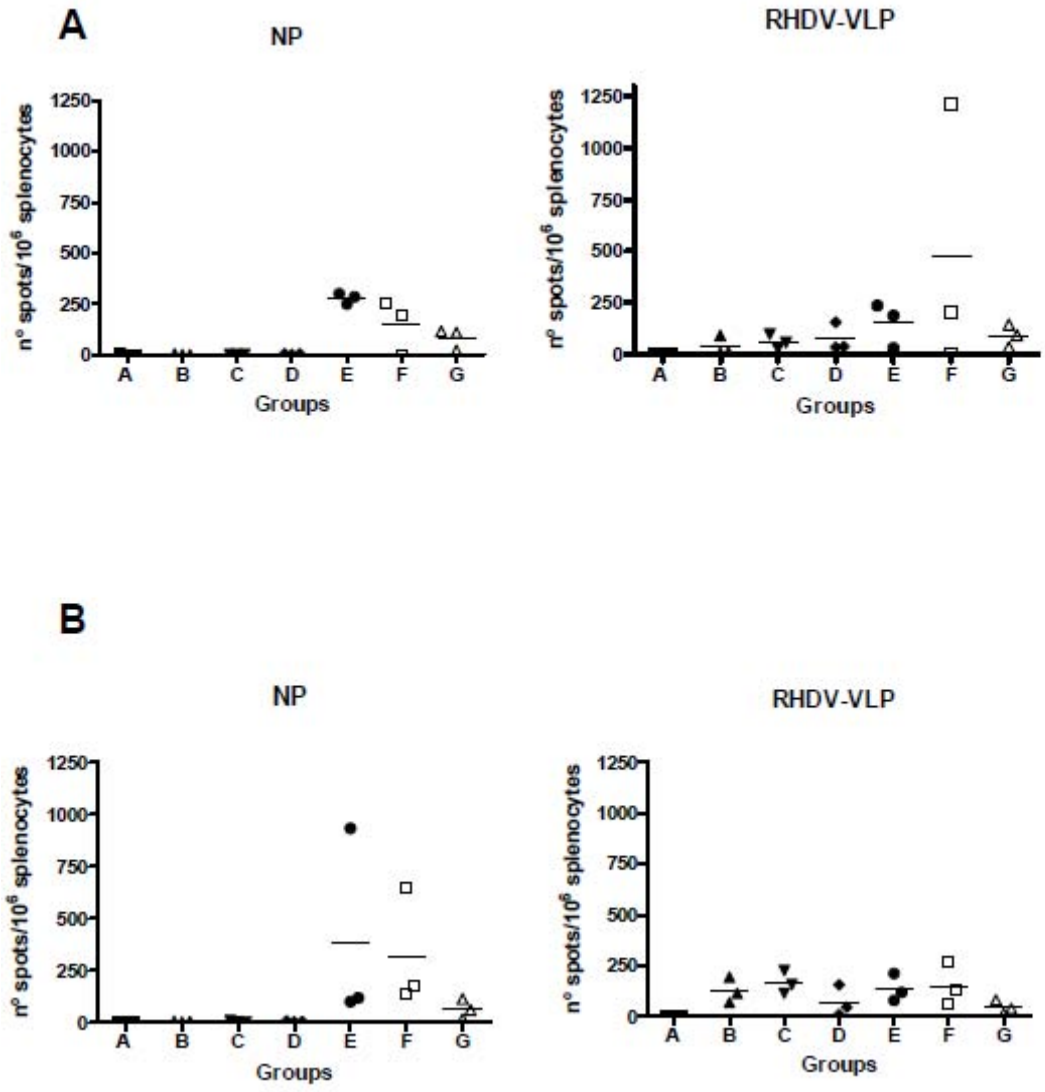


Fig. 17. Frequency of NP and RHDV-VLP-specific IFN-γ-producing cells in the spleen of immunized mice and day 7 (A) and 14 (B) after the second immunization. Groups of three animals were twice inoculated with 100 µg of the RHDV-NP-VLPs and specific IFN-γ-producing cells were measured by ELISPOT assay. The data shown are the results obtained in triplicates for each animal with the line representing the means of the group. Data shown are representative of two independent experiments.

Mice inoculated with chimeric RHDV-VLPs stimulated in the assay with PR8 induced a lower number of specific IFN-γ-secreting cells compared with the stimulation with the peptide. Also in this case, the parenteral route showed higher responses compared with intranasal immunization (**Fig. 18**). However, no statistical differences were found in all the chimeric VLP-immunized groups.

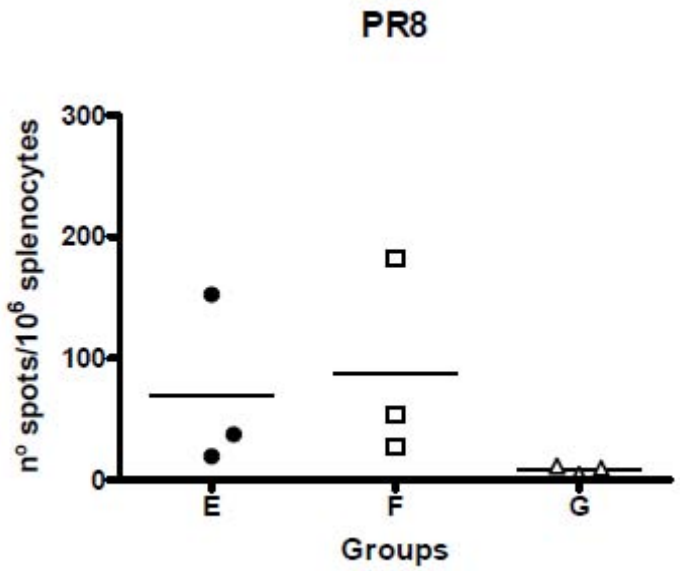
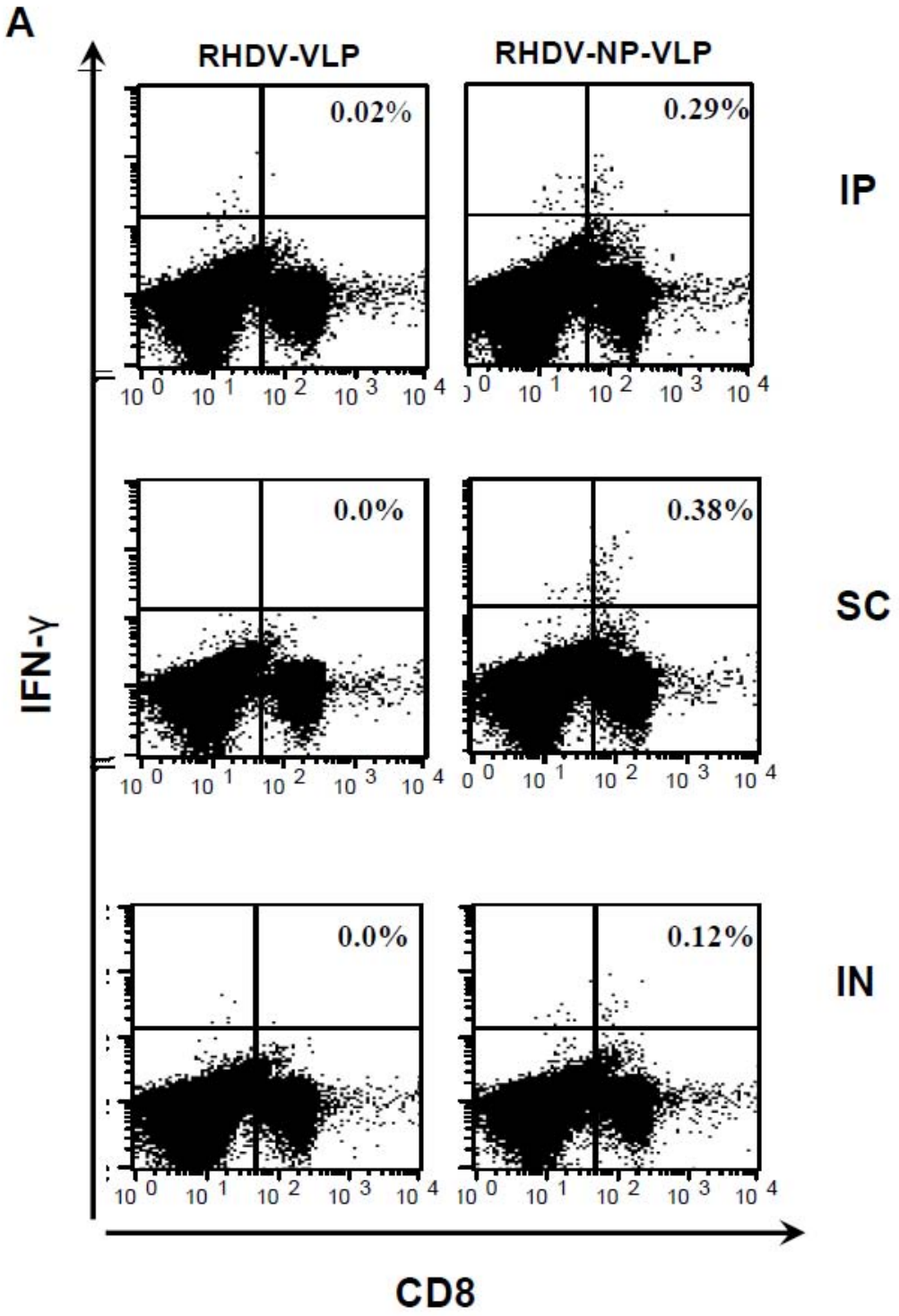


Fig. 18. Frequency of PR8-specific IFN-γ-producing cells in the spleen of immunized mice at day 14 after the second immunization. Groups of three animals were twice inoculated with 100 μg of the RHDV-NP-VLPs and specific IFN-γ-producing cells were measured by ELISPOT assay. The data shown are the results obtained in triplicate for each animal with the line representing the means of the group. Data are representative of one experiment.

By counterstaining with anti-CD8 Ab, the number of IFN-γ-producing $CD8^+$ cells was found to be higher on days 7 (**A**) and 14 (**B**) in the group inoculated SC with chimeric RHDV-VLPs compared with the other groups (**Fig. 19**). This analysis also confirmed that chimeric RHDV-VLPs induced an increase in the number of IFN-γ-producing $CD8^+$ cells when compared with native VLP-immunized mice that did not show specific responses (**Fig. 19**).


A
RHDV-VLP
RHDV-NP-VLP
0.02%
0.29%
IP
0.0%
0.38%
SC
0.0%
0.12%
IN
IFN-γ
CD8
10 0
10 1
10 2
10 3
10 4

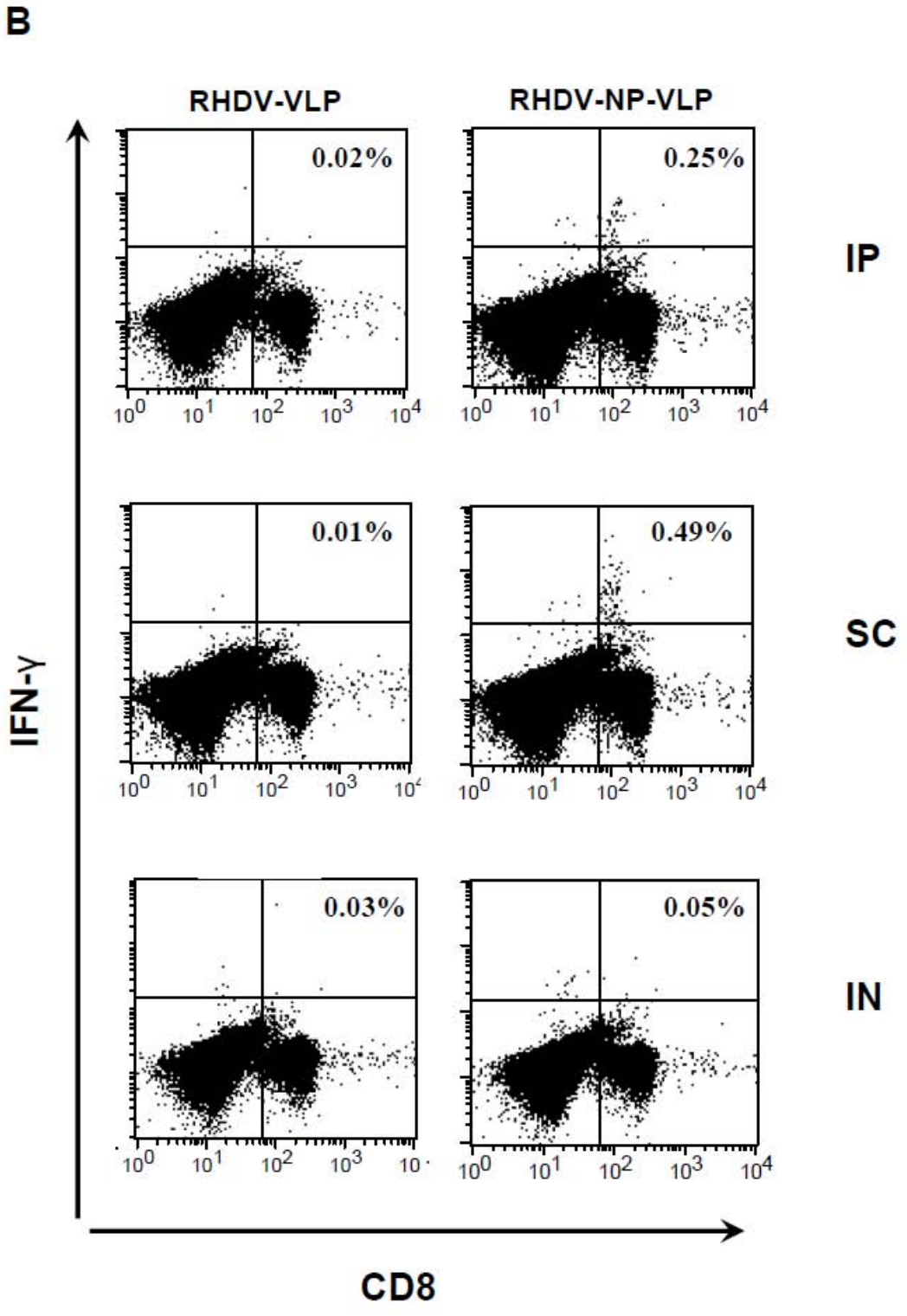


Fig. 19. ICCS assay. Frequency of influenza protein-primed IFN-γ-producing $CD8^+$ T cells in immunized mice. Three mice were immunized with the native vector RHDV-VLP or with chimeric RHDV-NP-VLPs. The splenocytes of these mice were used for an IFN-γ ICCS assay one week (**A**) or two weeks (**B**) after the last immunization. The typical percentage of IFN-γ-producing cells in $CD8^+$ T cells are shown in each dot plot.

Viral challenge

Infection with PR8 was not reproducible enough due to technical problems related to the intranasal infection. Indeed, most of the naïve mice or vector (RHDV-VLP) immunized mice inoculated with PR8 did not shown any symptoms or loss of weight (**Fig. 20**) and no viral load was detected in the lungs of selected mice on day 3 post infection (**Fig. 21**). Therefore, the level of protection induced by RHDV-NP-VLPs was left unassessed.

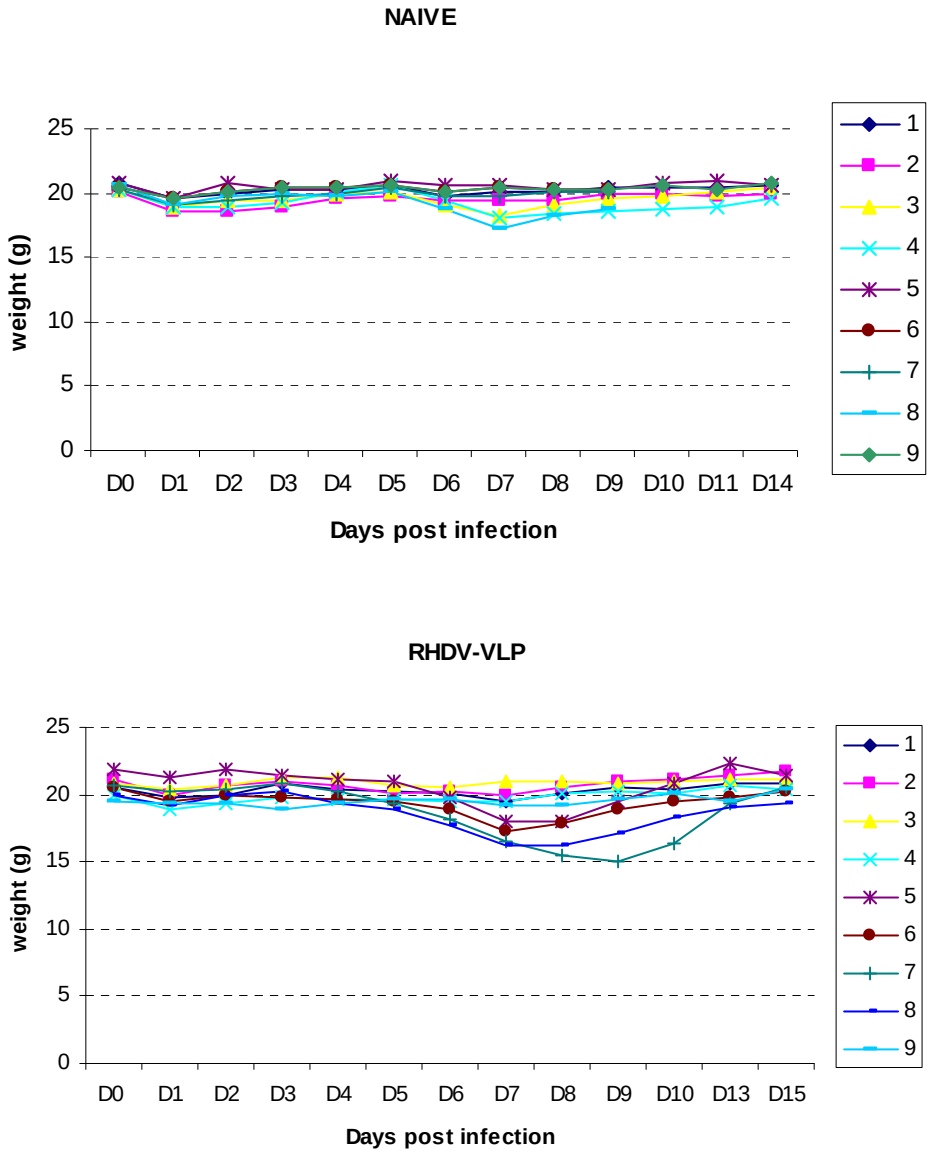


Fig. 20. Body weight of naïve and RHDV-VLP immunized mice after the PR8 infection.
Mice were infected with $4x10^3$ PFU/mice of influenza A/PR/8/34 in 20 µl PBS and body weight was analysed during all the experiment for 14-15 days. No loss of weight or only mild loss of weight was observed in mice infected with PR8.

