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Chimeric calicivirus-like particles elicit specific immune responses in pigs

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ABSTRACT

Virus-like particles (VLPs) have received considerable attention due to their potential application in veterinary vaccines and, in particular, VLPs from rabbit haemorrhagic disease virus (RHDV) have successfully shown to be good platforms for inducing immune responses against an inserted foreign epitope in mice. The aim of this study was to assess the immunogenicity of chimeric RHDV-VLPs as vaccine vectors in pigs. For this purpose, we have generated chimeric VLPs containing a well-known T epitope of 3A protein of foot-and-mouth disease virus (FMDV). Firstly, RHDV-VLPs were able to activate immature porcine bone marrow-derived dendritic cells (poBMDCs) *in vitro*. Secondly, pigs were inoculated twice in a two-week interval with chimeric RHDV-VLPs at different doses intranasally or intramuscularly. One intramuscularly treated group was also inoculated with adjuvant Montanide™ ISA 206 at the same time. Specific IgG and IgA antibodies against RHDV-VLPs were induced and such levels were higher in the adjuvanted group compared with other groups. Interestingly, anti-RHDV-VLP IgA responses were higher in groups inoculated intramuscularly than those that received the VLPs intranasally. Two weeks after the last immunisation, specific IFN-γ-secreting cells against 3A epitope and against RHDV-VLPs were detected in PBMCs by ELISPOT. The adjuvanted group exhibited the highest IFN-γ-secreting cell numbers and lymphoproliferative specific T cell responses against 3A epitope and RHDV-VLP. This is the first immunological report on the potential use of chimeric RHDV-VLPs as antigen carriers in pigs.

1. Introduction

Virus-like particles (VLPs) have received considerable attention due to their potential application in vaccines, gene therapy and drug delivery [1]. Indeed, VLPs are robust protein cages in the nanometer range exhibiting well-defined geometry and remarkable uniformity [1] that mimic the overall structure of the native virions. VLPs are appealing as vaccine candidates for their inherent properties of stability and non-replicative form, and have advantages in terms of immunogenicity and safety over previous approaches [2]. In fact, lacking the genome of the virus avoids any of the risks associated with virus replication, reversion, recombination or re-assortment [3]. The strong immunogenicity of VLPs, the high productivity of expression systems, their ability to achieve a rapid implementation at production scale and the cost-effectiveness have encouraged researchers in the field [4]. Additionally, VLPs have self-adjuvanting ability and stimulate strong B-cell-mediated and cellular immune responses [5].

The two most successful VLP-based vaccines 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 [6]. Also in veterinary vaccinology, new VLP-based vaccine candidates have been elaborated for different viruses including porcine parvovirus [4], porcine encephalomyocarditis [7], canine parvovirus [8,9], goose parvovirus [10], equine rhinitia A virus [11], bluetongue virus [12,13], chicken anaemia virus [14,15] and infectious bursal disease virus [16]. Although multiple candidates are in course of study in the veterinary field [17], only porcine circovirus type 2 (PCV2) VLP-based vaccine, Ingelvac CircoFLEX® developed by Boehringer Ingelheim (Germany) is commercially available. Besides, for the same virus, another sub-unit commercial vaccine, Porcilis PCV® (Intervet International, The Netherlands) is commercialised.

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In addition of being used to induce immune responses against the particle itself, animal VLPs have been successfully used as vaccine vectors for inducing immune responses against inserted foreign immunogenic epitopes (chimeric VLPs) [18–20] or for drug delivery [1].

VLPs derived from rabbit haemorrhagic disease virus (RHDV) have been shown to be highly immunogenic and protect rabbits from a lethal challenge with the virus [21–23]. These VLPs are formed from 180 copies of the single coat protein (VP60), assembled into an icosahedral capsid [24]. Recently, we have shown that chimeric RHDV-VLPs harbouring a model cytotoxic T-cell epitope at the N-terminal end of the VP60 monomers were able to stimulate a powerful specific cytotoxic response *in vivo* and to protect mice from viral challenge [18]. Moreover, a tumour antigen chemically coupled to RHDV-VLPs has been shown to delay or prevent the development of tumours [19]. The well-documented immunogenicity of VLPs is probably due to their interaction with dendritic cells (DCs) [25]. RHDV-VLPs enter both human and murine DCs by clathrin-dependent pinocytosis and phagocytosis. Win et al. found no evidence for receptor-mediated acquisition by DCs from either species, which may be related to the fact that RHDV does not naturally infect mice or humans [26].

Foot-and-mouth disease virus (FMDV) is a picornavirus that produces a highly transmissible and devastating disease in farm animals and other cloven-hoofed livestock [27]. FMDV shows a high genetic and antigenic variability, reflected in the seven serotypes and the numerous variants described to date [28]. Several T-cell epitopes frequently recognised by natural host lymphocytes have been identified in FMDV proteins. One of these T-cell epitopes, located in residues 21–35 of FMDV NS protein 3A, is efficiently recognised by lymphocytes from infected pigs. This epitope was capable to provide adequate T-helper co-operation when synthesised juxtaposed to the B-cell antigenic site VP1, inducing significant levels of serotype-specific anti-FMDV activity *in vitro* [29]. Its amino acid sequence is conserved among FMDV types A, O, and C, showing limited variation among isolates from the seven FMDV serotypes [30]. Cubillos et al. have shown the successful use of a dendrimeric peptide, using such 3A and VP1 epitopes, to protect pigs against a challenge with FMDV [31].

Taking into account the strong potential of RHDV-VLPs as vaccine vectors, our aim was to develop these VLPs as a delivery system for the multimeric presentation of immunogenic epitopes derived from pathogens relevant for animal health, in livestock species. As a first approach we generated chimeric RHDV-VLPs incorporating a well defined T-helper epitope [29] from FMDV 3A protein (RHDV-3A-VLPs), and analysed their ability to induce specific immune responses in pigs. The foreign epitope was inserted at the N-terminus of VP60 protein, which is predicted to be buried in the internal face of the VLPs. Firstly, the immunogenic potential of RHDV-VLPs in immature porcine bone marrow derived dendritic cells (poBMDCs) *in vitro* was studied. RHDV-VLPs were able to stimulate poBMDCs *in vitro*. Then, fifty conventional pigs were immunised with two doses at two week interval with chimeric RHDV-3A-VLPs using different routes. Acute phase proteins were also analysed in chimeric RHDV-3A-VLP immunised pigs since their responses comprise innate reactions [32]. Moreover, specific humoral and cellular responses against the inserted epitope and the vector were specifically induced. This is the first immunological report on the potential use of RHDV-VLPs as vectors for antigen presentation in pigs in which specific immunity against a foreign epitope was induced.

2. Materials and methods

2.1. Virus and cells

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, St. Louis, MO, USA) supplemented with 5% foetal calf serum (FCS), as described previously [33]. *Spodoptera frugiperda* cells (SF9) were used for generation of recombinant baculoviruses, plaque assays, and the preparation of high titre viral stocks. *Trichoplusia ni* cells (H5) were used for high level expression of recombinant proteins.

2.2. Construction of recombinant baculovirus transfer vectors

The baculovirus transfer vector chosen was plasmid pBacPAK8XB. This plasmid is a derivative of pBacPAK8 (Clontech, Takara Bio Europe), in which several restriction sites were eliminated from the multiple cloning site [24]. The full-length VP60 gene of RHDV was subcloned in pBacPAK8XB, generating plasmid pMVP60 [24]. The sequence coding the T-helper epitope AAIEFFEGMVHDSIK, derived from the 3A protein of FMDV [29], was inserted at the 5′ end of the VP60 gene by performing two sequential PCRs. First, two separate PCRs were performed using the primer pairs Bac1F/NT3A15R and NT3A15F/VP60PR (Table 1), 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 *Bgl*II restriction site of pBacPAK8XB generating pNT15. The inserted sequence in the resulting recombinant plasmid was verified by sequence analysis.

2.3. Generation of recombinant baculoviruses

The recombinant baculoviruses were produced using the BacPAK baculovirus expression system (Clontech, Takara Bio Europe) as described previously [24]. Briefly, monolayers of SF9 insect cells were cotransfected with recombinant transfer vectors and Bsu36I triple-cut AcMNPV DNA [34] using lipofectamine (Invitrogen). Recombinant baculoviruses were selected on the basis of their LacZ-negative phenotypes, plaque purified, and propagated as described elsewhere [35].

2.4. Expression and purification of the recombinant RHDV-VLPs

The recombinant constructs VP60 and NT15 were expressed in H5 insect cell-cultures infected with the corresponding recombinant baculoviruses, as previously described [33]. To purify the

Table 1
Oligonucleotide primers used for cloning.

Primer	Sequence (5′–3′)[a]
Bac1F	GACTCCAAGTGTGTGGGTGAAGTC
NT3A15F	TTTGAGGGC**ATG**GTACACGACTCCATTAAAGCCCGCACAGCGCCG
NT3A15R	GAGTCGTGTACCATGCCCTCAAAGAATTCAATGGCTGCCATATGAAGATCTTCTAGGATCGATCCG
VP60PR	TCCGAAGATC**TCA**GACATAAGAAAAGCCATTG

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

self-assembled VLPs, 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, consisting of 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 Applied Science) for 1 h at RT. Next, samples were adjusted to 2% Sarkosyl (sodium N-lauroylsarcosine, Sigma) and 5 mM EDTA in PBS-V, and incubated overnight (ON) at 4 °C. Subsequently, the cell lysates were clarified by low-speed centrifugation and the supernatant was 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, Sigma–Aldrich), 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 PBS-V with 15% Opti-prep (a 60% solution of iodixanol in water, Invitrogen). Pellets were 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, Thermo Scientific Inc., USA).

2.5. Porcine bone-marrow derived and human monocyte-derived dendritic cell generation

Porcine bone marrow (BM) haematopoietic cells were obtained from femurs of clinically healthy Large White × Landrace pigs of eight weeks of age. Porcine bone marrow derived dendritic cells (poBMDCs) were generated by an eight-day protocol as previously described by [36] with some modifications [37]. Briefly, bone marrow haematopoietic cells were resuspended in RPMI-1640 (Lonza, Walkesville, USA) culture medium containing 2 mM of L-glutamine (Invitrogen®, Barcelona, Spain), 100 U/ml of Polymixin B (Sigma–Aldrich Quimica, S.A., Madrid, Spain) 10% of FCS (Euroclone, Sziano, Italy) and 100 μg/ml of penicillin with 100 U/ml of streptomycin (Invitrogen®, Barcelona, Spain). Recombinant porcine GM-CSF (R&D Systems, Spain) was added at 100 ng/ml to the cells three times during the culture within 2 days intervals. Stimuli were added at day 8 and on day 9, mature poBMDCs were harvested and analysed by flow cytometry and cytokine production. Human monocyte derived dendritic cells (huMoDCs) were prepared as described previously [38]. Briefly, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll 1.077 (Sigma–Aldrich, Spain) density gradient centrifugation after leukapheresis of healthy donors performed at the Banc de Sang i Teixits (Barcelona). For isolation by adherence, PBMC were seeded in a concentration of 1×10^8 per 75 cm^2 tissue culture flasks (Corning, Spain) in 8 ml culture medium and incubated for 1–2 h at 37 °C, 5% CO_2. Non adherent cells were washed away extensively with PBS. To the adherent cells, 10 ml of X-VIVO 15 medium (BioWhittaker, Walkersville, MD) with 2% human serum (Sigma–Aldrich, Spain) was added, of which half was replaced on day one with culture medium supplemented with IL-4 (300 U/ml) and GM-CSF (450 U/ml) (Miltenyi Biotec, Spain). At day 3, all cells were harvested using cold PBS and seeded at 1×10^6 cells in 2 ml fresh culture medium per well of a 6-well plate. Stimuli were added at day 6 and mature DCs were harvested and analysed on day 7 by flow cytometry and cytokine production.

2.6. Stimulation of poBMDCs and huMoDCs

For *in vitro* studies, poBMDCs and huMoDCs were stimulated with different concentrations of RHDV-VLPs (10 and 50 μg/ml). We used as positive controls LPS (Sigma) at a concentration of 1 μg/ml for huMoDCs and 10 μg/ml for poBMDCs, and poly:IC (50 μg/ml, Sigma) for poBMDCs. Briefly, stimuli were plated with poBMDCs after 8 days of culture (5×10^5 cells/well) in 96-well plates or with huMoDCs after 6-day culture (10^6 cells/well) in 6-well plates. Activation of DCs was analysed by flow cytometry at 24 h post-stimulation or by cytokines release in supernatant at different time-points (4, 8, 16, 24 h post-stimulation) using specific ELISAs. Non-stimulated cells cultured in media served as controls. Data were obtained from four poBMDC experiments, corresponding each one to a different animal, and for huMoDCs from two different individuals.

2.7. Flow cytometry analyses of poBMDCs and huMoDCs

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 reagents were detected by hybridoma supernatants. Briefly, 5×10^5 cells/50 μl/well were labelled during 1 h at 4 °C 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), 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, bioNova cientifica, Madrid, Spain), anti-CD16-FITC (G7, Serotec), CTLA4-mIg (Ancell, MN, USA), purified anti-porcine CD86 (mouse mAb 5B9.88, Alexion Pharmaceuticals, Cheshire, CT, USA) [39] and anti-CD163 (2A10/11) were used. After incubation, they were washed with cold PBS with 2% FCS by centrifugation at 450 × *g*, 4 °C for 5 min. 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 h at 4 °C, they were 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).

To characterise and compare the phenotype of the huMoDC populations the following mAbs or appropriate isotype controls were used: anti-CD14, CD80, CD83, CD86, CCR7, MHC-I and MHC-II (all from BD Biosciences, Mountain View, CA). Primary antibodies were followed by staining with PE-labelled goat-anti-mouse (BD Bioscience). Flow cytometry was performed with FACSCanto II™ with FACSDiva software (BD Biosciences). The histogram analysis was performed with Weasel v3.0 (Walter and Eliza Hall Institute, Melbourne, Australia).