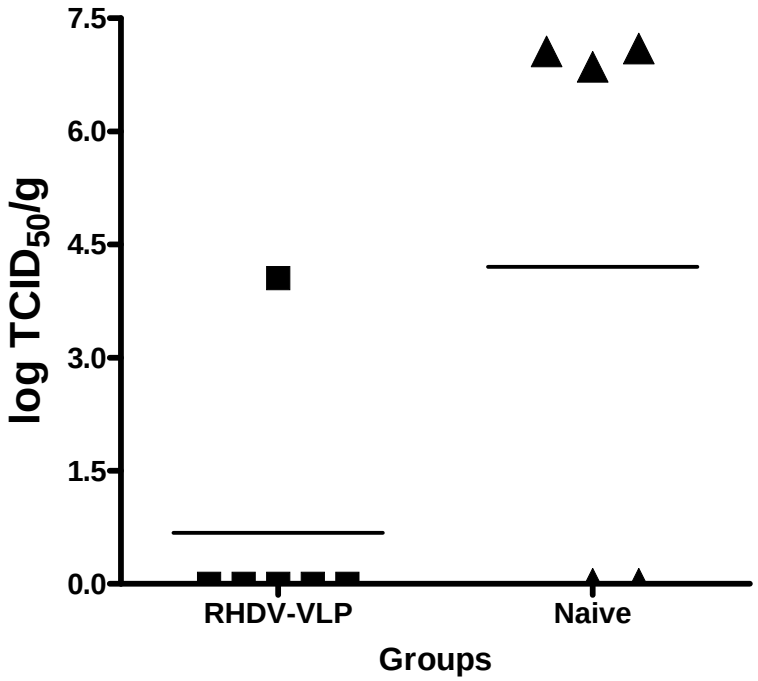


Fig. 21. Viral load in the lung of naïve and RHDV-VLP immunized mice at day 3 after PR8 infection. Mice were infected with $4x10^3$ PFU/mice of influenza A/PR/8/34 in 20 µl PBS. Viral load (log $TCID_{50}$/g) was determined in MDCK cells (four wells for each dilution). RHDV-VLP immunized mice and some naïve mice (negative control) did not show any viral load after PR8 infection. Data obtained from one experiment.

“Pig performance”

Jon Cattapan

Australia, 1978

CHAPTER 3

CALICIVIRUS-LIKE PARTICLES INDUCE ACTIVATION OF PORCINE DENDRITIC CELLS *IN VITRO*

INTRODUCTION

In this study we used native RHDV-VLPs, previously described in **chapter 1**.
The aim of this study was to analyze the immunogenic potential of RHDV-VLPs in a porcine system *in vitro*.

By studying the immunogenicity of RHDV-VLPs *in vitro* in pigs, a serendipitous finding occurred: RHDV-VLPs were able to stimulate the expression of long pentraxin 3 (PTX3) protein in porcine bone marrow derived dendritic cells. This interesting result paved the way for the first characterization of PTX3 in pigs.

The innate immune system constitutes the first line of defence against microorganisms and plays a primordial role in the activation and regulation of adaptive immunity. Components of the humoral arm include members of the complement cascade and soluble pattern recognition molecules (PRMs), such as collectins, ficolins and pentraxins (146, 147). Pentraxins are acute phase multifunctional proteins with multimeric organization and are phylogenetically conserved from arachnids to mammals (146, 148, 149). Long pentraxin 3 (PTX3) is the prototype of this family and is composed of eight identical protomers associated via disulphide bonds, forming an elongated asymmetric molecule with two different sized domains interconnected by a stalk (150). High homology is found between human and murine PTX3 promoters (151).
A variety of cell types express PTX3 upon exposure to inflammatory signals, such as cytokines (e.g. IL-1β, TNF-α), TLR agonists, microbial moieties (e.g. LPS, OmpA, lipoarabinomannans) or microorganisms (146, 148, 149). Myeloid DCs are the major source of PTX3, although this molecule is also expressed by a number of other cell types. In contrast, PTX3 expression is undetectable in T and B lymphocytes, natural killer (NK) cells and plasmacytoid DCs (146-148). Neutrophils are a reservoir of ready-to-use PTX3, released in minutes, whereas DCs and macrophages produce this molecule *de novo* in response to inflammatory signals (149, 152). PTX3 is also involved in resistance against some viral infections. Indeed, human and murine PTX3 binds influenza virus through interaction between viral haemagglutinin (HA) glycoprotein and the sialic acid residue present on PTX3. Here, the first characterization of SwPTX3 was

described using *in vitro* analysis with porcine bone-marrow derived DCs (poBMDCs). Knowing the importance of myeloid DC in PTX3 production, we investigated the response of poBMDCs after incubation with TLR, virus (swine influenza virus, SwIV) and RHDV-VLPs. Swine PTX3 production in poBMDCs upon SwIV infection and RHDV-VLPs was described for the first time using cross-reactive antibodies.

MATERIALS AND METHODS

Virus and cells

Derivatives of AcNPV were used to obtain the recombinant BVs expressing RHDV-VLPs as previously described in **chapter 1**. H5 cells were used as previously described in **chapter 2**.

Construction of recombinant baculovirus transfer vectors

The same construction strategy described in **chapter 1** was used.

Generation of recombinant baculoviruses

The generation of recombinant BVs was performed as previously described in the **chapter 1**.

Expression and purification of the recombinant RHDV-VLPs

For the purification of RHDV- VLPs generated for this study we used the same protocol than in **chapter 2**.

Porcine bone marrow derived dendritic cell generation

Porcine bone marrow (BM) hematopoietic cells were obtained from femurs of healthy Large white X Landrace pigs of eight weeks of age. BM cells were negative to: PCV2 by RT-qPCR as previously described (154) and by ELISA to influenza virus and *Actibobacillus pleuropneumoniae* (HIPRA, Amer Spain), *Mycoplasma hyopneumoniae* (OXOID, Cambrige UK), porcine PV and Aujeszky's disease virus (INGENASA, Madrid, Spain), porcine reproductive and respiratory syndrome virus (PRRSV) (IDEXX Europe B.V., Netherlands) and *Salmonella spp.* (SVANOVA Biotech AB, Uppsala, Sweden). PoBMDCs were generated by an eight-day protocol as previously described

by (155) with some modifications (156). Briefly, BM was extracted from femur and cell suspensions were cultured in RPMI 1640 complete medium (LONZA) containing 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 U/ml polymyxin B (Sigma-Aldrich, St. Louis, USA), and 100 ng/ml recombinant porcine granulocyte macrophage colony-stimulating factor (rpGM-CSF) (R&D Systems, Abingdon, Oxon, United Kingdom). Fresh medium was given every three days. The BM progenitors were incubated at 37°C in 5% CO_2 in Petri dishes for 8 days and then immature poBMDCs were stimulated as described below. On day 9, mature poBMDCs were harvested and analyzed by flow cytometry and cytokine production.

Stimulation of poBMDCs

PoBMDCs were stimulated with different concentration of RHDV-VLPs (10 and 50 µg/ml). LPS (10 µg/ml; Sigma) or poly:IC (50 µg/ml; Sigma) were used as positive control (TLR agonists). Briefly, stimuli were plated with poBMDCs obtained after 8 days in culture ($5x10^5$ cells/well) in 96-well plate. Activation of DCs was analyzed by flow cytometry at 24 hours post stimulation and by cytokines release in the supernatant at different time-points (4, 8, 16, 24 hours post stimulation) using specific ELISAs. Non-stimulated cells cultured in complete medium served as control. PoBMDCs data were obtained for each experiment from four different animals.

Additionally, poBMDCs were infected with H3N2 (A/Swine/Spain/SF32071/2007) swine influenza virus (SwIV) using a procedure modified from the previously described methodology by Rimmelzwaan *et al.* (157). Briefly, 10^6 poBMDCs were infected with 10^6 $TCID_{50}$ (multiplicity of infection, MOI = 1) of previously porcine trypsin type IX (Sigma) treated H3N2. Then, cells were incubated for 1 h at 37ºC 5% CO_2 for virus adsorption. After this time, cells were washed once with PBS with 2% FCS and 400 µl of RPMI 1640 complete medium was added. Also, poBMDCs were stimulated with 10 µg/ml of LPS (Sigma) and 50 µg/ml of RHDV-VLPs for 8 h and overnight (ON). Uninfected and unstimulated poBMDCs were used as negative control.

Flow cytometry analyses of poBMDCs

Flow cytometry analysis of poBMDCs was performed using an indirect labelling for CD172a, SLA-I, SLA-II, CD4, CD1c, CD11R3, CD11R1, SWC1, CD40, CD80/86, CD86 and CD163 and direct labelling for CD14 and CD16. Unless specified below, the

surface markers were detected by hybridoma supernatants. Briefly, $5x10^5$ cells/50µl/well were labelled during 1 h at 4ºC for each CD marker, using 50 µl of antibody solution. Anti-CD172a (SWC3, BA1C11), anti-SLAI (4B7/8), anti-SLAII (1F12), anti-CD1c (76-7-4), anti-CD4 (76-12-4), anti-CD11R1(MIL4, IgG1, Serotec, bioNova cientifica, Madrid, Spain), anti-CD11R3 (2F4/11), purified anti-human CD40 (G28.4, Biolegend, San Diego CA, USA), anti-SWC1 (76-6-7), anti-CD14-FITC (MIL2, Serotec), anti-CD16-FITC (G7, Serotec), CTLA4-mIg (Ancell, Minnesota, USA), purified anti-porcine CD86 (mouse mAb 5B9.88, Alexion Pharmaceuticals, Cheshire, CT, USA) (158) and anti-CD163 (2A10/11) were used. After incubation, they were washed with cold PBS with 2% FCS by centrifugation at 450g, 4ºC for 5 minutes. Then, the secondary antibody R-phycoerythryn anti-mouse IgG (Jackson ImmunoResearch, Suffolk, UK) diluted 1:300 was added when required. Cells were incubated for 1 hour at 4ºC, washed as before and resuspended in PBS with 2% FCS. Stained cells were acquired on Coulter® EPICS XL-MCL cytometer and analysed by EXPO 32 ADC v.1.2 program. A gate strategy was applied in 85% of living cells using the forward and side scatter (FS/SS).

Cytokine ELISAs

Cytokine levels in conditioned cell supernatants were assayed by ELISAs for porcine IFN-α, TNF-α, IL-18, IL-6 and IL-10**.** Different time-points (4, 8, 16, 24 h) were tested. For each ELISA, triplicate wells of stimulated or un-stimulated cells supernatants were used and all the results were analyzed with KC Junior Program (Bio Tek Instruments, Inc) using the filter Power Wave XS reader.

To detect IFN-α an in-house ELISA for anti-IFN-α (around 4 U/ml of detection limit) was performed using commercial antibodies purchased from PBL Biomedical Laboratories (Piscataway, NJ, USA) as previously described (159).

For TNF-α, IL-6 and IL-10 detection, a DuoSet® ELISA Development system for porcine TNFα/TNFSF1A, IL-6 and IL-10 respectively (R&D Systems, Abingdon, UK) was used following the manufacturer's instructions. The limit of detection was around: 148 pg/ml for TNF-α, 70 pg/ml for IL-6 and 50 pg/ml for IL-10.

For IL-18 secretion the kit Pig IL-18 Module Set BMS672MST (Bender Med Systems, Vienna, Austria) was used following the manufacturer's instructions. The limit of detection was around 74.5 pg/ml of IL-18.

PTX3 and anti-PTX3 antibodies

Recombinant human and murine PTX3 were purified under endotoxin-free conditions by immunoaffinity from the supernatants of stably transfected CHO cells, as previously described (160).

Polyclonal rabbit antiserum was obtained by immunization with purified recombinant human PTX3. Specific IgG anti human PTX3 were subsequently purified by immunoaffinity. Rat monoclonal antibodies MNB4 and 16B5 were raised against human PTX3 and shown to respectively recognize epitope (87-99) in the N-terminal domain and epitope (306-312) in the C-terminal domain (161). 6B11 and 2C3 monoclonal antibodies were raised against murine PTX3 by immunization of *ptx3-/-* mice (162). The epitopes recognized by 6B11 and 2C3 have not been identified. Biotinylated antibodies were obtained following standard protocols.

***In silico* study for SwPTX3**

PTX3 sequences from human (GenBank accession number: NP_002843.2) and swine (predicted; GenBank accession number: XP_003132554.1) were retrieved from the National Centre for Biotechnology Information (NCBI) database. Sequences were subjected to blast analysis [http://www.ncbi.nlm.nih.gov/BLAST] and Bioedit software (Ibis Biosciences, CA, USA) to find the homology between them. Amino acids recognized by MNB4 and 16B5 antibodies in the human PTX3 sequence were compared to the predicted swine PTX3 sequence.

Western blot analysis for PTX3

PoBMDCs were infected overnight with influenza virus, or stimulated overnight with LPS or with RHDV-VLPs. After incubation, cells were washed with 1x PBS and lysed with lysis buffer containing 50 mM Tris-HCL, pH8, 150 mM NaCl, 2mM EDTA, 10 % Triton X-100 and a protease inhibitor cocktail (Sigma) at 4°C. After centrifugation at 12.000 x g for 15 min at 4ºC, supernatant (lysate) was collected.

Each lysate was generated from $3x10^6$ total cells/sample with an average of total protein around 1.7 mg/ml. Fifteen microlitres of cell lysate were subjected to denaturing SDS-PAGE (NuPAGE® Novex 4-12% Bis-Tris gel; Invitrogen Corporation) followed by Western Blot analysis. Gels were electroblotted to a Hybond ECL™ nitrocellulose membrane (GE Healthcare) followed by blocking in 5% skimed milk. After incubation

of membranes with primary antibody MNBA or 16B5 (1:10000 dilution) at room temperature (RT) for 1 h, three washes with tris-buffered saline + 0.1% Tween 20 (TBS-Tween) 10 min each followed. Anti-rat secondary antibody HRP conjugate (Invitrogen; 1:100000 dilution) was added and membrane incubated at RT for 1 h followed by three washes. Proteins were revealed with ECL Advance™ Western Blotting Detection Kit (GE Healthcare) as described by the manufacturer. RPMI 1640 complete medium (LONZA) with 10% of FCS, used for cell culture, was included as background control and the isotype control and first antibody omission were used as negative controls.

Proteins were visualized using Fluorochem HD2 chemiluminescencent workstation (Alpha Innotech) as described by the manufacturer.

Immunofluorescence analysis for PTX3

PoBMDCs were fixed with 4% paraformaldehyde, permeabilized in 0.5% Triton X-100 and incubated with blocking solution (3% bovine serum albumin (BSA)/1x PBS) for 1 hour at RT. Labelling of PTX3 producing cells was performed using three different antibodies: biotinylated anti-mouse PTX3 antibody (6B11) (162) or rat anti-human PTX3 (MNB4 and 16B5) (161). These primary antibodies (1:100 dilution) were incubated ON at 4ºC. Detection of influenza infected cells was determined using mouse anti-NP antibody (HB65 ATCC), incubated ON at 4ºC; anti swine leukocyte antigen class II (SLAII-DR, clone 1F12) (INIA, Madrid) (163) antibody for cell phenotyping was used for 1 h at RT.

Rat anti-HuPTX3 antibodies were detected using goat anti-rat IgG conjugated with Alexa 488 or Cy3 fluorophores (1:200 dilution) (Invitrogen) and 6B11 with TRITC-conjugated streptavidin (1:300 dilution) (ZYMED® Laboratories, California). The rest of primary antibodies against cell markers and influenza virus were detected using goat anti-mouse IgG secondary antibodies coupled to Alexa 555 (Invitrogen) or Cy2 fluorophores (1:200 dilution) (Jackson ImmunoResearch Europe Ltd). Finally, nuclei were counterstained with DAPI and mounted in Fluoprep (BioMérieux, France). Negative control procedures were applied for all immunofluorescent tests. Specifically, in each specimen, negative controls to assess false positivity, autofluorescence and cross-reactivity including lack of primary antibody were performed.

Microscopy and image analysis for PTX3

Fluorescent preparations were viewed on a Nikon eclipse 90i epifluorescence microscope equipped with a DXM 1200F camera (Nikon Corporation, Japan). To assess the co-localisation between different fluorescent labels, image stacks of sections were captured using the Leica TCS SP5 confocal microscope (x 63/NA 1.4 objective). Images were processed by using the LAS AF Lite program from Leica and Image J v1.42k software [http://rsb.info.nih.gov/ij]. All sections were systematically examined in full and pictures were taken as representative areas for each section.