2.8. Cytokine ELISAs

Cytokine levels in conditioned cell supernatants were assayed by ELISA 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-cell supernatants were used and all the results were analysed 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 [40]. For TNF-α, IL-6 and IL-10 detection, a DuoSet® ELISA Development system (R&D Systems, Abingdon, UK) was used following 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 manufacturer's instructions (limit of detection was around 74.5 pg/ml). Supernatants of human DC cultures were collected after stimulation for 24 h. IL-10, IFN-α (Bender

Table 2
Experimental design.

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

MedSystems) and TNF-α (Mabtech) were analysed by ELISA according to the manufacturer's guidelines using Spectramax 340PC 384 reader (Molecular Devices). The detection limits were respectively 4.7 pg/ml, 7.8 pg/ml, 4.7 pg/ml and 15.6 pg/ml.

2.9. Experimental design

At the age of 6–7 weeks, fifty male conventional pigs (Large White × Landrace) were selected from a high health status farm located in the Northern part of Spain; these pigs were porcine reproductive and respiratory syndrome virus (PRRSV), influenza virus and *Mycoplasma hyopneumoniae* negative at the beginning of the experiment. Animals were clinically healthy when the study began. 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) containing 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 2). 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 organised as summarised in Table 2. Group B was intranasally inoculated with 20, 60 and 180 μg per dose of chimeric RHDV-3A-VLPs on day 0. Group C and D animals were intramuscularly inoculated in the right neck muscle with abovementioned amounts of chimeric RHDV-3A-VLPs and 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 immunisation reactions and samples of blood and saliva (using Salivette® Cotton Swab from SARSTEDT, Spain) were collected at day 0, 14 and 28 after the beginning of the experiment. Fourteen days after the second immunisation pigs were euthanised 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).

2.10. 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 analyser (Olympus AU400, Hamburg, Germany) as previously described [41].

Porcine pentraxin 3 (PTX3) concentration in sera was determined as previously described [42] by sandwich ELISA against murine PTX3 (MuPTX3) with the following antibodies: 2C3 and biotinylated 6B11. Values of OD were analysed at 450 nm and we count as positive sera those giving OD values above the cut-off of the assay (0.09).

2.11. Detection of specific anti-RHDV-VLP antibodies by ELISA

Antibodies against RHDV-VLP were examined in serum samples collected at 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), ON at 4 °C. Duplicate four-fold dilution series of each serum sample were made, starting at 1/50. Fifty microlitre 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-HRPO by monoclonal antibodies specific for these isotypes, supplied by Serotec, and using as secondary antibody a goat anti-mouse IgG (H + L)-HRP (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 chromophore, 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, respectively. Antibody titres (total Ig and isotypes) were therefore expressed as the reciprocal $\log_{10}$ of serum dilutions giving the absorbance recorded in the control wells (serum collected day 0) plus twice standard deviation. These data were calculated by interpolation.

2.12. 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 supplied by D. Andreu and B.G. De la Torre, Pompeu Fabra University, Spain), in PBS buffer and ON at 4 °C. Duplicate serum samples diluted 1/50 were assayed and specific antibodies were detected with horseradish peroxidase conjugated with protein G (Pierce), and colour development was obtained after addition of TMB substrate. We count as positive sera those giving OD values above the cut-off of the assay (0.2).

2.13. ELISPOT assay

Two and four weeks after the first immunisation, (PBMC) were collected and analysed for specific IFN-γ production by ELISPOT set following manufacturer's instructions (Becton Dickinson, UK). PBMCs were isolated by Histopaque-1.077® gradient and plated in duplicate at 5×10^5/100 μl/well in RPMI-1640 supplemented with 10% FCS into 96-well plates (MultiScreen® MAHAS4510 Millipore) coated ON at 4 °C with 5 μg/ml 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 use as negative control. Plates were cultured for 72 h at 37 °C, then incubated with 2 μg/ml of

biotinylated anti-IFN-γ mAb (P2C11, Becton Dickinson), followed by streptavidin–horseradish peroxidase conjugates (Jackson Immunoresearch Lab.). 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.

2.14. Lymphoproliferation assay

Proliferation assays of swine lymphocytes were performed as described previously [43]. Blood was collected in 5 μM EDTA and used immediately for the preparation of PBMCs [44]. Assays were performed in 96-well round-bottomed microtitre plates (Nunc). Briefly, 2.5×10^5 PBMCs 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 various concentrations of (i) RHDV-VLP at 20 μg/ml and (ii) 3A synthetic peptide, at 20 μg/ml. Cultures with medium alone or with mock-infected cells 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).

2.15. Pathological analysis

The objective of the pathologic studies at the site of injection (right neck muscle, *brachiocephalicus*) was to establish any reaction due to the intramuscular immunisation. 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 haematoxylin 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 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 fibres and fibrosis were observed.

2.16. Statistical analysis

All statistical analyses were 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.

3. Results

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

In order to analyse the potential of RHDV-VLPs as a delivery system for foreign T cell epitopes in pigs, we produced recombinant baculoviruses expressing a RHDV VP60 construct harbouring an epitope derived from the 3A protein of FMDV (AAIEFFEGMVHDSIK) fused to the N-terminus. Expression was verified by SDS-10% PAGE (data not shown). Preparations were characterised by electron microscopy to confirm that the chimeric VP60 construct self-assembled into VLPs (data not shown).

3.2. Maturation of poBMDCs after RHDV-VLP stimulation

Once chimeric RHDV-VLPs expressing 3A epitope were generated, their immunogenicity was studied by analysing whether or not RHDV-VLPs were able to stimulate poBMDCs *in vitro*. Consistent with previous reports [36,37], poBMDCs phenotype at day eight 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 homogeneous and they constituted our starting culture for further experiments. Porcine BMDCs maturation induced by RHDV-VLPs was evaluated after 24 h of stimulation. Pulsing poBMDCs with RHDV-VLPs resulted in increased levels of maturation markers SLA-II, CD80/86 and CD86 that in some cases reached higher levels that after LPS stimulation (Fig. 1). A dose-dependent up-regulation of SLA-II and CD80/86 was found (Suppl. Fig. 1). No differences were found in all other surface markers assessed (data not shown).

3.3. RHDV-VLPs induce secretion of cytokines by DCs

To investigate whether RHDV-VLPs were capable of inducing cytokine production in DCs, 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 different concentration of RHDV-VLPs at different time-points. LPS or 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. 2) and the differences with un-stimulated DCs showed a strong statistical tendency ($0.07 < p$ value < 0.05). 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 inflammatory cytokine as early as 4 h after stimulation. Moreover, RHDV-VLP-stimulation was lower than LPS or poly:IC induction (Fig. 2) with a statistical tendency ($0.08 < p$ value < 0.05). RHDV-VLPs did not induce DCs to secrete IFN-α, IL-10 and IL-18, at least not above the detection limit of the assay (data not shown).

3.4. Detection of acute phase proteins in serum

Then, groups of conventional pigs were inoculated through different routes and with different amount of chimeric RHDV-VLPs as stated in Section 2 and Table 2. Firstly, acute phase proteins such as haptoglobin (Hp) and PTX3 were analysed to determine whether they could influence immune responses in the treated animals. Protein levels were tested in sera after each RHDV-3A-VLP injection. No differences were found in Hp production between the various cohorts studied at day 14 and day 28 (data not shown). On the contrary, PTX3 levels in sera were higher in animals from groups C1 and D1 compared with control animals at day 14, but then PTX3 decreased at day 28 and only animals in group D1 remained statistically higher than control animals (Table 3). Moreover, animals in group C1 exhibited PTX3 levels statistically higher when compared with all animals in group B at day 14, although these differences were no longer observed at day 28 (Table 3). All the other groups showed no significant differences at either day 14 or 28 (data not shown).

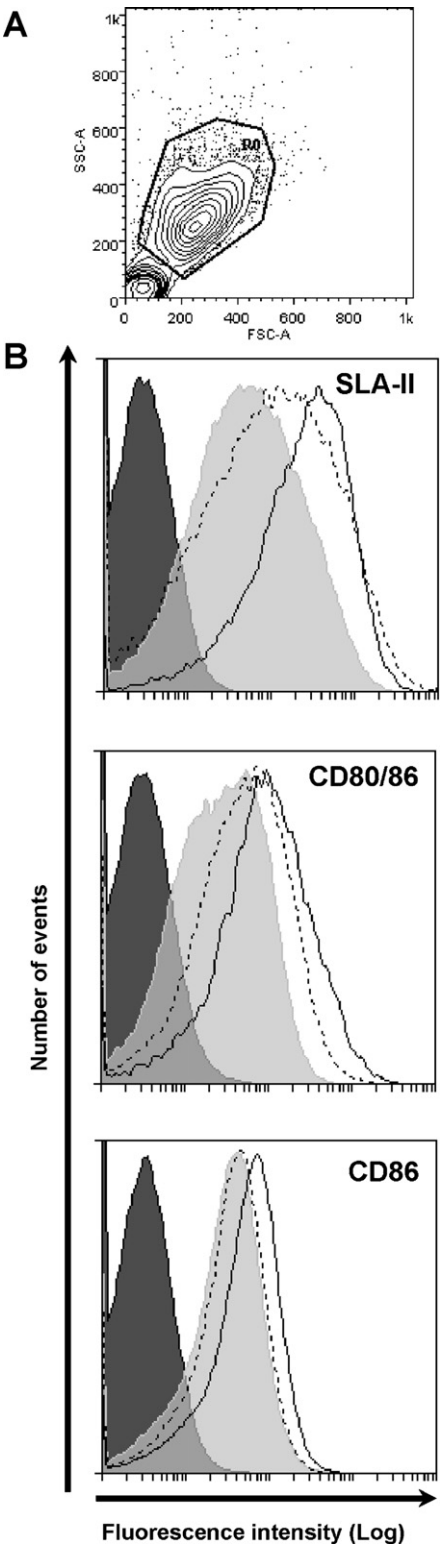

Fig. 1. Phenotypic maturation of poBMDCs after exposure to RHDV-VLPs or LPS. PoBMDCs were incubated with either RHDV-VLPs (50 μg/ml) or LPS (10 μg/ml) and after 24 h of culture, surface expression of SLA-II, CD80/86 and CD86 was determined by flow cytometry using PE-conjugated secondary mAb. (A) Dot-plot shows the gating strategy based on FSC/SSC. R0 region indicates poBMDCs. (B) Histograms show expression patterns of 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 of a minimum of five independent experiments are shown.

Table 3
Statistical analysis of PTX3 in sera. p values resulted from non-parametric Mann–Whitney test comparing PTX3 concentration between different groups at day 14 and 28.

	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

NS, no significative differences.
* p values < 0.05 are considered statistically significant.

3.5. RHDV-VLP and 3A specific antibody responses after two immunisations

Serum samples were collected from each VLP-immunised pig group before immunisation and on day 14 and 28 after the first VLP inoculation. As shown in Fig. 3A, total Ig antibodies specific for RHDV-VLPs were readily detectable at two weeks after priming in all pigs immunised by IM route with adjuvant and at all doses tested. Total specific antibody levels increased rapidly up to more than 4 $\log_{10}$ after the last immunisation in those pigs. Immunisation with RHDV-VLPs by IM without adjuvant required the administration of at least two doses to induce significant anti-RHDV-VLP antibodies. Throughout this experiment, pigs immunised using the IM route plus adjuvant produced significantly higher RHDV-VLP total antibody titres than the other groups at days 14 (p value < 0.05) and 28 (p value < 0.01). No statistical differences were found within animals in groups C and D when comparing the effect of different doses (p value > 0.1). Pigs inoculated IN showed the lowest antibody responses against the vector and no statistical differences were found between doses (p value < 0.1).

In 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. 3B–D). In this case, also animals in group C showed the highest antibody production with significant differences (p value $\leq$ 0.01) compared with animals from other groups at day 28. No statistical differences were found at this time point within animals from group C between the subgroups receiving different doses (p value > 0.1). At day 14, specific isotype responses in animals from group C were higher compared with the other groups (p value < 0.05) (Fig. 3), but pigs inoculated IN and IM without adjuvant did not show significant differences between them (p value > 0.1) (Fig. 3B–D). Considering the IgG1 responses of animals in group C at day 14 (Fig. 3B), a dose effect was found: the lower dose was statistically different than the highest (p value = 0.02). No statistical differences were found in the other groups caused by the different doses (p value > 0.1).

Interestingly, pigs immunised by parenteral route with chimeric RHDV-3A-VLPs were able to induce anti-RHDV-VLP IgA in serum (Fig. 3D). Increased serum anti-RHDV-VLP IgG1, IgG2 and IgA levels were detected after the first immunisation in the intranasally treated group. However, no further enhancement was found after boosting (Fig. 3B–D). Only the group inoculated with 60 μg IN showed an increased IgA response in serum after the boost (Fig. 3D). The specific RHDV-VLP antibodies remained undetectable in serum of the negative control group for the duration of the experimental procedure (data not shown). Regarding specific humoral responses to FMDV peptide 3A, only the animals in groups C1 (3/5 animals; OD values: 0.4; 0. 39 and 0.26) and C2 (2/5 animals; OD values: 0.3 and 0.5) have shown detectable antibodies in serum after the second immunisation.