Statistical analysis

All statistical analysis was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). For all analyses, pig was used as the experimental unit. The significance level (α) was set at 0.05 with statistical tendencies reported when $P<0.10$. The Shapiro Wilk´s and the Levene test were used to evaluate the normality of the distribution of the examined quantitative variables and the homogeneity of variances, respectively. A non-parametric test (Mann–Whitney) was chosen to compare the different values obtained for all the immunological parameters between groups all the sampling times.

RESULTS

Maturation of poBMDCs after RHDV-VLP stimulation

Consistent with previous reports (155, 156) poBMDCs phenotype at day 8 of culture was CD172a^{+},SLAI^{+}, SLAII^{+}, CD1^{+}, CD4^{-}, CD11R1^{-}, CD14^{+}, CD16^{+}, CD40^{-}, CD80/86^{+} and CD163^{low}. At this time, the population of poBMDCs was rather homogenous and they constituted our starting culture for further experiments. The poBMDCs maturation induced by RHDV-VLPs was evaluated after 24 hours of stimulation by flow cytometry analysis of the cell surface markers. Pulsing poBMDCs with RHDV-VLPs resulted in increased levels of maturation markers SLA-II, CD80/86 and CD86 (**Fig. 22**), but the levels of up-regulation were not statistically different as compared to unpulsed cells. Additionally, the LPS stimulation of surface markers was, in some cases, lower than RHDV-VLP stimulation (**Fig. 22**). A dose-dependent up-regulation of SLA-II and CD80/86 was found (**Fig. 23**). No differences were found in all the other surface markers (data not shown).

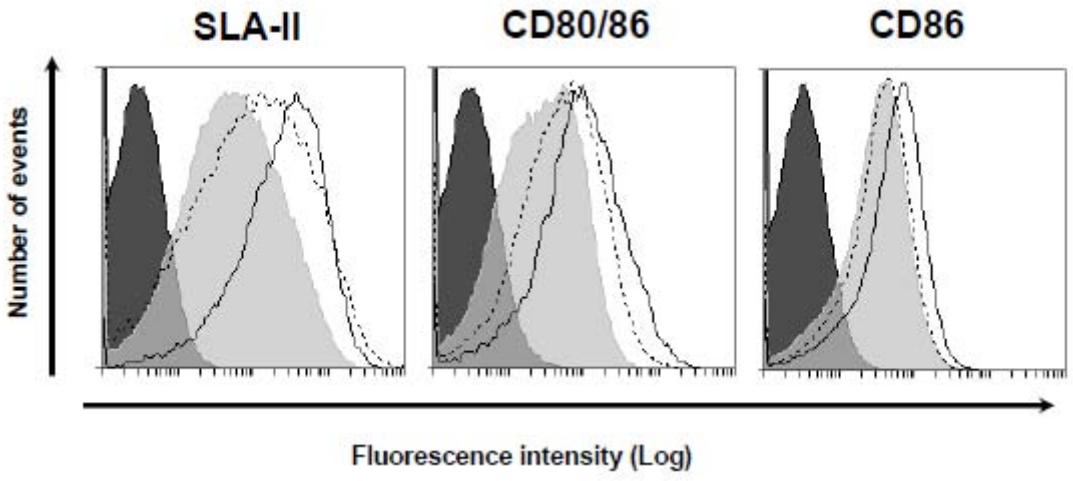


Fig. 22. Phenotypic maturation of poBMDCs after exposure to RHDV-VLPs or LPS. PoBMDCs were incubated with either RHDV-VLPs (50 µg/ml) or LPS (10 µg/ml) and after 24 h of culture, surface expression of SLA-II, CD80/86 and CD86 was determined by flow cytometry using PE-conjugated secondary mAb. Histograms show expression patterns on poBMDCs gated for FS/SS. RHDV-VLP-pulsed poBMDCs (solid line) and LPS-exposed poBMDCs (dotted line) express higher levels of all markers as compared to un-stimulated poBMDCs in steady state (grey histograms) or isotype control (black histograms). Results of one representative experiment are shown out of a minimum of five independent experiments.

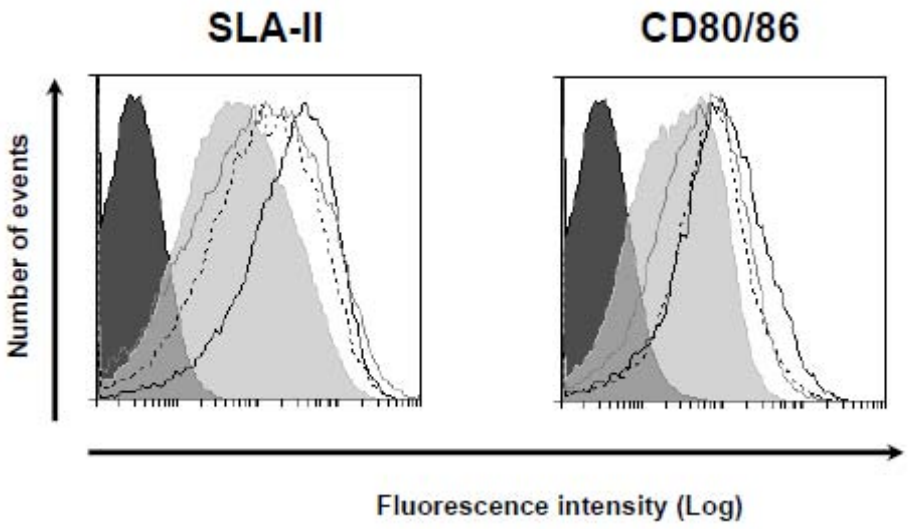


Fig. 23. Phenotypic maturation of poBMDCs after exposure to RHDV-VLPs or LPS. PoBMDCs were incubated with either RHDV-VLPs (10, 50 µg/ml) or LPS (10 µg/ml) and after 24 h of culture, surface expression of SLA-II and CD80/86 was determined by flow cytometry using PE-conjugated secondary mAb. Histograms show expression patterns of poBMDCs gated for FS/SS. PoBMDCs pulsed with 50 µg/ml (solid black line) or with 10 µg/ml (dotted black line) and LPS-exposed poBMDCs (solid grey line) express higher levels of all markers as compared to un-stimulated poBMDCs (grey histograms) or isotype control (black histograms). The responses to RHDV-VLPs is dose-dependent. Results of one representative experiment are shown out of a minimum of five independent experiments.

RHDV-VLPs induced secretion of cytokines by DCs

To investigate whether RHDV-VLPs were capable of inducing cytokine production in poBMDCs, levels of TNF-α, IL-18, IL-6, IL-10, IL-12 and IFN-α were determined in supernatants of poBMDCs cultured in the presence or absence of RHDV-VLPs at different time-points. LPS and poly:IC were used as positive controls. RHDV-VLPs induced the production of TNF-α and IL-6, with maximum levels between 16 and 24 h (**Fig. 24**) and the differences with un-stimulated DCs showed a statistical tendency ($0.05 < p < 0.07$) . When kinetics of cytokine production was analyzed, secretion of IL-6 and TNF-α in response to the different RHDV-VLPs occurred more slowly than that induced by LPS or poly:IC, which rapidly induced pro-inflammatory cytokines as early as 4 h after stimulation. Moreover, RHDV-VLP cytokine induction was lower than LPS or poly:IC induction (**Fig. 24**) with a statistical tendency ($0.05 < p < 0.08$). RHDV-

VLPs did not induce DCs to secrete IFN-α, IL-10 or IL-18, at least not above the detection limit of the assay (data not shown).

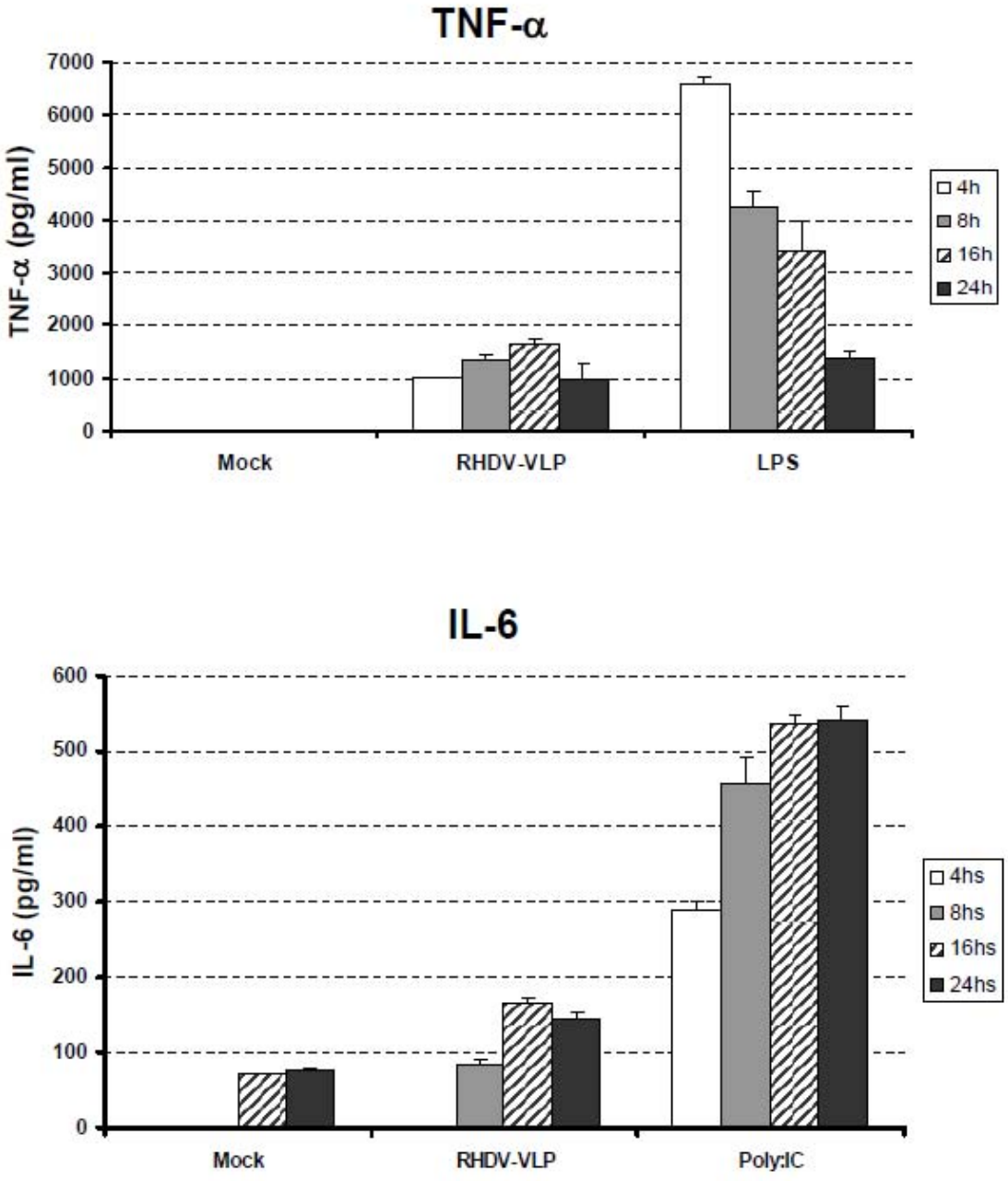
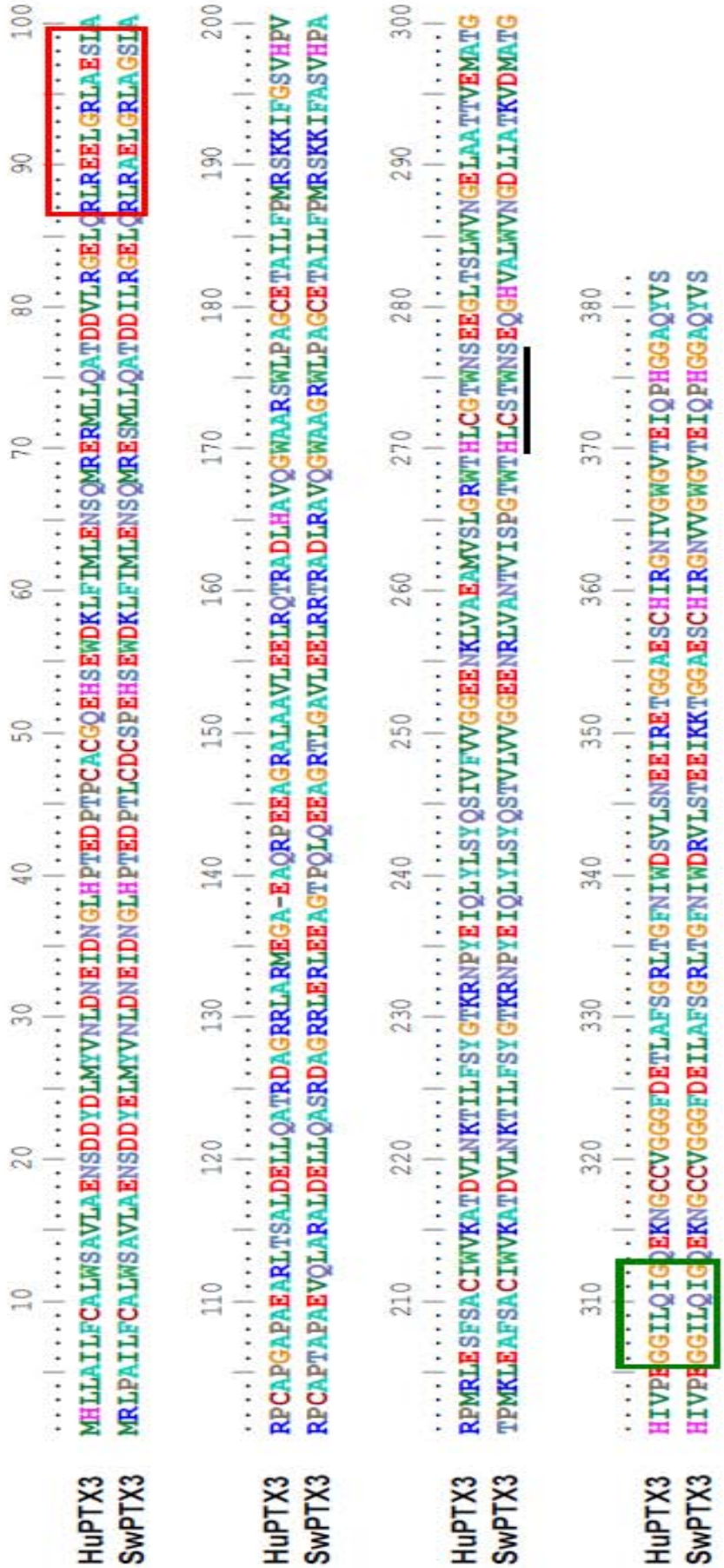


Fig. 24. Cytokine production of poBMDCs pulsed with VLPs and LPS or poly:IC *in vitro*. Immature poBMDCs were pulsed with RHDV-VLPs (50 µg/ml) or LPS (10 µg/ml) or poly:IC (50 µg/ml). After 4, 8, 16 and 24 h of culture, secreted TNF-α and IL-6 were quantified by commercially available ELISAs. Cytokine levels in culture supernatants are shown in pg/ml (mean ± standard deviation). As controls, poBMDCs were cultured in medium alone (Mock). The limit of detection was around 148 pg/ml for TNF-α and 70 pg/ml for IL-6. Results of triplicate wells of one representative experiment are shown out of a minimum of three independent experiments.

In silico comparison and Western blot analysis for SwPTX3

An alignment analysis of HuPTX3 and the predicted SwPTX3, full length aa sequences (Clustal W., European Bioinformatics Institute) was performed to analyse the homology between both sequences. Results in **figure 25** showed that the alignment of the two proteins exhibited an aa homology of 84%. The C-terminal pentraxin-like domain of SwPTX3 predicted protein was well conserved compared to HuPTX3, sharing 86% of identity. The alignment of human and the predicted SwPTX3 aa sequences showed that epitope (302-312) was fully conserved in both species (**Fig. 25**), suggesting that 16B5 could be cross-reactive for SwPTX3, while the N-terminal extremity exhibited a lower percentage of identity (83%) between both species. In particular, the alignment of both aa sequences showed that epitope (87-99) is not totally conserved in both species. Glutamic acid (E) at positions 90 and 96 is replaced by Alanine (A) and Glycine (G) respectively (**Fig. 25**). Thus, the cross-reactive activity of MNB4 might not be complete. Importantly, the SwPTX3 aa predicted sequence contains the canonical ‘‘pentraxin consensus signature’’ (HxCxS/TWxS) (**Fig. 25**).

Fig. 25. Alignment analysis of amino acid (aa) residues for predicted swine PTX3 full length (SwPTX3) and human PTX3 (HuPTX3). Green square indicates the 16B5 binding epitope in the C terminal part, which is fully conserved in both sequences. Red square indicates the MNB4 binding epitope in the N terminal part. This epitope is not totally conserved and Glutamic acid (E) at positions 90 and 96 is replaced by Alanine (A) and Glycine (G) respectively. The pentraxin canonical signature (H·C·S/TW·S/T) is underlined with a black line.


HuPTX3
SwPTX3
HuPTX3
SwPTX3
HuPTX3
SwPTX3
HuPTX3
SwPTX3

To gain further insight into the cross-reactivity of both monoclonal antibodies (16B5 and MNB4) in porcine samples, SwPTX3 production in SwIV infected (**Fig. 26**, lane 2), LPS (**Fig. 26**, lane 3) and RHDV-VLP (**Fig. 26B**, lane 6) stimulated poBMDCs was analysed by Western blot. Myeloid DCs were selected because they are described as a major source of PTX3 (146, 148). 16B5 (**Fig. 26**) and MNB4 (data not shown) antibodies were used as primary antibodies. The same results were obtained from both antibodies in Western blot, but 16B5 gave a stronger signal than MNB4 (data not shown), which was reasonable considering the sequence homology of each binding epitope (**Fig. 26**). Basal level of SwPTX3 was also detected in unstimulated or uninfected cell lysate (data not shown) similarly as shown by other works (152, 164).
Swine PTX3 shows an approximate molecular weight (MW) of 40 KDa (**Fig. 26**), as compared to the human (**Fig. 26,** lane 5) and murine PTX3 (**Fig. 26,** lane 1) proteins which are detected at an approximate MW of 45KDa, as previously described ((160) and (165) respectively).