IgA were also detected in saliva of RHDV-3A-VLP immunised pigs. Titres followed a similar pattern as in sera but with lower

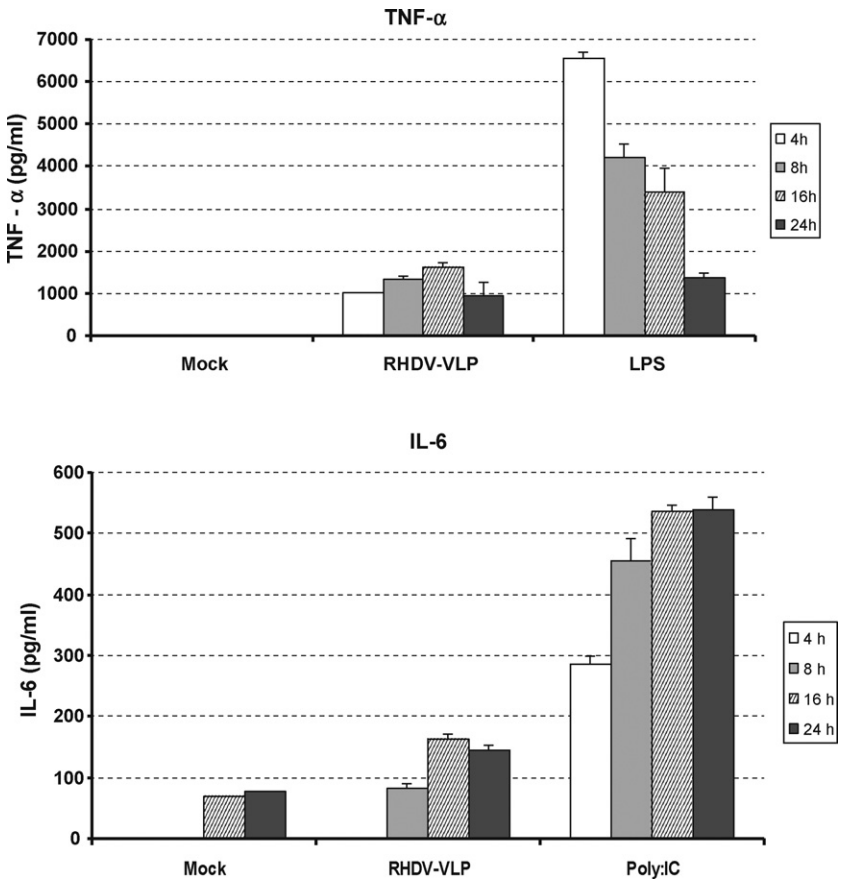

Fig. 2. Cytokine production of poBMDCs pulsed with VLPs and LPS or poly:IC *in vitro*. Immature poBMDCs were pulsed with RHDV-VLPs (50 μg/ml) or LPS (10 μg/ml) or poly:IC (50 μg/ml). After 4, 8, 16 and 24 h of culture, secreted TNF-α and IL-6 were quantified by commercially available ELISAs. Cytokine 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 of a minimum of three independent experiments are shown.

anti-RHDV-VLP antibody levels (Fig. 3E); indeed, animals in group C reached around 2.2 $\log_{10}$ titres at day 28. Significant differences were found between animals in group C and B (p value < 0.03), and D1 and B (p value < 0.05) at day 28. Animals from group D1 showed the same responses as adjuvanted group (no statistical difference, p value > 0.1) and also, no differences between doses were found in animals from group C. Interestingly, mucosal immunisation did not elicit high amount of local specific RHDV-VLP IgA antibodies, whereas parenteral inoculation could stimulate higher production of specific RHDV-VLP mucosal IgA locally in saliva (Fig. 3E). At day 14 no statistical differences were observed between all the groups (p value > 0.1). The negative control group remained negative in saliva for specific RHDV-VLP antibodies during the experimental procedure (data not shown).

3.6. *RHDV-VLP and 3A specific cellular immune responses elicited by chimeric RHDV-3A-VLP immunisation*

To get further insight into the immune responses induced by RHDV-3A-VLPs, cell-mediated immune responses in pigs immunised with chimeric RHDV-3A-VLPs were studied by analysing porcine PBMCs isolated after each immunisation. 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 specific 3A IFN-γ-secreting cells. Indeed, 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. 4A). Animals in 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 value > 0.1) (Fig. 4A). Animals in group C showed a high statistical difference with animals from group B (p value = 0.01), whereas only a strong tendency was found between animals from group C1 or C2 and group D (0.09 < p value < 0.06). An interesting finding was that RHDV-3A-VLP immunisation was able to induce specific RHDV-VLP IFN-γ-secreting cells also after the first inoculation in all the groups, mainly in animals from group C1 (Suppl. Fig. 2). Likewise, specific 3A IFN-γ-secreting cells were detected at day 14 in all the groups, mainly in animals from group C1 (Suppl. Fig. 2). Responses at day 14 were lower that at day 28 and no statistical differences were found between the subgroups receiving different doses of RHDV-3A-VLPs (p value > 0.1). As expected, control pigs (Fig. 4A and Suppl. Fig. 2) or pigs prior to immunisation did not show any significant response.

The data were also consistent with the measurement of the lymphoproliferation assay indicating that the RHDV-3A-VLPs were capable of inducing cellular immune responses against the foreign antigen and the vector. Lymphoproliferation assay results had the same pattern as the ELISPOT results and animals immunised with the adjuvant were able to induce higher responses against the vector and the peptide compared with the other groups (Fig. 4B). Stimulation indexes (SI) from animals from group C had lower levels against the peptide 3A compared with the vector RHDV-VLP (Fig. 4B) but no statistical differences were detected as a result of the different doses within the same group (p value > 0.1). Conversely, different results were obtained when PBMCs at day 28 were stimulated with RHDV-VLP. In this case, animals from group C showed the