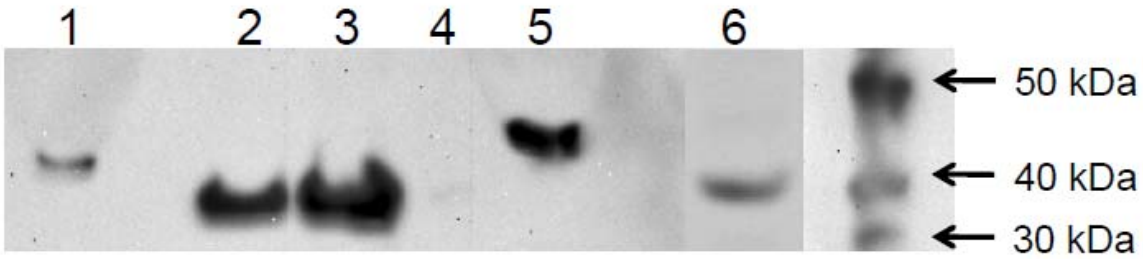


Fig. 26. Western blot with SDS-PAGE. Western blot using 16B5 as primary antibody, revealed distinct masses for PTX3 protein corresponding to around 40 kDa (lanes 2, 3 and 6). No band was detected with the negative control (cell culture medium) (lane 4). Murine PTX3 protein (lane 1) and HuPTX3 protein (lane 5) showed previously described molecular weight of approx. 45 kDa. Detection of SwPTX3 was also tested in samples from SwIV-infected (lane 2), LPS-stimulated (lane 3) and RHDV-VLP-stimulated (lane 6) poBMDCs cell lysate after overnight incubation. Representative results from three independent experiments.

Immunofluorescence for PTX3

The porcine BMDCs ability to produce SwPTX3 was also assessed by immunofluorescence using not only the cross-reactive antibodies against HuPTX3 (MNB4 and 16B5) as primary antibodies but also using an antibody against MuPTX3 (6B11) after 8 h of incubation (**Fig. 27**). A basal level of SwPTX3 staining was detected in the uninfected or unstimulated cells (**Fig. 27A**) which increased in poBMDCs infected with SwIV (**Fig. 26B**). However SwPTX3 staining was observed not only in cells positive for NP (infected cells) but also in cells negative for NP staining (**Fig. 27B**). Moreover, PTX3 staining was increased in LPS-stimulated poBMDCs (**Fig. 27C**) and in RHDV-VLP-stimulated poBMDCs (**Fig. 27D**).

Similar results were obtained using 16B5 or the 6B11 as primary antibody (data not shown). The fact that the 6B11 antibody has shown the same recognition pattern than the other anti-HuPTX3 antibodies (data not shown) indicated a certain degree of cross-reactivity of 6B11 for SwPTX3.

For additional three-dimensional (3D) figures see **Annexes** (**Figure 28 and 29**).

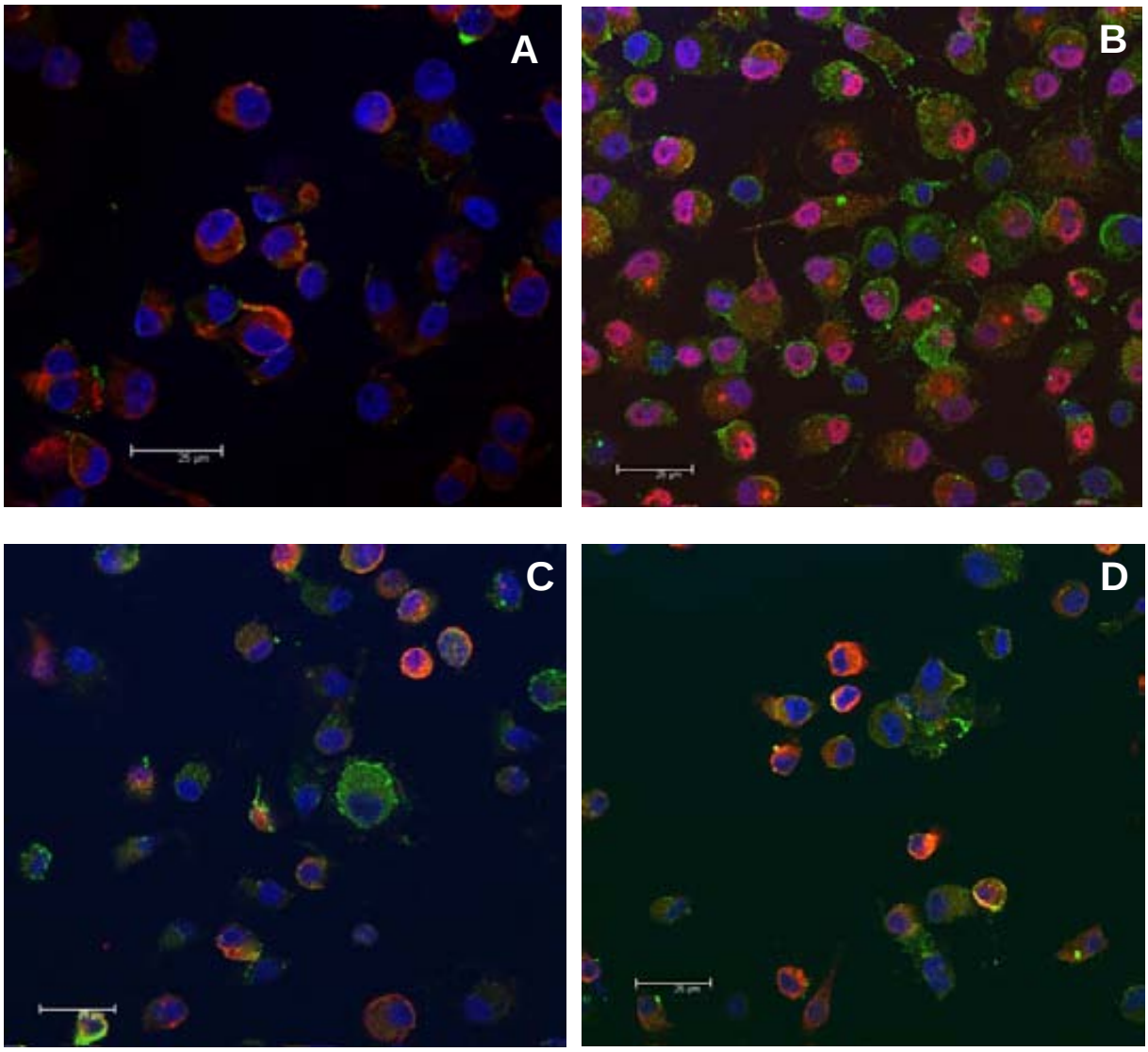


Fig. 27. PTX3 expression in poBMDCs using anti human PTX3 MNB4 antibody. PTX3 immunostaining appears in green and nuclei in blue (DAPI). (A) Unstimulated or uninfected poBMDCs (experiment control) at 8 hours. Red: SLAII positive cells. Bar = 25 µm. (B) SwIV infected poBMDCs at 8 hours. Red: NP staining of influenza virus positive cells. Fuchsia: co-localization of blue (nucleus) and red (NP). Bar = 25 µm. (C) LPS-stimulated poBMDCs at 8 hours. Red: SLAII positive cells. Yellow: co-localization of green (PTX3) and red (SLAII). Bar = 25 µm. (D) RHDV-VLPs-stimulated poBMDCs at 8 hours. Red: SLAII positive cells. Yellow: co-localization of green (PTX3) and red (SLAII). Bar = 25 µm. Representative results from three independent experiments.

CHAPTER 4

CHIMERIC CALICIVIRUS-LIKE PARTICLES ELICIT SPECIFIC IMMUNE RESPONSES IN PIGS *IN VIVO*

INTRODUCTION

After demonstrating the immunogenicity of RHDV-VLPs in porcine DCs *in vitro,* we proceed investigating the immunologic properties of these particles as vaccine vectors in pigs.

For this purpose we generated chimeric RHDV-VLPs, carrying a well defined T epitope of foot and mouth disease virus (FMDV) in the N-terminus of VP60 protein.

FMDV is a picornavirus that produces a highly transmissible and devastating disease in farm animals and other cloven-hoofed livestock (53). FMDV shows a high genetic and antigenic variability, reflected in the seven serotypes and the numerous variants described to date. FMD control in endemic regions is implemented mainly by using chemically inactivated whole-virus vaccines. Viral infection and immunization with conventional vaccines usually elicit high levels of circulating neutralizing antibodies that correlate with protection against the homologous and antigenically related viruses (166). Despite its wide use, immunization with chemically inactivated vaccines has disadvantages, such as the risk of virus release during vaccine production, the problems for serological distinction between infected and vaccinated animals and the short-lasting immunity generated (167, 168).

Several T-cell epitopes frequently recognized by natural host lymphocytes have been identified in FMDV proteins. One of these T-cell epitopes, located in residues 21 to 35 of FMDV non-structural (NS) protein 3A, efficiently recognized by lymphocytes from infected pigs, was capable to provide adequate T-helper co-operation when synthesized juxtaposed to the B-cell antigenic site in VP1, and induced significant levels of serotype-specific anti-FMDV activity *in vitro* (169). Its amino acid sequence is conserved among the FMDV types A, O, and C, showing limited variation among isolates from the seven FMDV serotypes (170). Cubillos *at al.* (171) have shown the successful use of a dendrimeric peptide, using such 3A and VP1 epitopes, for the protection of pigs against a subsequent challenge with FMDV.

MATERIAL and METHODS

Virus and cells

Derivatives of AcNPV were used to obtain the recombinant baculoviruses as previously described in **chapter 1**. SF9 were used for generation of recombinant BVs as described in **chapter 1** and H5 were used as described in **chapter 2**.

Construction of recombinant baculovirus transfer vectors

The BV transfer vector chosen was plasmid pBacPAK8XB. This plasmid is a derivative of pBacPAK8 (Clontech), in which several restriction sites were eliminated from the multiple cloning site (106). The full-length VP60 gene of RHDV was subcloned in pBacPAK8XB, generating plasmid pMVP60 (106). The sequence coding the T-helper epitope AAIEFFEGMVHDSIK, derived from the 3A protein of FMDV (169), was inserted at the 5' end of the VP60 gene by performing two sequential PCR reactions. First, two separate PCR reactions were performed using the primer pairs Bac1F/NT3A15R and NT3A15F/VP60PR (**Table 6**) (See **Annexes**), and plasmid pMVP60 as template. The PCR products obtained were gel purified, denatured and annealed together in a secondary PCR reaction in which the extended template was amplified using the external primers Bac1F/VP60PR. The PCR product obtained was cloned into the unique BglII restriction site of pBacPAK8XB generating pNT15. The inserted sequence in the resulting recombinant plasmid was verified by sequence analysis.

Generation of recombinant baculoviruses

The generation of recombinant BVs was performed as previously described in the **chapter 1**.

Expression and purification of the recombinant RHDV-VLPs

The recombinant constructs VP60 and NT15 were expressed in H5 insect cell-cultures infected with the corrresponding recombinant BVs, as previously described in (120) and in **chapter 1**.

For the purification of RHDV- VLPs generated for this study we used the same protocol than in **chapter 2**.

Experimental design

At the age of 6-7 weeks, fifty male conventional pigs (Large White x Landrace) were selected from a high health status farm located in the North-eastern part of Spain; these pigs were PRRSV, influenza virus and *M. hyopneumoniae* serologically negative before the beginning of the experiment. Pigs received non-medicated commercial feed ad libitum and had free access to drinking water. Animals were housed in an experimental farm (CEP, Torrelameau, Lleida, Spain) in four pens (12-14 piglets per pen). The space available for the animals was 0.75 m^2/pig. The building was equipped with manual mechanisms to control ventilation.

At the beginning of the experiment pigs were identified, double ear-tagged and randomly distributed into four groups, namely A (n = 5), B (n = 15), C (n = 15) and D (n = 15) balanced by weight (**Table 7**).

Table 7. Experimental design

Group		Route	Doses (µg/pig)
A		-	- (n=5)
B	1	**IN**	20 (n=5)
	2		60 (n=5)
	3		180 (n=5)
C	1	**IM+ADJ**	20 (n=5)
	2		60 (n=5)
	3		180 (n=5)
D	1	**IM**	20 (n=5)
	2		60 (n=5)
	3		180 (n=5)

IN: intranasal route. IM: intramuscular route.
ADJ: adjuvant.

Pigs of group A remained untreated and were used as negative controls. Groups B, C and D were inoculated twice with 1 ml of RHDV-3A-VLPs in a two-week interval. Subgroups were organized as summarized in **Table 7**. Group B was intranasally immunized with 20, 60 and 180 µg per dose of chimeric RHDV-3A-VLPs on day 0.

Group C and D animals were intramuscularly immunized in the right neck muscle with the above mentioned amounts of chimeric RHDV-3A-VLPs, although pigs from group C received the different doses emulsified with the adjuvant Montanide™ ISA 206 (SEPPIC) in 1 ml with equal proportions. Pigs were monitored daily for immunization reactions and samples of blood and saliva (using Salivette® Cotton Swab from SARSTEDT, Spain) were collected on days 0, 14 and 28 after the beginning of the experiment. Fourteen days after the second immunization pigs were euthanized with an intravenous overdose of sodium pentobarbital.

The experiment received prior approval from the Ethical Committee for Animal Experimentation of the Institution (Universitat Autònoma de Barcelona). The treatment, housing and husbandry conditions conformed to the European Union Guidelines (The Council of the European Communities 1986, EU directive 86/609/EEC).

Detection of haptoglobin and pentraxin 3 in serum

Haptoglobin (Hp) was quantified by a spectrophotometric method (haemoglobin binding assay) with commercial reagents (Tridelta Development Limited, Ireland) and performed on an automatic analyzer (Olympus AU400, Hamburg, Germany) as previously described (172).

PTX3 concentration in sera was determinated as previously described (162) by sandwich ELISA against murine PTX3 (MuPTX3) with the following antibodies: 2C3 and biotinylated 6B11. Values of OD were obtained at 450 nm. We count as positive sera those giving OD values above the cut-off of the assay (0.09).

Detection of specific anti-RHDV-VLP antibodies by ELISA

Antibodies against RHDV-VLP were examined in serum samples collected on days 14 and 28 by ELISA. Briefly, Maxisorp 96-well ELISA plates (Nunc) were coated with RHDV-VLP (50 ng/well), in carbonate/bicarbonate buffer (pH 9.6), overnight at 4ºC. Duplicate four-fold dilution series of each serum sample were made, starting at 1/50. Fifty microliter volumes were used throughout. Specific antibodies were detected with horseradish peroxidase conjugated with protein G (Pierce).

RHDV-VLP-specific IgG1, IgG2 (in sera) and IgA (in sera and saliva) were measured following the same procedure described but replacing the protein G-HRP by monoclonal antibodies specific for these isotypes, supplied by Serotec, and using as secondary antibody a goat anti-mouse IgG (H+L)-HRP (Zymed, Invitrogen). In the case

of saliva, two consecutive incubations with sample were performed before adding the commercial monoclonal antibody to porcine IgA, in order to increase the sensitivity of the assay. Colour development was obtained after addition of the substrate chromofore, OPD (total Ig analyses) or TMB (isotyping), and stopped by adding a H_2SO_4 solution. Plates were read in an automatic microplate reader (Fluostar Omega) at 492 and 450 nm. Antibody titers (total Ig and isotypes) were therefore expressed as the $\log_{10}$ of the last reciprocal serum dilution giving a value higher than twice absorbance recorded in the control wells (serum collected day 0). These data were calculated by interpolation.

Detection of specific anti-FMDV epitope T-3A antibodies by ELISA

Serum samples were examined for the presence of antibodies against NSP 3A T-cell epitope, displayed in the RHDV-VLP. Assay was performed in 96-well Immobilizer Amino Plates (Nunc) coated with 4 μg/well of T-3A synthetic peptide (kindly supply by D.Andreu and B.G. De la Torre, Pompeu Fabra University, Barcelona, Spain), in PBS buffer, and incubated overnight at 4ºC. Duplicate 1/50 dilution of each serum sample was made. Specific antibodies were detected with horseradish peroxidase conjugated with protein G (Pierce), and colour development was obtained after addition of TMB substrate. We counted as positive sera those giving OD values above the cut-off of the assay (0.2).

ELISPOT assay

Two and four weeks after the first immunization, PBMCs were collected and analyzed for specific IFN-γ production by ELISPOT set following the manufacturer's instructions (Becton Dickinson, UK). PBMCs were isolated by Histopaque-1.077® gradient and plated in duplicate at 5 x 10^5/100 μl/well in RPMI-1640 supplemented with 10% FCS into 96-well plates (MultiScreen® MAHAS4510 Millipore) previously coated overnight at 4ºC with 5 μg/ml with anti-pig IFN-γ-specific capture mAb (P2G10, Becton Dickinson UK) 100 μl/well. For the *in vitro* antigen recall, 35 μg/ml of 3A peptide or 20 μg/ml RHDV-VLPs were used as stimuli. As positive control, cells were incubated with 10 μg/ml phytohaemagglutinin (PHA) (Sigma) and cells incubated in the absence of antigen were used as negative control. Plates were cultured for 72 h at 37ºC, then incubated with 2 μg/ml of biotinylated anti-IFN-γ mAb (P2C11, Becton Dickinson, UK), followed by streptavidin-horseradish peroxidase conjugates (Jackson Immunoresearch Lab., Europe). The presence of IFN-γ-producing cells was visualised

using 3-Amino-9-Ethylcarbazole (AEC) substrate (Sigma). The background values (number of spots in negative control wells) were subtracted from the respective counts of the stimulated cells and immune responses were expressed as number of spots per million of PBMCs.