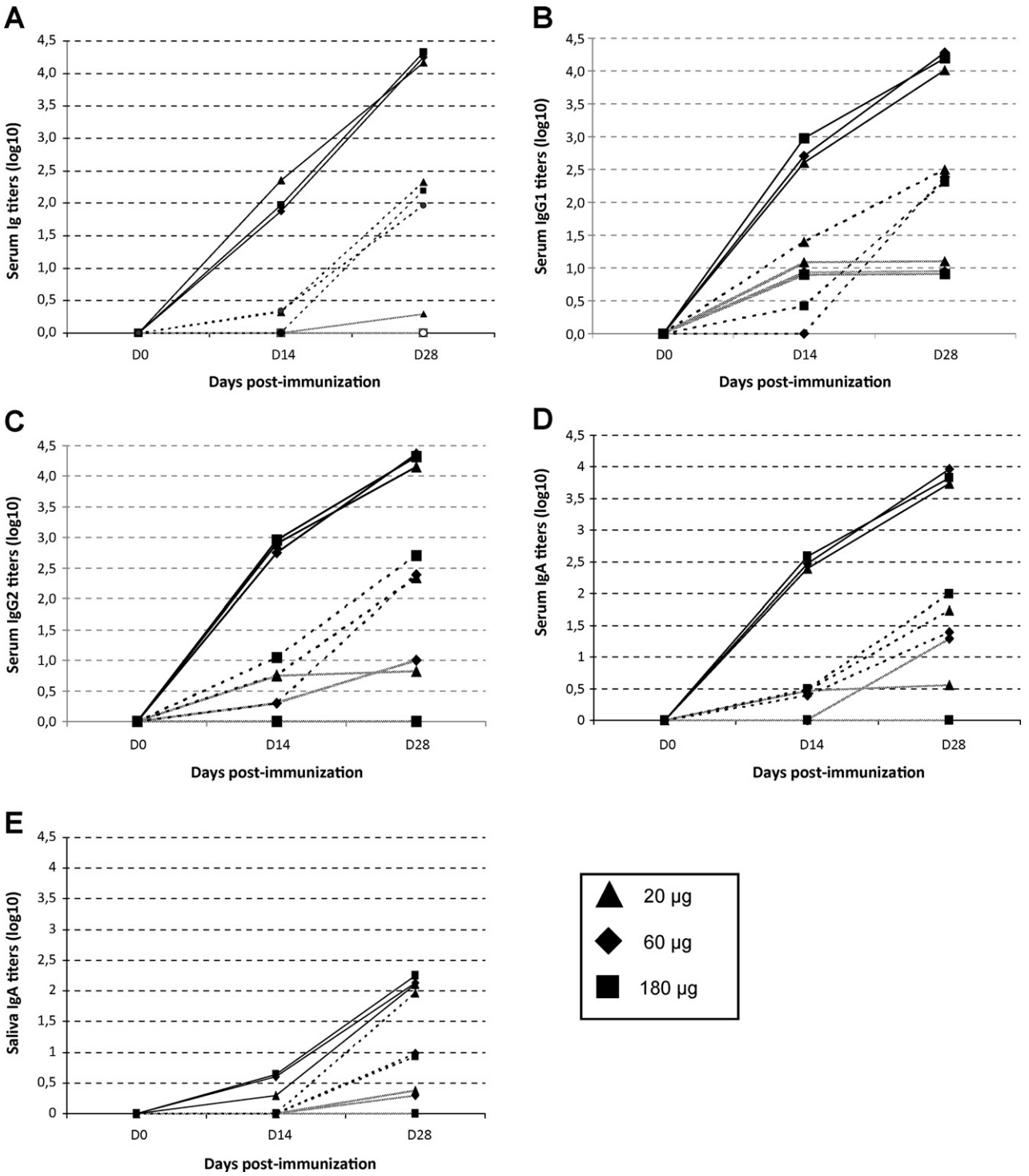

Fig. 3. Specific humoral responses of RHDV-3A-VLP immunised pigs against the vector RHDV-VLP in serum and saliva at day 14 after the second immunisation. (A) Anti-RHDV-VLP total Ig antibodies in serum. (B) Anti-RHDV-VLP IgG1 antibodies in serum. (C) Anti-RHDV-VLP IgG2 antibodies in serum. (D) Anti-RHDV-VLP 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 (rhomb) and 180 μg (square), summarised in the legend. 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.

highest responses, but also a significant dose effect whose 20 μg-dose–response was significantly higher (p value = 0.02) compared to the 180 μg-dose response (Fig. 4C). Moreover, animals immunised IN with 180 μg showed comparable cellular responses than pigs vaccinated IM with 180 μg of RHDV-3A-VLPs (Fig. 4C). No stimulation was observed in PBMCs from control pigs or pigs prior to RHDV-3A-VLP immunisation (data not shown).

3.7. Pathological analysis

Macroscopically, no injection site reactions were observed after immunisation and all swine were healthy during the immunisation 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

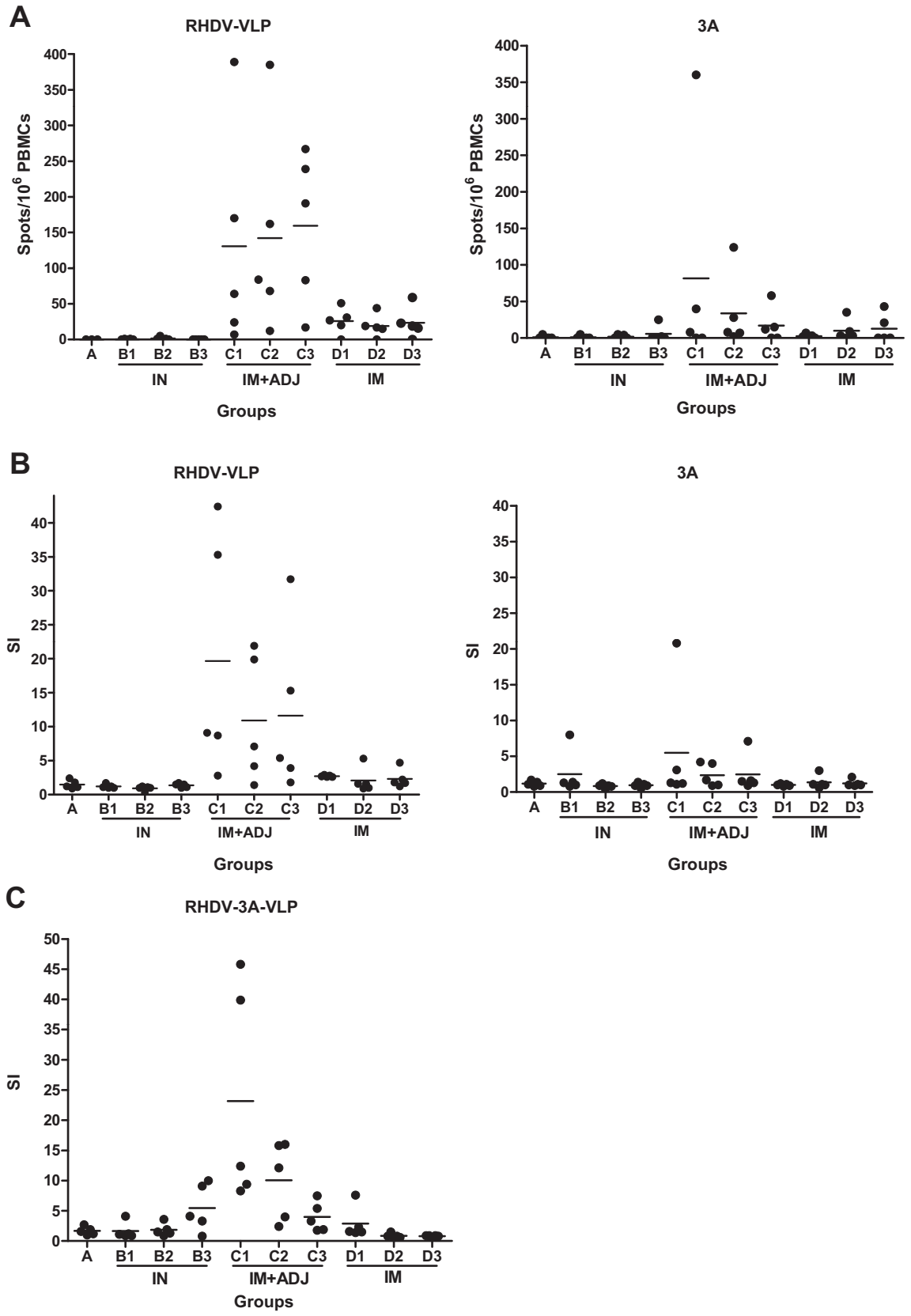

Fig. 4. Specific cellular responses of RHDV-3A-VLP immunised pig against the vector RHDV-VLP, against the peptide 3A and against chimeric RHDV-3A-VLP at day 28. Pigs are divided in different groups, depending on the inoculation route: intranasal (IN), intramuscular + adjuvant (IM + ADJ) and intramuscular (IM). 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 Section 2) of each animal. Results of triplicate wells of one representative experiment are shown.