Lymphoproliferation assay

Proliferation assays of swine lymphocytes were performed as described previously (173). Blood was collected in 5 μM EDTA and used immediately for the preparation of PBMCs (174). Assays were performed in 96-well round-bottomed microtiter plates (Nunc). Briefly, 2.5 x 10^5 PBMC per well were cultured in triplicate, in a final volume of 200 μl, in complete RPMI, 10% FCS, 50 μM 2-mercaptoethanol, in the presence of 20 μg/ml of RHDV-VLP, RHDV-3A-VLP or 3A synthetic peptide. Cultures with medium alone were included as controls. Cells were incubated at 37°C in 5% CO_2 for 4 days. Following incubation, each well was pulsed with 0.5 μCi of [*methyl*-3H]thymidine for 18 h. The cells were collected using a cell harvester and the incorporation of radioactivity into the DNA was measured by liquid scintillation counting with a Microbeta counter (Pharmacia). Results were expressed as stimulation indexes (SI), which were calculated as the mean counts per minute (cpm) of stimulated cultures/mean cpm of cultures grown in the presence of medium alone (peptide) or mock-stimulated cells (RHDV-VLPs).

Pathological analysis

Pathological studies were analyzed by Dr. Jorge Martínez from the Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona, Spain.

The objective of the pathologic studies at the site of injection (right neck muscle, *brachiocephalicus*) was to establish any inflammatory reaction due to the intramuscular immunization. Tissue samples from the inoculation point were fixed in 10% buffered formalin and routinely processed for histopathology. Sections 4 μm thick were cut, stained with hematoxylin and eosin (H-E) and observed in a blinded-fashion method.

Lesions were classified regarding to the severity of the inflammatory reaction. Thus, lesions scores were as follows: 0 (no lesions); 1 (mild): small accumulation of inflammatory cells in the perimuscular adipose tissue; 2 (moderate): muscular tissue was mildly infiltrated by inflammation; 3 (severe): extended areas of muscular tissue were infiltrated and loss of muscular fibers and fibrosis were observed.

Statistical analysis

All statistical analysis was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). For all analyses, pig was used as the experimental unit. The significance level (α) was set at 0.05 with statistical tendencies reported when $P<0.10$. The Shapiro Wilk´s and the Levene test were used to evaluate the normality of the distribution of the examined quantitative variables and the homogeneity of variances, respectively. A non-parametric test (Mann–Whitney) was chosen to compare the different values obtained for all the immunological parameters between groups all the sampling times.

RESULTS

Generation of chimeric RHDV-VLPs carrying 3A T epitope of FMDV

In order to analyze the potential of RHDV-VLPs as a delivery system for foreign T cell epitopes we produced recombinant baculoviruses expressing VP60 construct with the epitope 3A of FMDV (AAIEFFEGMVHDSIK) in the N-terminus.

The expression of VP60 construct in H5 insect cell cultures infected with the corresponding recombinant baculovirus was verified by SDS-10% PAGE, with similar results as shown in **figure 7** in **chapter 1**. In addition, self-assembling into VLPs was studied by electron microscopy, obtaining similar results as the one shown in **figure 8** in **chapter 1**.

Detection of acute phase proteins in serum

Hp and PTX3 were analyzed in sera after each RHDV-3A-VLP injection.

No statistical differences were found in Hp level in all the groups on days 14 and day 28 (range between 0.3 and 1.3 mg/ml). On the contrary, PTX3 levels in sera were higher in group C1 (OD=0.12 ± 0.02, n=5) and D1 (OD=0.13 ± 0.09, n=5) compared with control animals on day 14, but then PTX3 decreased at day 28 and only group D1 (OD=0.24 ± 0.4, n=5) remain statistically higher compared with control animals (**Table 8**). Moreover, group C1 exhibited PTX3 levels statistically higher when compared with all groups B (background level) on day 14, although these differences were no longer observed at day 28 (**Table 8**). All the other groups did not show significant differences on day 14 and day 28 (OD at background levels).

Table 8. Statistical analysis of PTX3 production in sera.

	D14			D28		
	C1	**D1**	**D2**	**C1**	**D1**	**D2**
A	+(0.07)	+(0.07)	NS	NS	*(0.04)	NS
B1	*(0.02)	*(0.02)	+(0.07)	NS	NS	NS
B2	*(0.02)	*(0.02)	+(0.07)	NS	NS	NS
B3	*(0.02)	*(0.02)	+(0.07)	NS	NS	NS

* $p < 0.05$. + $0.05 < p < 0.1$. NS: no significant differences.

RHDV-VLP and 3A specific antibody responses after two immunizations

Serum samples were collected from each VLP-immunized pig group before immunization and on days 14 and 28 after the first VLP inoculation. As shown in **Fig. 28A**, total Ig antibodies specific for RHDV-VLP were readily detectable at two weeks after priming in all pigs immunized by the IM route with adjuvant and at all doses tested. Total antibody levels increased up to more than 4 $\log_{10}$ two weeks after the last immunization in those pigs. Immunization with RHDV-3A-VLPs by the IM route without adjuvant required the administration of at least two doses to induce significant levels of specific antibodies to RHDV-VLPs. Throughout this experiment, pigs immunized using the IM route plus adjuvant produced significantly higher RHDV-VLP total antibody titres than the other groups on days 14 ($p < 0.05$) and 28 ($p < 0.01$). No statistical differences were found within groups C and D when comparing the effect of different doses ($p > 0.1$). Pigs inoculated intranasally did not show any significant antibody responses against the vector and no statistical differences were found between doses ($p > 0.1$).

In the sera, kinetics of the different anti-RHDV-VLP Ig isotypes and subtypes (IgG1, IgG2, IgA) followed a similar pattern than total RHDV-VLP-specific antibodies (**Fig. 28**: **B, C, D** respectively). In this case, also the group C showed higher antibody production with significant differences ($p \leq 0.01$) compared with the other groups on day 28. No statistical differences were found at this time point within group C between the subgroups receiving different doses ($p > 0.1$). On day 14, group C specific isotype responses were higher compared with groups B and D ($p < 0.05$) (**Fig. 28**), but pigs inoculated IN and IM without adjuvant did not show relevant significant differences between them ($p > 0.1$) (**Fig. 28: B, C, D**). Considering the IgG1 responses of group C on day 14 (**Fig. 28B**) a dose effect was found: the lower dose was statistically different than the highest ($p = 0.02$). No statistical differences were found in the other groups between different doses ($p >0.1$).

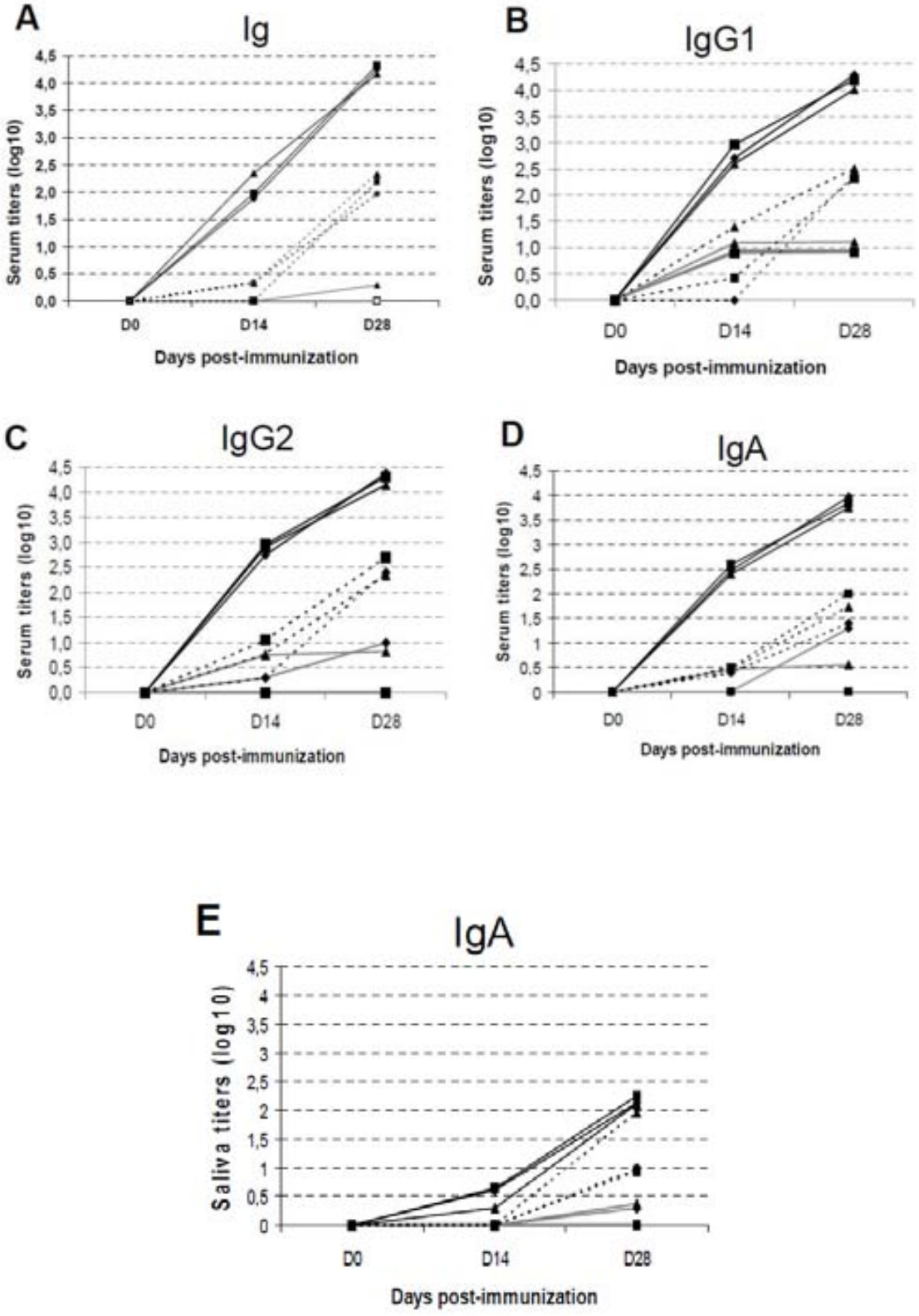
Interestingly, pigs immunized by the parenteral route with chimeric RHDV-3A-VLPs were able to induce systemic IgA anti RHDV-VLPs in the serum (**Fig. 28D**). The group immunized intranasally, showed an increase in serum specific RHDV-VLP IgG1, IgG2 and IgA after the first immunization, but no further enhancement was found after the boost (**Fig. 28**: **B, C, D**). Only the group inoculated with 60 μg IN showed an increased IgA response in the serum after the boost (**Fig. 28D**). Specific RHDV-VLP antibodies

remained undetectable in the serum of the negative control group for the duration of the experimental procedure (data not shown).

Regarding the specific humoral responses to FMDV peptide 3A, only the groups C1 (3/5 animals; values: 0.4, 0.39 and 0.26) and C2 (2/5 animals; values: 0.3 and 0.5) had shown detectable total Ig antibodies in the serum after the second immunization (data not shown).

IgA were also detected in the saliva of RHDV-3A-VLP immunized pigs. Titres followed a similar pattern than in the sera but with lower anti-RHDV-VLP antibody levels (**Fig. 28E**); indeed, group C could reach only around 2.2 $\log_{10}$ titres on day 28. Significant differences were found between group C and B ($p < 0.03$), and D1 and B ($p < 0.05$) on day 28. Group D1 showed the same responses than the adjuvanted group ($p >0.1$) and also, no differences in dose were found in groups C. Interestingly, mucosal immunization did not elicit high amount of local RHDV-VLP-specific IgA antibodies, whereas parenteral immunization could stimulate a higher production of RHDV-VLP-specific mucosal IgA locally in the saliva (**Fig. 28E**). At day 14, no statistical differences were observed between all the groups ($p > 0.1$). The negative control group remained negative in the saliva for RHDV-VLP-specific antibodies throughout the experimental procedure (data not shown).

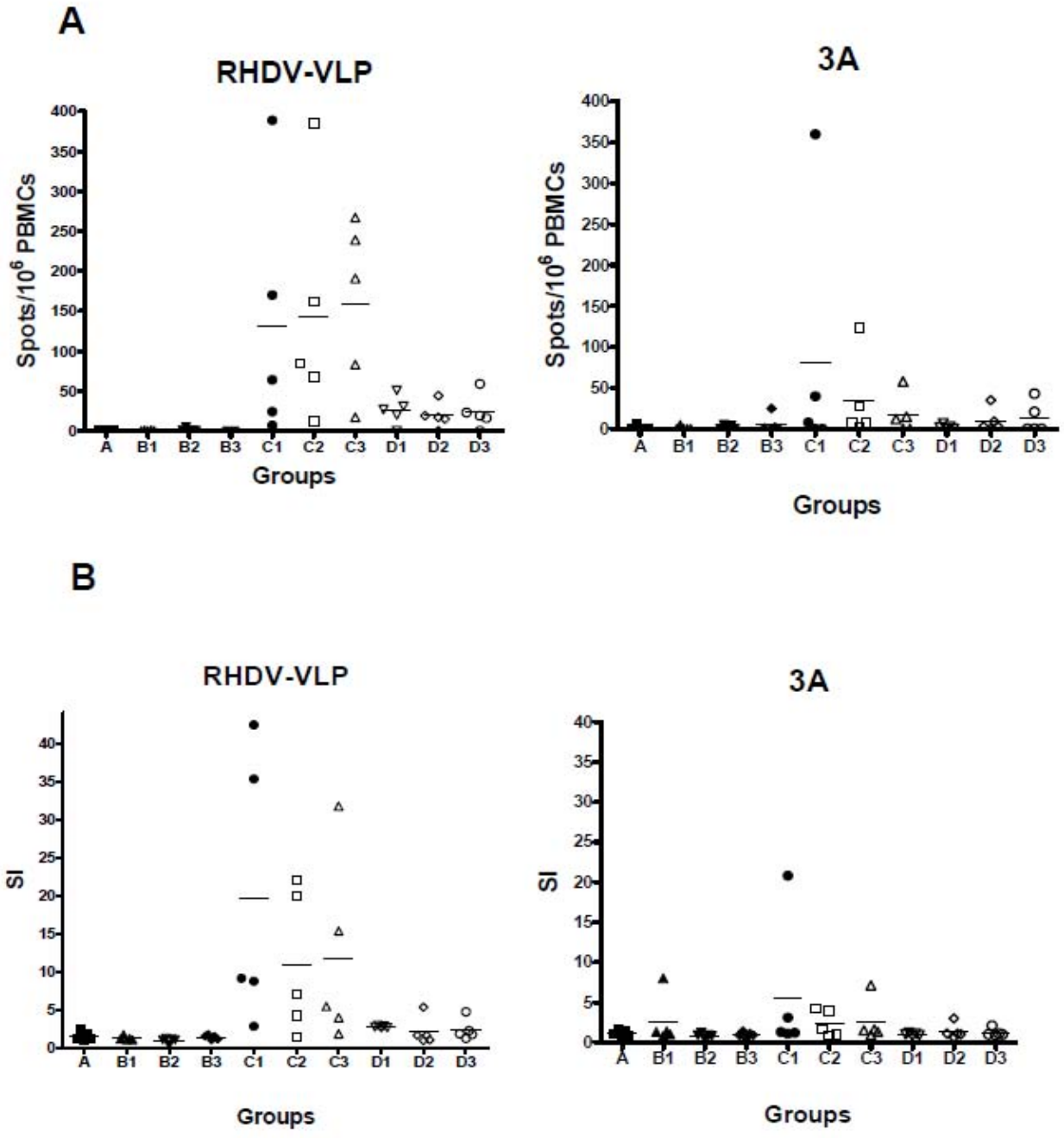
Fig. 28. Specific humoral responses of RHDV-3A-VLP immunized pigs against the vector RHDV-VLP in serum and saliva at day 0, 14 and 28. **A**) Anti-RHDV-VLP total Ig antibodies in serum. **B**) Anti-RHDV-VLP IgG1 antibodies in serum. **C**) Anti-RHDV-VLP IgG2 antibodies in serum. **D**) Anti-RHDV-VLP IgA antibodies in serum. **E**) Anti-RHDV-VLP IgA antibodies in saliva. Pigs are divided in different groups depending on the inoculation route: B (IN) (grey line), C (IM+ADJ) (solid black line) and D (IM) (dotted line). Pigs are also divided depending on the VLP dose: 20 µg (triangle), 60 µg (diamond) and 180 µg (square). Titres are expressed as reciprocals of the last dilution of sera (Log_{10}), calculated by interpolation to give an A_{492} of 1.0 OD unit. Each value corresponds to geometric mean of all the animals (duplicate wells) of each group.