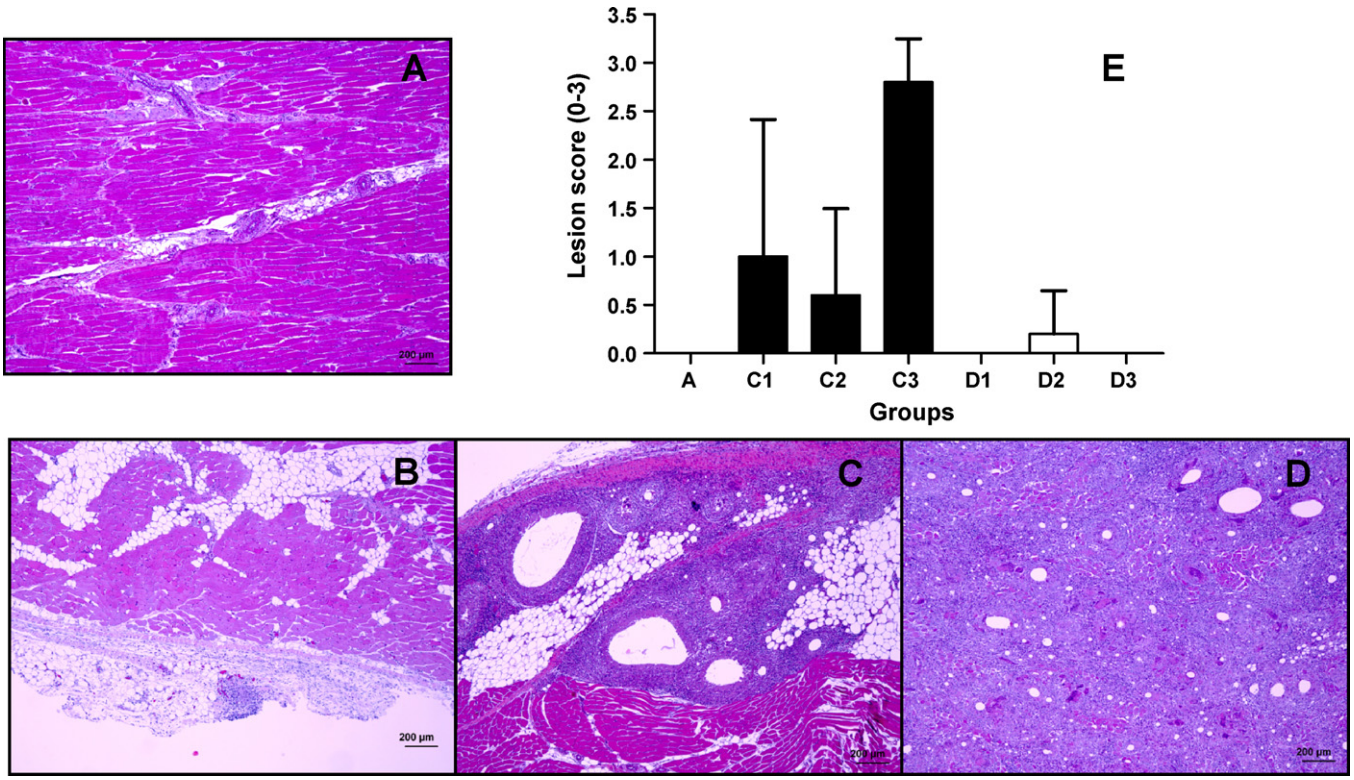

Fig. 5. Histopathological analysis of injection point (*brachiocephalicus* muscle) of pigs at day 14 after the second immunisation (H–E stain, bar = 200 μm). Lesions were classified as (A) absence; (B) mild: small focus of granulomatous inflammation located in the perimuscular adipose tissue; (C) moderate: granulomatous reaction is partially occupying the perimuscular fat with mild infiltration of the adjacent muscular tissue; (D) severe: adipose and muscular tissues are infiltrated and almost substituted by a diffuse granulomatous inflammation. (E) In the graphic is shown the pathological score (0–3) for all the groups indicated as mean (bars) ± standard deviation (lines).

reaction composed by 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. Few lymphocytes, plasma cells and eosinophils were present in the periphery of the lesion (Fig. 5).

From the thirty-three studied pigs (3 control pigs and 30 intramuscularly injected), 23 (69.7%) showed no histopathological lesions (Fig. 5A), 2 (6.1%) had mild lesions (Fig. 5B), 3 (9.1%) moderate lesions (Fig. 5C) and 5 (15.1%) severe lesions (Fig. 5D). 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. 5). Statistical differences were found between animals in group C3 and group A (p value = 0.02) or all group D (p value < 0.007). Moreover, the animals from the groups immunised with the highest dose of chimeric RHDV-VLPs showed significant higher lesion score compared with other groups immunised with other doses (p value < 0.05).

3.8. Maturation of huMoDCs after RHDV-VLP stimulation and cytokine production

Once chimeric RHDV-VLPs potential as vaccine vector was established in pigs, as it was previously established in mice [18], the question was whether these properties could be applied in other systems. In the mice system as well as in the swine system, there was a direct relationship between activation of BMDC *in vitro* and induction of significant specific responses *in vivo*. Therefore, human monocyte derived DCs (huMoDCs) were tested *in vitro* as a surrogate model for possible human use. At day six of culture, the un-stimulated huMoDCs phenotype (data not shown) was consistent with previous observations [38]. After stimulation with RHDV-VLPs, huMoDCs exhibited a similar pattern of activation than poBMDCs with up-regulation of MHC-II and CD86 (Fig. 6). The activation level was dose-dependent (data not shown). In this system, LPS stimulation of surface markers was higher or equal to RHDV-VLP stimulation (Fig. 6). No differences were found when assessing all the other surface markers indicated (data not shown). No increased expression of maturation markers could be detected in un-stimulated DCs (data not shown). However, RHDV-VLPs did not induce huMoDCs to secrete IL-10, IL-12, TNF-α and IFN-α or the production did not result in appreciable levels (data not shown).

4. Discussion and conclusions

In this work, the potential of RHDV-VLPs as vaccine vectors for antigen presentation in pigs was investigated for the first time, using a well-known T helper epitope derived from the 3A protein of FMDV. New subunit vaccines are getting a foothold in veterinary vaccinology and VLPs are one of the most appealing approaches, opening up new frontiers in animal vaccines. VLPs are protein shells that mimic the overall structure of the virions and are often antigenically indistinguishable from native virus, maintaining a non-replicative form. 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, drugs and other small molecules to the immune system. VLPs derived from RHDV have shown to be a suitable vector for the presentation of foreign T-cell epitopes in mice [18,19], but this knowledge has not been translated into applications suitable for relevant animal health diseases affecting livestock animals.