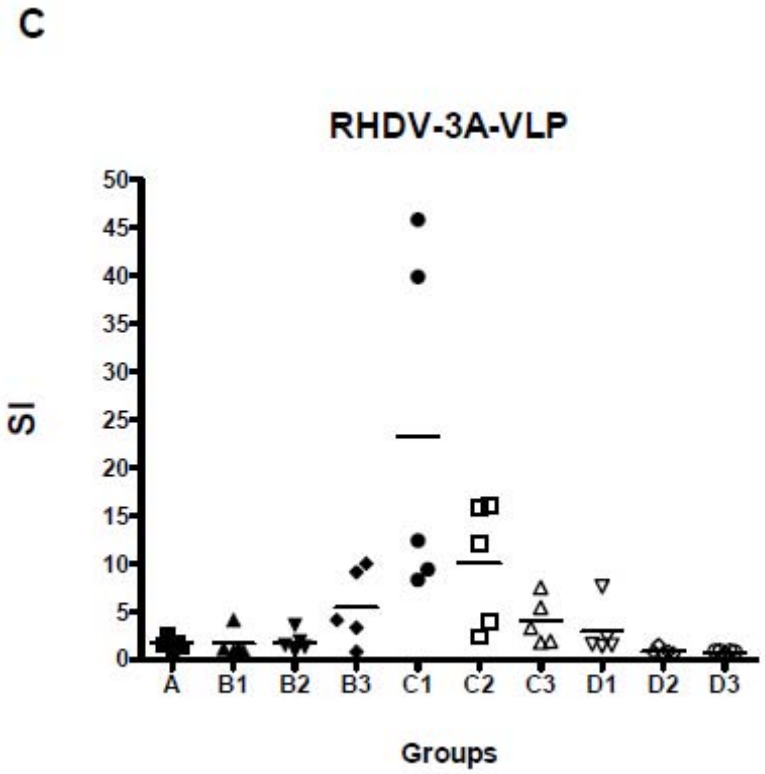

A
Ig
Serum titers (log10)
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
D0
D14
D28
Days post-immunization
B
IgG1
Serum titers (log10)
Days post-immunization
C
IgG2
Serum titers (log10)
Days post-immunization
D
IgA
Serum titers (log10)
Days post-immunization
E
IgA
Saliva titers (log10)
Days post-immunization

RHDV-VLP and 3A specific cellular immune responses elicited by chimeric RHDV-3A-VLP immunization

To get further insight into the immune responses induced by RHDV-3A-VLPs, cell-mediated immune responses in pigs immunized with chimeric RHDV-3A-VLPs were studied by analysing porcine PBMCs isolated after each immunization. Taking into account that 3A sequence is an immunodominant T cell epitope, it was conceivable to assume that a good vaccine vector carrying such epitope would induce 3A-specific IFN-γ-secreting cells. Indeed, two weeks after the last inoculation of animals with RHDV-3A-VLPs, specific IFN-γ-secreting cells against 3A but also against the vector RHDV-VLP were detected in PBMCs of pigs by ELISPOT (**Fig. 29A**). Group C showed the highest production of IFN-γ-secreting cells against RHDV-VLP and 3A compared with the other groups. However, no statistical difference was found between the different adjuvanted doses of RHDV-3A-VLPs ($p > 0.1$) (**Fig. 29A**). Considering the response against the vector, group C showed the highest statistical difference as compared to group B ($p = 0.01$) whereas only a tendency was found between group C1 or C2 and group D ($0.09 < p < 0.06$).

Fig. 29. Specific cellular responses of RHDV-3A-VLP immunized pig against the vector RHDV-VLP, against the peptide 3A and against chimeric RHDV-3A-VLP at day 28. Specific RHDV-VLP and 3AT IFN-γ-producing cells are detected by ELISPOT (**A**). The background values (number of spots in negative control wells) were subtracted from the respective counts of the stimulated cells and the immune responses were expressed as number of spots per million of PBMCs for each animal. Shown are the results of duplicate wells of one representative experiment. Specific RHDV-VLP (**B**), 3A (**B**) and RHDV-3A-VLP (**C**) T-cell proliferation is detected by lymphoproliferation assay. Data are shown as SI (stimulation indexes, see Materials and methods) for each animal. Results shown are the mean of triplicate wells for each animal.


A
RHDV-VLP
3A
Spots/10^6 PBMCs
Groups
A
B1
B2
B3
C1
C2
C3
D1
D2
D3
B
RHDV-VLP
3A
SI
Groups



An interesting finding was that RHDV-3A-VLP immunization was able to induce specific RHDV-VLP IFN-γ-secreting cells also after the first inoculation in all the groups, mainly in group C (**Fig. 30**). Likewise, specific 3A IFN-γ-secreting cells were detected on day 14 in all the groups, mainly in group C (**Fig. 30**). Responses on day 14 were lower than on day 28 and no statistical differences were found between the different doses of RHDV-3A-VLPs. As expected, control pigs (**Fig. 29 and 30**) or pigs prior to immunization did not show any significant response (data not shown).

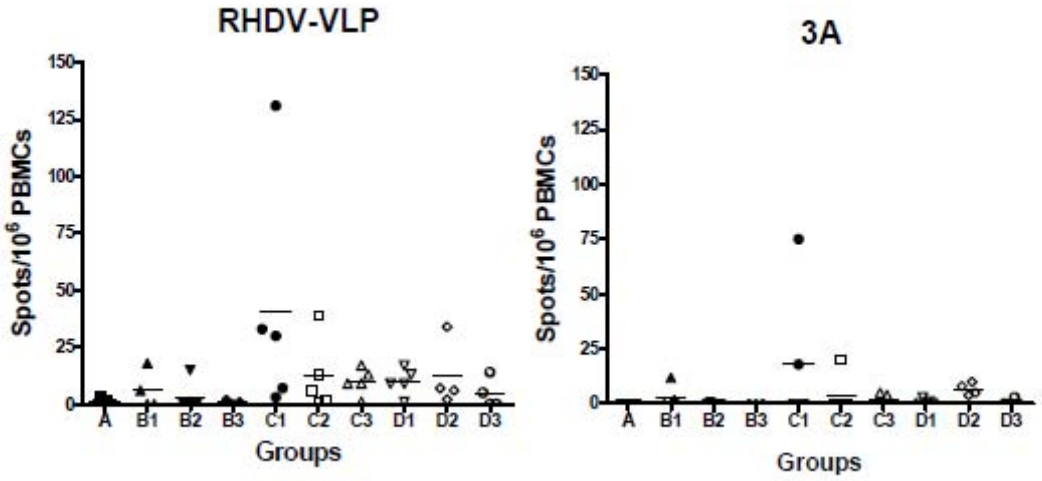


Fig. 30. Specific cellular responses of RHDV-3A-VLP immunized pig against the vector RHDV-VLP and against the peptide 3A at day 14. Specific RHDV-VLP and 3AT IFN-γ-producing cells are detected by ELISPOT. The background values (number of spots in negative control wells) were subtracted from the respective counts of the stimulated cells and the immune responses were expressed as number of spots per million of PBMCs for each animal. Shown are the results of duplicate wells of one representative experiment.

The IFN-γ ELISPOT data were also consistent with the results from the lymphoproliferation assay indicating that the RHDV-3A-VLPs were capable of inducing a cellular immune response against the foreign antigen and the vector. Lymphoproliferation assay results had the same pattern than ELISPOT results and animals immunized in the presence of adjuvant were able to induce higher responses against the vector and the peptide compared with the other groups (**Fig. 29B**). Group C SI against the peptide 3A were lower then those against the vector RHDV-VLP (**Fig. 29B**) but no statistical differences were detected between the different doses of the same group ($p > 0.1$). Conversely, different results were obtained when PBMCs on day 28 were stimulated with RHDV-3A-VLP. In this case, group C also showed the highest responses, but a significant dose effect was observed with 20 µg-dose response being significantly higher ($p = 0.02$) compared to the 180 µg-dose response (**Fig. 29C**). Moreover, animals immunized IN with 180 µg (group B3) showed comparable responses than pigs vaccinated IM with 180 µg of RHDV-3A-VLPs plus adjuvant (group C3) (**Fig. 29C**). No stimulation was observed with PBMCs from control pigs (**Fig. 29**) or pigs prior to RHDV-3A-VLP immunization (data not shown).

Pathological analysis

No injection site reactions were observed macroscopically after immunization and all swine were healthy during the immunization period. Histopathological analysis of the inoculation point revealed a similar type of lesion in those pigs with microscopical alterations. This consisted in a focal inflammatory granulomatous reaction composed of abundant macrophages and multinucleated giant cells surrounding droplets of foreign lipid material. In the cytoplasm of some multinucleated giant cells, small accumulations of fragmented, fibrillar and eosinophilic material could be observed. A few lymphocytes, plasma cells and eosinophils were present at the periphery of the lesion (**Fig. 31**).

From the thirty three studied pigs (3 control pigs and 30 intramuscularly injected), 23 (69.7%) showed no histopathological lesions (**Fig. 31A**), 2 (6.1%) had mild lesions (**Fig. 31B**), 3 (9.1%) moderate lesions (**Fig. 31C**) and 5 (15.1%) severe lesions (**Fig. 31D**). The average and standard deviation of the histopathological scores by groups were as follows, group A: 0 ± 0, group C1: 1 ± 1.4, group C2: 0.6 ± 0.89, group C3: 2.8 ± 0.45, group D1: 0 ± 0, group D2: 0.2 ± 0.45, group D3: 0 ± 0 (**Fig. 31**). Statistical differences were found between group C3 and group A ($p = 0.02$) or all group D ($p < 0.007$). Moreover, the highest dose of group C showed the higher lesion score compared with other doses ($p < 0.05$).

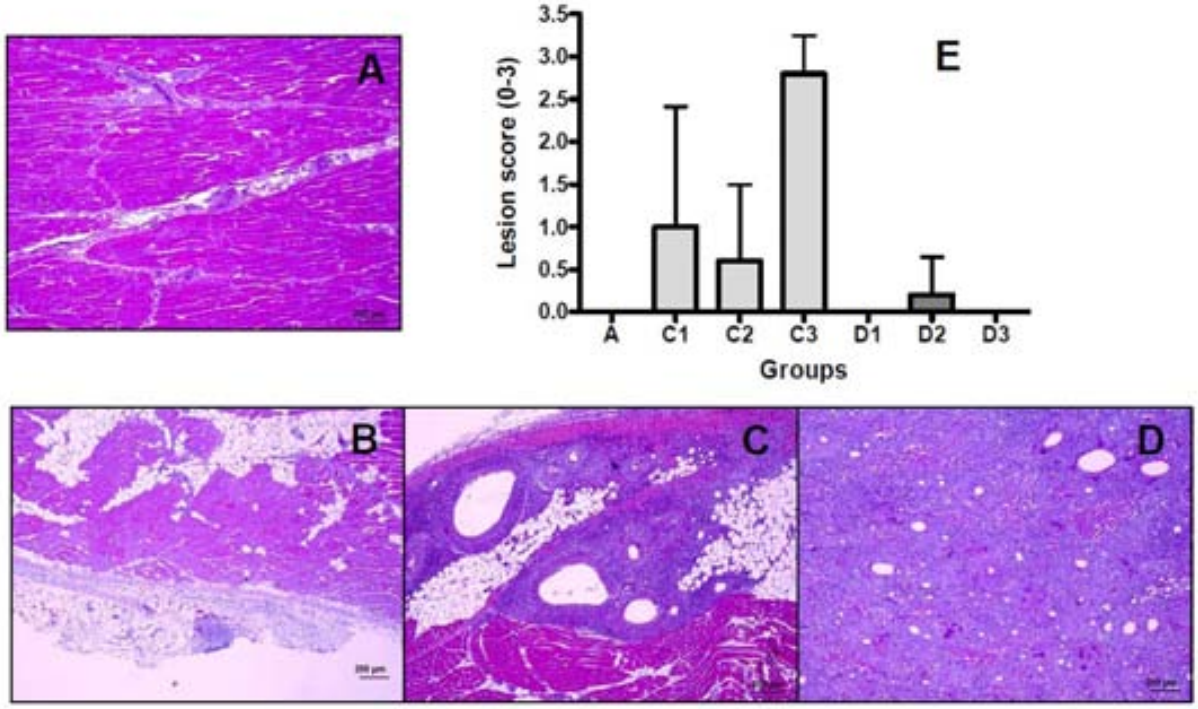


Fig. 31. Histopathological analysis of the tissue at the injection point (*brachiocephalicus* muscle) of pigs at day 14 after the second immunization (H-E stain, bar = 200 µm). Lesion were classified in (**A**) absence (score=0); (**B**) mild: small focus of granulomatous inflammation located in the perimuscular adipose tissue (score=1); (**C**) moderate: granulomatous reaction is partially occupying the perimuscular fat with mild infiltration of the adjacent muscular tissue (score=2); (**D**) severe: adipose and muscular tissues are infiltrated and almost substituted by a diffuse granulomatous inflammation (score=3).(**E**) In the graphic is shown the pathological score (0-3) for all the groups indicated as mean (bars) ± standard deviation (lines).

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION

Classical approaches to viral vaccine development, mainly in the veterinary field, involve chemical inactivation of virions or infected tissue (termed “killed” vaccines), attenuation of virulence during passage in cell culture or animal hosts (termed “live-attenuated” vaccines), or immunization with portions of the pathogen that can induce specific immune responses. (termed “subunit” vaccines). At the beginning of veterinary vaccinology the balance between protection and safety was reached by using whole virus, inactivated or killed. Currently, a large number of successful vaccines are based on whole viruses but there are intrinsic disadvantages involved with the use of this type of vaccination. Live-attenuated viral vaccines are unstable and there is a potential for genetic reversion, reassortment and mutation. Killed vaccine, induce weaker immune responses and a regimen of doses is generally required, increasing the expenses (discussed in (3)). Nowadays, vaccine development has been empiric, often characterized by an ‘isolate, inactivate, inject’ paradigm of development. Such an approach ignores both pathogen and host variability and as a result significant limitations ensue such as inadequate immune protection, inability to develop vaccines against hypervariable viruses and an insufficient understanding of how protective immune responses develop (175). Considering the necessity to develop new effective, immunogenic and inducing long-lasting immunity vaccine to avoid the intrinsic disadvantages of classical vaccines, new advances are being developed in vaccinology.

The era of VLP-based human vaccines seems to be in its boom, considering that two vaccines are already licensed and several of them are in clinical trial phase. Veterinary VLP-based vaccines started to go hand in hand with this tendency and several candidates are investigated in animal models, even though most of them are not commercially available. The strong potential and immunogenicity, the stability and flexibility of these particles have been demonstrated and the advantages of this technology explain the frenetic research in the field.

Some ethical issues, mainly related to baculovirus expression system, remain without elucidation but the effectiveness of the vaccination and the fast industrial scale production has outweighed the constraints. Thus, new stimuli are given in veterinary vaccinology to test new candidates to challenge animal viral disease.

Chimeric RHDV-VLPs-OVA elicit protective anti-viral cytotoxic responses without adjuvant in mice

We have developed a system for the generation of chimeric VLPs derived from RHDV that have shown a 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 were also 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 (75, 76, 124). 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 (74). Several mechanisms responsible for the processing of exogenous antigens and presentation in the MHC class I pathway have been described (176), which are generally called cross-presentation. Cross-presentation is the process whereby APCs acquire, process and present exogenous antigen as peptides bound to MHC-I molecules to $CD8^+$ T cells (reviewed in (177)). In the present study, we demonstrated that although chimeric RHDV-VLPs are exogenous antigens, they are presented by BMDCs in the context of MHC-I as evidenced by stimulation of hybridoma cells B3Z *in vitro*.

$CD8^+$ 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 (178, 179). Second, virus-specific $CD8^+$ T cells are potent producers of anti-viral cytokines, in particular IFN-γ, which may attenuate viral replication (180). 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 (181). VLPs

from porcine parvovirus have been previously reported to be processed by MHC-I pathway by cross-presentation (124) for activation of $CD8^+$ T cells which are essential for the clearance of intracellular pathogens such a virus (68, 182). The stimulation of this effective response is based on the induction of high frequency, efficiency and avidity CTLs (183). 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 both chimeric VLPs induced a reduction in viral levels upon a VV-OVA challenge, but immunization with chimeric RHDV-VLP-2 at the highest dose tested was able to totally resolve VV-OVA infection (Fig. 16). Considering that chimeric RHDV-VLP-2 was the most efficient construct at inducing specific CTLs and IFN-γ producing cells (Fig. 14 and 15), 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 OVA antibodies which did not correlate with protection (Fig. 16). OVA specific humoral responses were dose dependent. 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. Peacey *et al.* (113) reported a procedure to chemically conjugate whole proteins and peptides to the surface of VLPs from RHDV. 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 (114). Our results are in agreement and further extend those of Peacey *et al.* (114) obtained with antigen conjugated to RHDV-VLPs. Firstly, the data provided in the present 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 (114). Finally, we 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 was 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 (97, 184), and potential neutralizing antibody recognition sites (109, 185), characteristics in principle appropriate for an efficient insertion site intended for foreign B cell epitopes. This was not 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 (186). B cell epitopes must be inserted at exposed loops, in order to generate an efficient specific immune humoral response (187). 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 (71). 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 (188). 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. Interestingly, in our system, specific humoral responses against the vector were elicited for all the constructs with similar magnitude and these responses did not seem to influence the subsequent following protection; indeed, RHDV-VLP-2 was able to protect mice despite the presence of vector antibodies. These data are not surprising since Ruedl *et al*. (61) have shown that pre-existing VLP-specific antibodies did not affect the induction of effector cells. Thus, carrier suppression by VLP-specific antibodies may be considered low influential in VLP-based immunization also in our study and we can hypothesize it neither has a major influence in other species.

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.

Differential $CD8^+$ T cell responses generated by chimeric RHDV-FLU-VLPs depending on the route of administration

In this second study, current results suggested that immunization with chimeric RHDV-VLPs carrying the NP CTL epitope of influenza virus induces different responses depending on the

route of delivery. The parenteral delivery of chimeric VLPs has been shown to be more effective than mucosal delivery to elicit NP-specific IFN-γ-producing cells and also NP-specific $CD8^+$ T cells. The induction of PR8-mediated IFN-γ-producing cells was less effective compared with the NP-mediated response. The responses against the inserted CTL peptide increased during the experiment and were higher on day 14 after the second dose of chimeric VLPs; on the contrary the responses against the vector did not substantially increased were more homogeneous between the different routes on day 14 after the second immunization.

By evaluating the cellular responses 14 days after the second immunization, we could investigate the memory response elicited against the inserted epitope. Chimeric VLPs were able to elicit a memory response against NP epitope but further studies have to be performed to evaluate if this memory response is long-lasting and protective. Indeed, PR8 challenge had several technical problems and for this reason we could not analyze the protective effect of chimeric RHDV-NP-VLPs in mice using different immunization routes. Probably, our failure to properly infect mice with PR8 was due to the procedure for infection and to infectious dose. Inadequate anesthesia and some problems with the delivery route could account for the failure in the infection.

RHDV-VLPs induce activation of porcine cells *in vitro*

VLPs derived from RHDV have been shown to be a suitable vector for foreign epitopes in mice as described in the previous studies, but this knowledge has not been yet translated into larger animals for diseases affecting those animals. Thus, we investigated for the first time the potential immunogenicity of these RHDV-VLPs *in vitro* with porcine cells.

Firstly, the results showed that RHDV-VLPs have the ability to stimulate immature poBMDCs *in vitro* by up-regulating SLA-II molecule as well as co-stimulatory molecules (CD80/86, in particular CD86). The kinetics of the induction of cell surface markers was similar for LPS and RHDV-VLPs, because it required 24 h for the activated phenotype to be displayed. Moreover, RHDV-VLPs induced the release of IL-6 and TNF-α by poBMDCs in the culture supernatant. In our *in vitro* porcine system, the induction of surface markers in DC was dose-dependent and the DC activation resulted from specific recognition of VLPs and not from co-purified BV in the preparation. Indeed, BV replication was tested by incubating VLP preparations in H5 cells for more than one week; no cytopathic effect was detected after the observed period (data not shown). Ours results confirmed previous studies performed in mice

and humans with other VLPs. Lenz *et al*. (76) have shown that human papillomavirus VLPs have the ability to stimulate immature murine BMDCs with comparable results. Likewise, human DCs pulsed with rodent polyomaviruses VLPs were maturated by up-regulation of CD86, MHC-I and MHC-II and were found to secrete IL-12 (75). The fact that all these VLPs, including RHDV-VLPs, induce the production of TNF-α and IL-6, suggests that the interaction between RHDV-VLPs and DCs activates the NF-kB transcription factor pathway, which stimulates both cytokines (189, 190).

An important finding was that RHDV-VLPs were able to stimulate the expression of PTX3 protein in poBMDCs. This interesting result paved the way for the first characterization of PTX3 in pigs. To the authors' knowledge, this is the first characterization of swine PTX3 and this study further support the long pentraxin 3 homology between mammals.

Sequences of HuPTX3 and predicted SwPTX3 were retrieved from the NCBI database and the alignment of the two proteins showed a high percentage of identity. The C-terminal pentraxin-like domain of swine PTX3 predicted protein is well conserved compared with human PTX3. All the members of pentraxin family share an eight aa-long conserved sequence (HxCxS/TWxS, in which x is any amino acid) in the pentraxin domain, called pentraxin signature (149, 191) and swine predicted PTX3 also showed this signature. Using anti human PTX3 antibodies 16B5 and MNB4, recognizing respectively the C-terminal and the N-terminal domains, the molecular weight observed on Western blot under reducing condition is approximately 40 kDa, comparable to the predicted molecular weight of the human protein (160). MNB4 antibody has shown less affinity with porcine samples in western blot, close to the detection limit, probably due to the changes in the aa sequence present in the swine predicted protein.

An important finding was the description for the first time that these antibodies with mapped epitopes against HuPTX3 cross-react with porcine samples, representing a good tool in SwPTX3 research, since nowadays there are no species-specific antibodies against the porcine protein. Western blot and immunohistochemistry determined that SwPTX3 protein is expressed in poBMDCs, after infection with SwIV and also after stimulation with LPS and RHDV-VLPs. An interesting finding was that RHDV-VLPs have shown to be a good inducer of SwPTX3 in poBMDCs. This fact support previous findings about the strong immunogenicity of these particles in mice (chapter 1 and 2) and in pigs (chapter 3).

RHDV-3A-VLPs elicit specific immune responses in pigs *in vivo*

The last study focused on investigating for the first time the potential immunogenicity and the ability of these RHDV-VLPs as vaccine vectors in pigs, using the well-known T epitope of the 3A protein from FMDV and generating chimeric RHDV-3A-VLPs. To determine the effects of RHDV-3A-VLP dosage on the induction of immunity and the effects of adjuvant presence on antigen sparing, groups of pigs were intranasally or intramuscular immunized with different doses of chimeric VLPs alone or in the presence of Montanide^{TM} ISA 206.

Acute phase proteins were first determined to evaluate an unspecific reaction to the immunization. Hp is released rapidly by the liver during the course of innate reaction (192), whereas PTX3 is produced locally mainly by DCs (147). Our results showed that VLPs immunization did not alter Hp serum concentration. These results indicated that in our experimental system the sanitary condition, stress status and housing of pigs did not influence Hp concentration, as shown in another study in pig (192); in the same way, RHDV-VLPs immunization did not alter Hp concentration during the study. On the contrary, PTX3 concentration in the serum was slightly increased at early stages of immunization, mainly in the intramuscularly inoculated group, when compared with intranasal group. This effect could be due to the immunogenicity of RHDV-VLPs that induce PTX3 production in poBMDCs (in our system the intramuscular injection seems to be more effective in the stimulation of immune responses).

Considering the humoral responses, higher increases in antibody levels against the vector were observed in groups immunized IM in the presence of adjuvant after the second immunization. Only adjuvanted animals were able to elicit anti-FMDV antibodies two weeks after the second immunization, although the response to the carrier was higher than to the 3A FMDV epitope. These results are not unexpected since the 3A epitope is mainly a T helper epitope and our results showed the insertion site in the VLPs, predicted to be buried, is not the best location to enhance a humoral response in the absence of adjuvant (Fig. 12 and 13). Despite this, the presence of the adjuvant seemed to enhance the capacity to induce anti-FMDV antibodies. When different routes of delivery were tested, intranasal versus intramuscular immunization, different responses were also obtained. The parenteral injection of chimeric VLPs was more effective than the mucosal administration for eliciting specific IgG or IgA antibody responses in the serum, but also in the saliva. Taking into consideration the kinetic analysis of antibody induction by different VLP doses, it is noteworthy that the time for reaching the highest antibody levels was more dependent on the presence of adjuvant

rather than on the VLPs dosage. Only in the saliva of IM-immunized pigs, the lowest dose of non adjuvanted VLPs was able to reach the response of adjuvanted group at day 28. Considering the general picture, our results provide evidence that immune responses induced after homologous prime-boost immunization with chimeric RHDV-3A-VLPs depend to a higher extent on the presence of an adjuvant than on the VLP-dosage in pig. The addition of an adjuvant plays an important role in enhancing the immune responses and the strategy has been shown to be effective in the induction of high humoral responses. However, further studies are needed to determine the protective capacity of these responses, including using an FMDV neutralizing B-cell epitope similar to the one successfully used in dendrimeric peptides (171) but mostly by performing a challenge experiment.

Adjuvants stimulate the immune system, but can also lead to unintended stimulations and different adverse reactions, which can result in undesiderable side effects such a fever and granulomatous inflammation. The acceptable level of side effects has been determined depending on the species in which the adjuvant is applied. The decision to use the water in oil in water (double emulsion) Montanide™ ISA 206 adjuvant was based on the immunogenicity results previously shown in pigs (193). Interestingly, no macroscopic lesions were observed after immunizations and animals were maintained at a healthy status during the length of the experimental period. The histopathological studies showed a local infiltration of immune cells detected at the injection site, mainly with the higher dose of chimeric RHDV-VLPs. Thus, this local reaction may indicate that the adjuvant might promote the immune response increasing the recruitment of professional APC to the immunization site, therefore increasing the delivery of antigen to APCs, improving the uptake of the antigen by DCs and enhancing the efficiency of vaccination. Montanide™ ISA 206 was also used in another previous study using two doses of FMDV-VLPs. In that model both FMDV-specific antibodies and neutralizing antibodies were generated in VLP-vaccinated animals, although their levels were lower than those generated by the commercial vaccine (53). Thus, our chimeric VLPs are another possible candidate to add to the list of FMDV-VLP-based vaccines, since 3A epitope is shared by different serotypes. Also, the possibility to differentiate between vaccinated and infected animals is of great interest, mainly to control FMD. Chimeric FMDV-VLPs induce serological responses compatible with its use as DIVA vaccine, since they do not elicit antibodies against the viral proteins not inserted in the vector. Such proteins can be used as diagnostic targets for FMDV infection versus FMDV vaccination.

The level of cellular immune response was determined by measuring IFN-γ, one of the markers for T helper type 1 (Th1) responses. Driving the immune response towards Th1 responses may be an attractive feature of RHDV-VLPs as immunity associated with a Th1 response is thought to be essential for the control of intracellular pathogens immunization. Interferon-γ stimulates MHC expression in antigen-presenting cells and efficiently inhibits FMDV replication (194). Chimeric VLP immunized animals were able to elicit IFN-γ producing cells against the vector and the FMDV epitope, mainly in the adjuvanted group, indicating that Montanide has an adjuvanting effect for the establishment of an effector anti-viral T cell response. Also regarding cellular responses, the parenteral injection of chimeric VLPs was more effective than the mucosal administration; this confirmed our previous results in mice (Fig. 17, 18 and 19). For the development of a potent immunization, a prolonged immune response is required to provide protection against a subsequent infection. Hence, the potential of chimeric RHDV-VLPs immunization to generate a memory T cell response was investigated after two weeks. Results show that immunization with chimeric RHDV-VLPs were sufficient to induce a specific cellular memory response. Although induction of neutralizing antibodies is considered to be the most important immune correlate to FMDV protection, specific T cells are also induced in convalescent and conventionally vaccinated animals and are relevant for protection (195). Chimeric RHDV-VLPs immunised animals exhibited similar SI in the lymphoproliferation assay to the SI depicted in the protected animals in a previous study with this T epitope (171). Therefore, it would be possible to speculate that chimeric RHDV-VLP-immunised animals showing high SI might be protected from FMDV infection. Nevertheless, an additional challenge study is necessary to assess the efficacy of RHDV-3A-VLP immunisation, since the aim of this study was only to investigate the immunogenicity of the vector in pigs.

In summary in this study we could demonstrate the potential and immunogenicity of RHDV-VLPs in pig and, in this way, its suitability as appealing vaccine vectors for veterinary viral vaccinology.

In conclusion, we demonstrated the high immunogenic properties that RHDV-VLPs have in different systems and their potential as vaccine vectors against viral diseases.

Finally, it would be fair to mention the statement by Ludwig and Wagner about VLPs: “In this light it appears almost ironical that viruses as such may serve a good purpose in the biotechnological era exploiting their weapons to beat them at their own game” (9).

CONCLUSIONS

The principal conclusions from this thesis are:

1. Chimeric RHDV-VLPs-OVA were generated using baculovirus expression system. They expressed the T cell epitope of OVA model antigen in two different positions, the N-terminus and a predicted exposed loop.

2. Chimeric RHDV-VLPs-OVA were immunogenic *in vitro* and *in vivo* in a murine system. In particular, chimeric RHDV-VLPs-OVA were able to stimulate murine BMDCs and to protect mice from a viral challenge with VV-OVA. The location of the antigen determined the nature of immune response generated.

3. Using chimeric RHDV-NP-VLPs, different VLP-immunization routes elicited different cellular responses against a CTL epitope. Parenteral immunization was the most effective for induction of cellular responses in mice.

4. RHDV-VLPs were immunogenic *in vitro* in porcine cells. In this context, RHDV-VLPs were able to mature porcine BMDCs and to stimulate PTX3 production, which was characterized for the first time in pigs.

5. Chimeric RHDV-3A-VLPs were immunogenic *in vivo* in pigs. These chimeric particles were able to induce specific humoral and cellular responses against the vector and the foreign inserted epitope.

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ANNEXES

FIGURES

Chapter 3

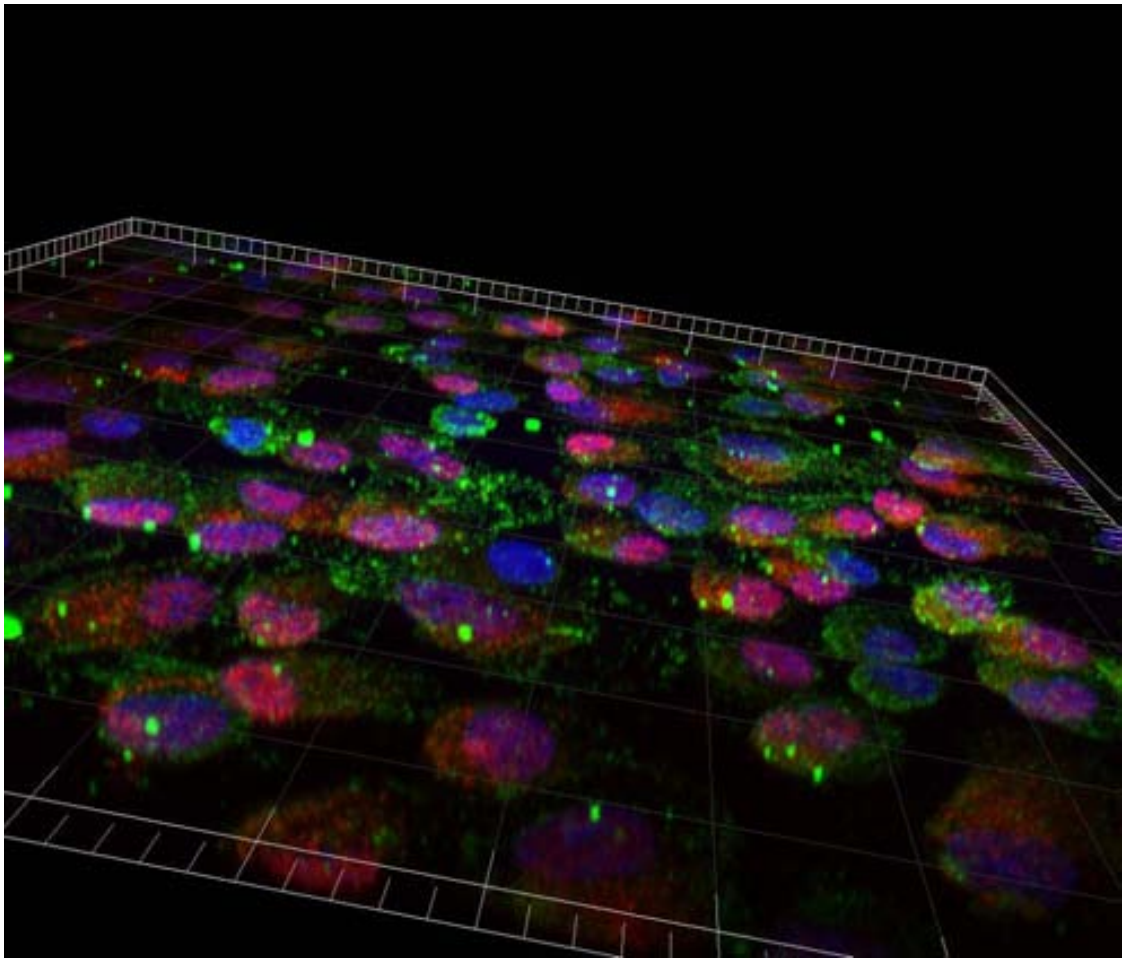

Fig. 28. PTX3 expression in SwIV infected poBMDCs using anti human PTX3 MNB4 antibody. Three-dimensional confocal image of poBMDCs infected for 8 h. PTX3 immunostaining appears in green and nuclei in blue (DAPI). Red: NP staining of influenza virus positive cells. Fuchsia: co-localization of blue (nucleus) and red (NP). Magnification 63X.

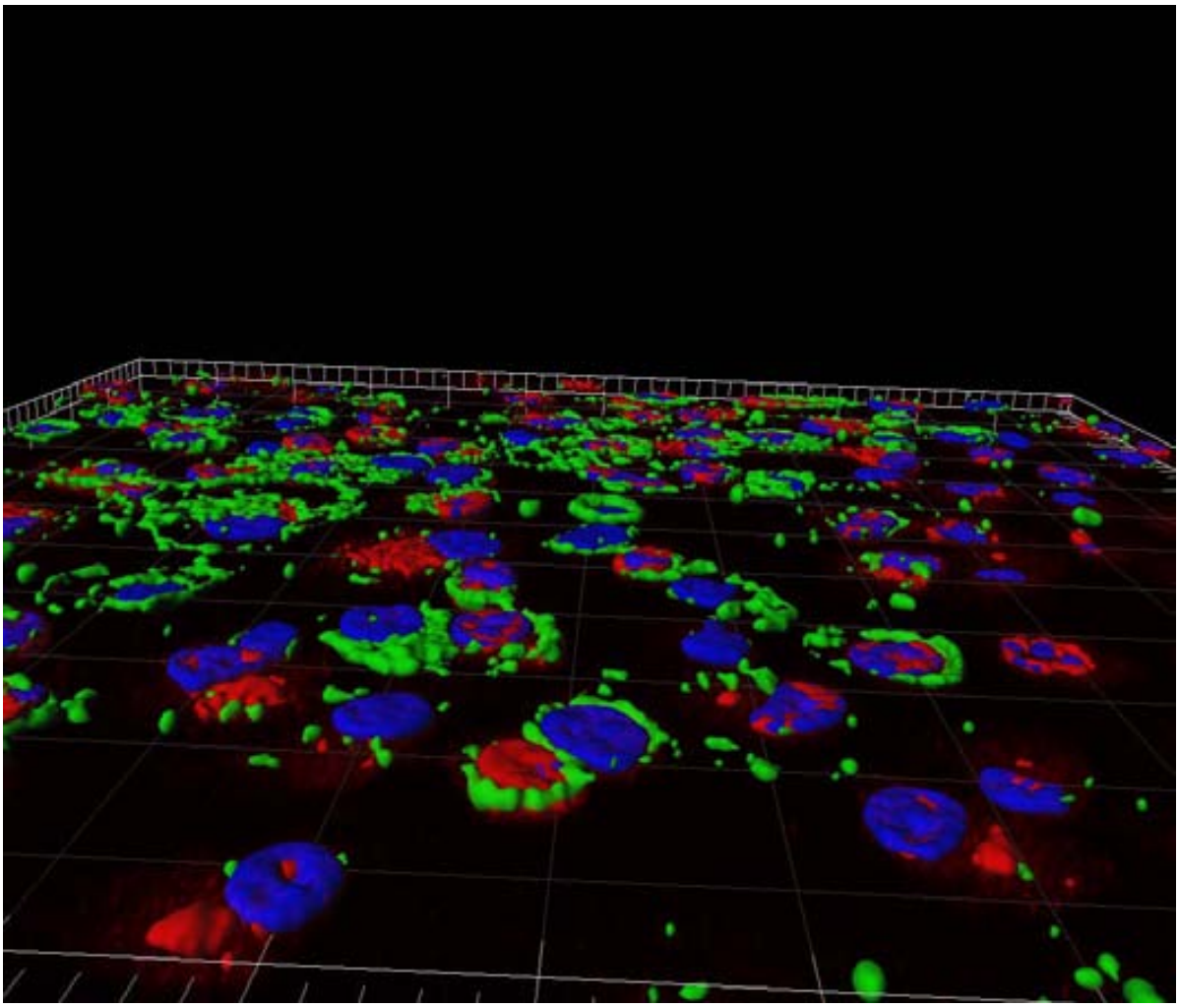

Fig. 29. PTX3 expression in SwIV infected poBMDCs at 8 h using anti human PTX3 MNB4 antibody. Isosurface representation of SwIV infected poBMDCs at 8 h within a 3D volumetric x-y-z data field. PTX3 immunostaining appears in green and nuclei in blue (DAPI). Red: NP staining of influenza virus positive cells. Magnification 63X.

TABLES

Table 3. Oligonucleotide primers used for cloning (**Chapter 1**).

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

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

Table 4. Oligonucleotide primers used for cloning (**Chapter 2**).

Primer	**Sequence (5' to 3')[a]**
NP366R	GATCCTGATTCCATAGTCTCCATATTTTCATTGGAAGCAATTTGAACA
NP366F	GATCTGTTCAAATTGCTTCCAATGAAAATATGGAGACTATGGAATCAG
VP60GSR	GGTACCAGATC**TCA**GGATCCGACATAAGAAAAGCCATTGGCTG

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

Table 6. Oligonucleotide primers used for cloning (**Chapter 4**).

Primer	**Sequence (5'to 3')**[a]
Bac1F	GACTCCAAGTGTGTGGGTGAAGTC
NT3A15F	TTTGAGGGC**ATG**GTACACGACTCCATTAAAGCCCGCACAGCGCCG
NT3A15R	GAGTCGTGTACCATGCCCTCAAAGAATTCAATGGCTGCCATATGAAGATCTTCT AGGATCGATCCG
VP60PR	TCCGAAGATCT**TCA**GACATAAGAAAAGCCATTG

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

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

E. Crisci [a], H. Almanza [b], I. Mena [b], L. Córdoba [a], E. Gómez-Casado [c], J.R. Castón [d], L. Fraile [a], J. Bárcena [b], M. Montoya [a,*]

[a] Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
[b] Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, Madrid, 28130 Spain
[c] Department of Biotecnología, INIA, Autopista A-6, Km 7, 28040-Madrid, Spain
[d] Department of Estructura de Macromoléculas, Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma de Madrid, Cantoblanco, 28049, Madrid, Spain

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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

* Corresponding author. Fax: +34 93 581 4490.
E-mail address: maria.montoya@cresa.uab.es (M. Montoya).

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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.

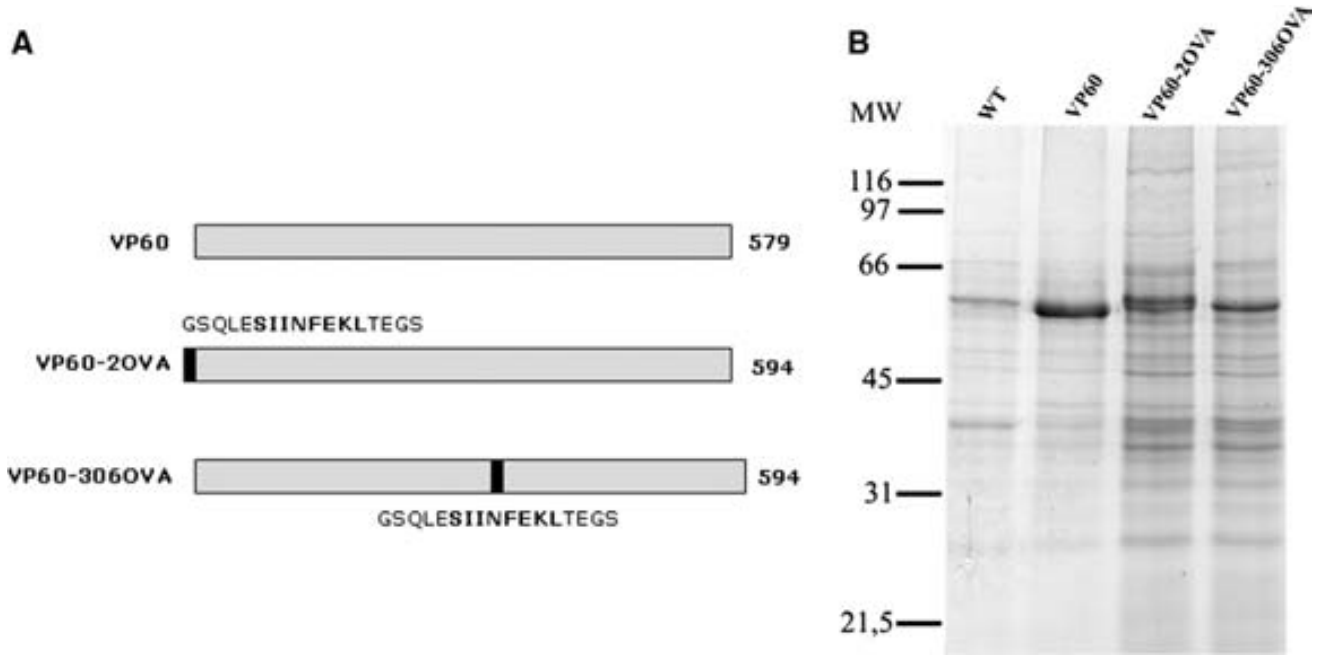

Fig. 1. Schematic representation and expression of the recombinant VP60 constructs used in this study. (A) Scheme of each construct showing names and numbers of amino acid residues. The chimeric proteins VP60-2OVA and VP60-306OVA harbour the depicted foreign peptide sequence containing de OVA derived 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.

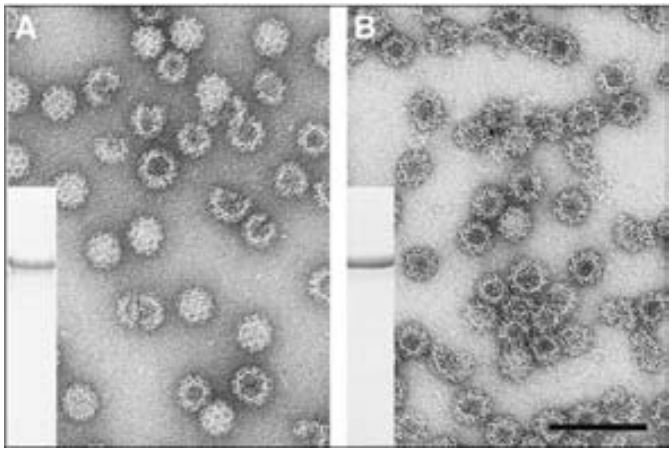

Fig. 2. Analysis of chimeric VP60 particles by negative staining and SDS-PAGE. Electron microscopy of negatively stained purified chimeric VP60 particles (A) VP60-2OVA and (B) VP60-306OVA. Purified particles were analyzed for protein content by SDS-10% PAGE and stained with Coomassie brilliant blue (insets).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

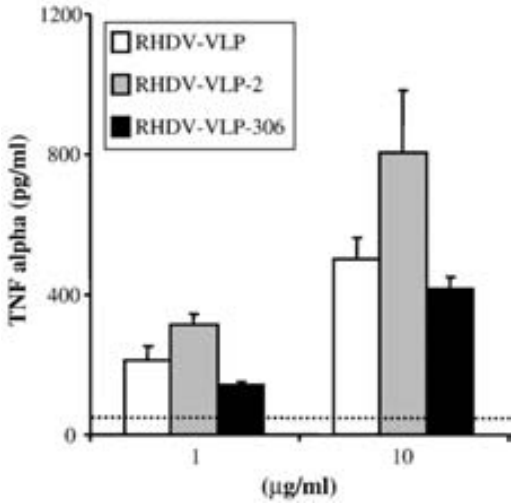

Fig. 3. Bone marrow derived DCs secreted TNF-α after incubation with control and chimeric RHDV-VLPs at different concentrations. The column colours indicate the different RHDV-VLPs: RHDV-VLP (white), RHDV-VLP-2 (grey) and RHDV-VLP-306 (black). Dotted line indicates background level of TNF-α secretion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

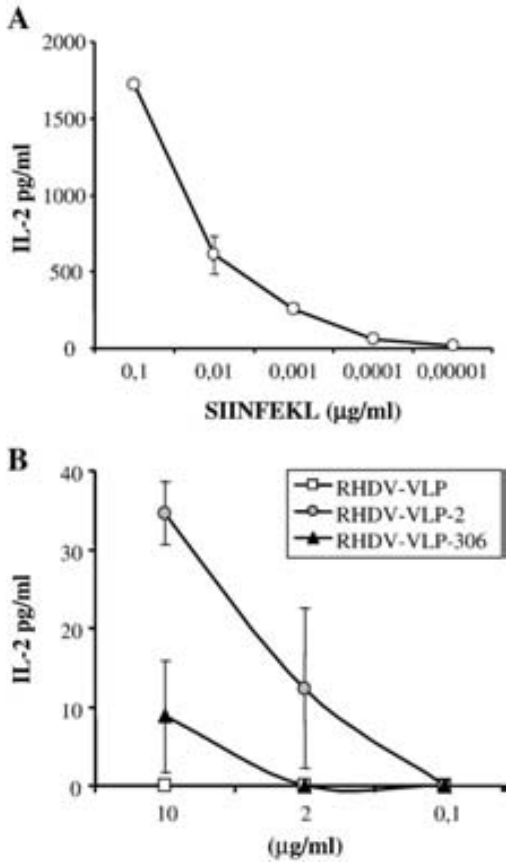

Fig. 4. Mouse DCs are able to process and present SIINFEKL peptide for $CD8^+$ specific recognition *in vitro* in a dose dependent manner. (A) C57Bl/6 BM-DCs were incubated for 6–7 h in the presence of the indicated concentration of synthetic SIINFEKL peptide, and IL-2 released was measured by ELISA. (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 ($54.2\% \pm 15$) and 8 µg ($8.9\% \pm 3.2$) dose of inoculation, whereas RHDV-VLP-306 was only able to generate detectable CTL activity at the higher dose ($23.3\% \pm 10$), 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).

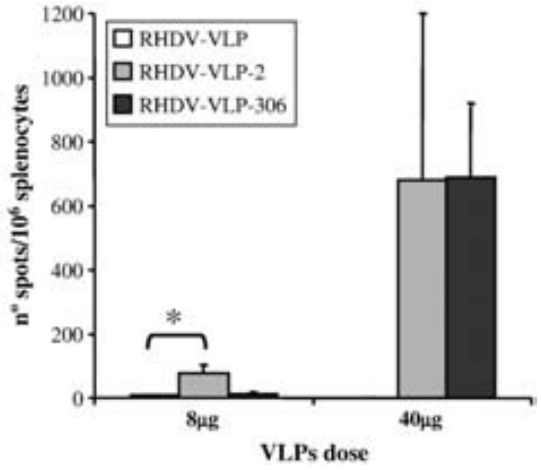

Fig. 5. Frequency of SIINFEKL-specific IFN-γ-producing cells in the spleen of treated mice. Groups of three mice were twice inoculated with 8 or 40 µg of the RHDV-VLPs and specific IFN-γ-producing cells were measured by ELISPOT assay. The column colours indicate the different RHDV-VLPs: RHDV-VLP (white), 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.

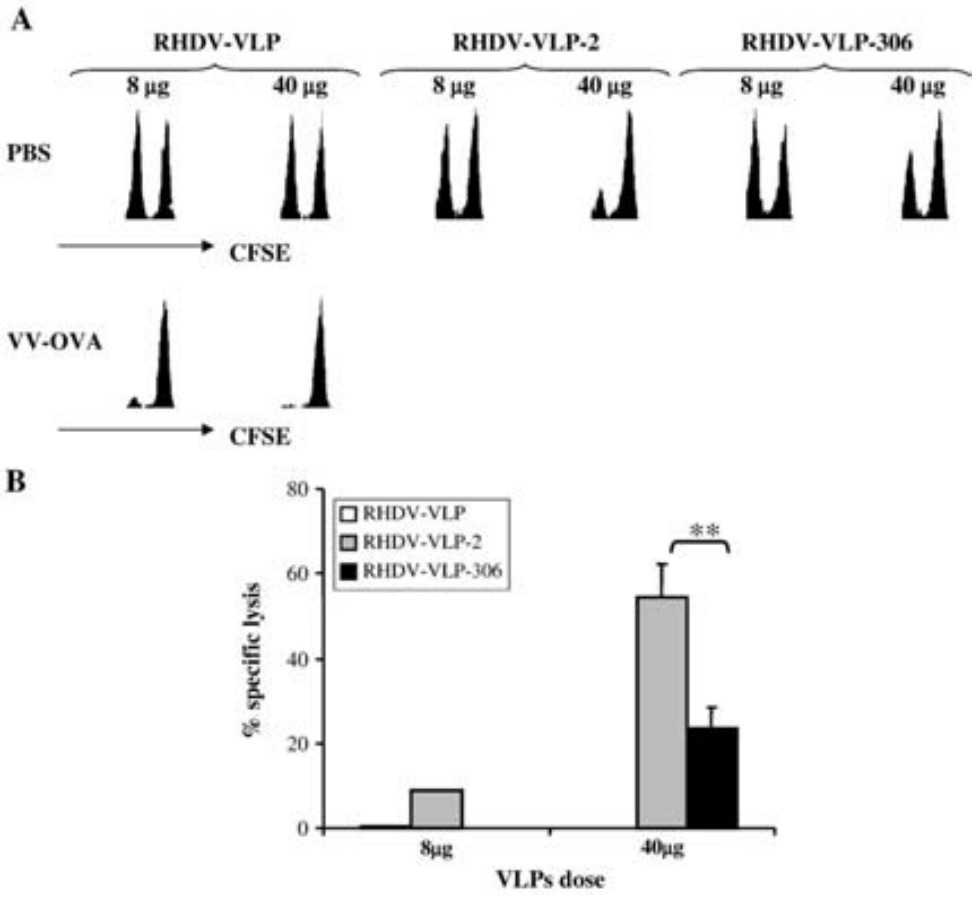

Fig. 6. OVA-specific CTL cell responses in VLP immunized mice by measuring SIINFEKL-specific cytotoxic activity using *in vivo* CTL assay as described in Materials and methods. Groups of three mice were twice inoculated with 8 or 40 µg of the different RHDV-VLPS: RHDV-VLP, RHDV-VLP-2 and RHDV-VLP-306. 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.

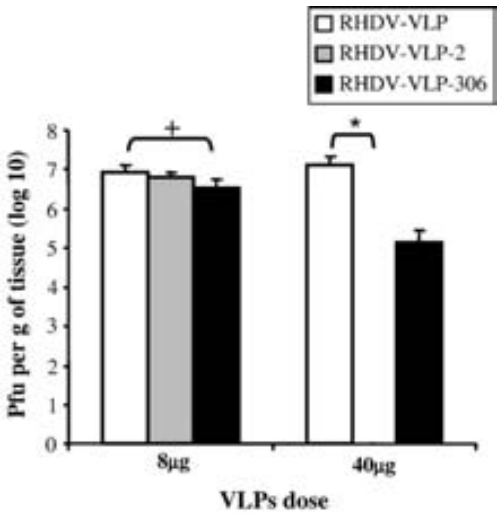

Fig. 7. Viral titers per gram of ovaries in mice immunized with the different RHDV-VLPs and subsequently challenged with VV-OVA. Mice were infected with VV-OVA 6 days before analysis. Columns indicate viral titers after immunization with 2 doses of either 8 or 40 mg of RHDV-VLPs after VV-OVA challenge. 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 *BstE*II 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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Es muy complicado resumir tanto tiempo en tan poco espacio y espero que todos los amigos cercanos hayan sido reagrupados en estas pocas palabras….MUCHISIMAS GRACIAS a TODOS… OS QUIERO…

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It is lovely to think that such a short period of time can create such strong friendship…and I'm really happy about it…

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It's complicate to summarize all in a page but I hope I grouped all in these few words…
Thanks to internet I feel closer all of you…
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