Firstly, the results show that RHDV-VLPs have the ability to stimulate immature poBMDCs *in vitro* by up-regulating SLA-II molecule

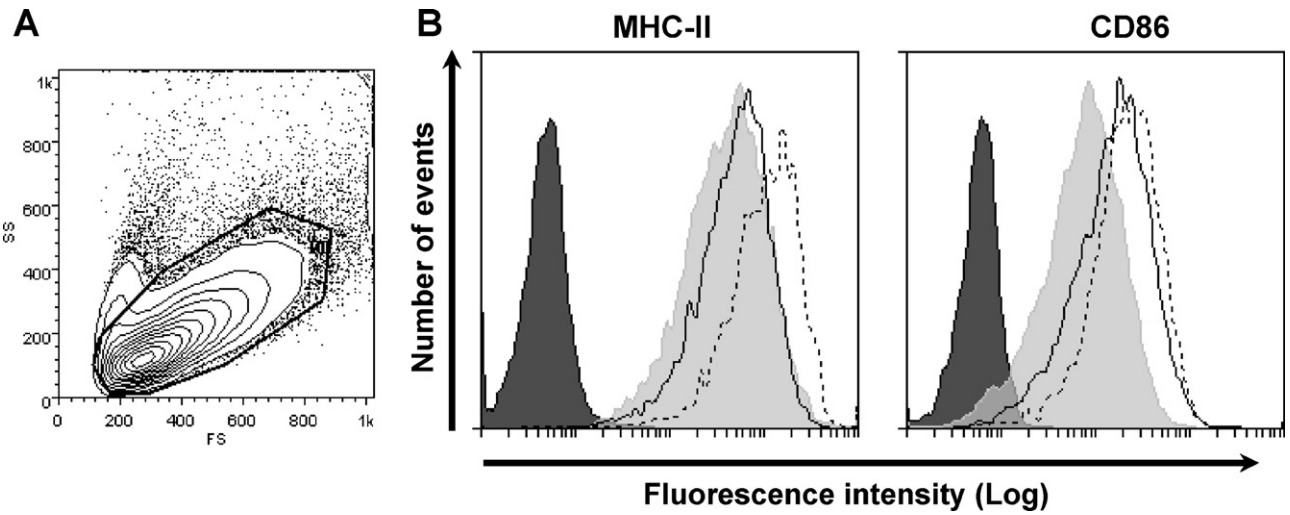

Fig. 6. Phenotypic maturation of huMoDCs after exposure to RHDV-VLPs or LPS. huMoDCs were incubated with either RHDV-VLPs (50 μg/ml) or LPS (1 μg/ml), and after 24 h of culture, surface expression of MHC-II and CD86 was determined by flow cytometry using PE-labelled secondary mAb. (A) Dot-plot shows the gating strategy based on FSC/SSC. R0 region indicates huMoDCs. (B) Histograms show expression patterns on huMoDCs gated for FS/SS. RHDV-VLP-pulsed huMoDCs (solid line) and LPS-exposed huMoDCs (dotted line) express higher levels of all markers as compared with un-stimulated poBMDCs in steady state (grey histograms) or isotype control (black histograms). Results of one representative experiment out of two independent experiments.

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-(by poBMDCs in culture supernatant. In our *in vitro* porcine system, the induction of surface markers and cytokine secretion in DC was dose-dependent. Our results confirmed previous studies performed in mice and humans with other VLPs. Lenz et al. have shown that human papillomavirus VLPs have the ability to stimulate immature murine BMDCs with comparable results [45]. Likewise, human DCs pulsed with rodent polyomavirus VLPs were maturated by up-regulation of CD86, MHC-I and MHC-II and were found to secrete IL-12 [46]. 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 through the NF-κB transcription factor pathway, which stimulates both cytokines [47]. To exclude any possible interference of contaminants (*e.g.* LPS) in our VLPs preparations, porcine DC cultures were maintained in conditioned medium with Polymixin B, an antibiotic which will attach to any traces of LPS in the medium. In fact, when Polymixin B was not removed from the culture, addition of 1 μg/ml LPS in the medium as positive control did not activate DC *in vitro* (data not shown). This conditioned medium was maintained during the DC cultures, avoiding any interference with possible minor concentrations of LPS. Furthermore, several *in vitro* experiments were performed including a mock VLP preparation (prepared from insect cells infected with wild type baculovirus). The VLPs used for comparison were RHDV-VLP-2 and RHDV-VLP-306, used in Crisci et al. [18]. When murine bone marrow derived-DC were incubated with different stimuli overnight, mock VLP showed the lowest level of TNF-α production, comparable with TNF-α production of PBS-V (the dilution buffer of the VLP samples) and un-stimulated DC (data not shown).

Secondly, we tested the immunogenicity of chimeric VLPs carrying the T helper epitope derived from the FMDV 3A protein *in vivo* in pigs. Acute phase proteins were first determined to evaluate an unspecific reaction to the immunisation. Our results show that VLP immunisation did not alter Hp serum concentration, whereas PTX3 induction was present only at early stage of immunisation. Hp is released rapidly by liver during the course of innate reaction [32], whereas PTX3 is produced locally mainly in DCs [48]. 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 [32]; in the same way, the immunisation did not alter Hp concentration during the study. On the contrary, PTX3 concentration in serum increased at early stages of immunisation, 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 porcine BMDCs (Crisci et al., submitted for publication) and/or to the fact that in our system the intramuscular injection seems to be more effective in the stimulation of immune responses.

Taking into consideration humoral responses, higher increases in antibody levels against RHDV were induced in groups containing adjuvant after the second immunisation. Only adjuvant-immunised animals were able to elicit anti-FMDV antibodies two weeks after the second immunisation and the response to the carrier were higher than to the 3A epitope. These results were not unexpected since the 3A protein-derived epitope is mainly a T helper epitope and the insertion site on the VLPs, predicted to be buried, might not be the optimal location to enhance a humoral response. Even though, presence of adjuvant seemed to enhance the capacity to induce anti-FMDV antibodies. When different routes of delivery were tested, intranasal *versus* intramuscular immunisation, different responses were also obtained. The parenteral injection of chimeric VLPs was more effective than the mucosal administration for eliciting specific antibody responses; indeed, higher anti-RHDV-VLP IgA responses in saliva were observed in groups inoculated intramuscularly. 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 VLPs dosage. The lowest dose of VLPs was able to induce in saliva similar responses to the one exhibited by animals in the adjuvant-immunised group at day 28. However, considering the general picture, our results provide evidence that immune responses induced after homologous prime-boost immunisation with chimeric RHDV-VLPs are more dependent on presence or absence of adjuvant than on the VLP-dosage in pig. The addition of an adjuvant plays an important role by enhancing immune responses. This strategy has shown to be effective in high humoral responses induction. However, further studies are required to get

insight into the protective capacity of these responses, for example including a FMDV neutralising B-cell epitope as the one successfully used in dendrimeric peptides [31].

Adjuvants stimulate immune system, but can lead to unintended stimulations and different adverse reactions, which can result in unwanted side effects such as fever and granulomatous inflammation. The acceptability of the side effects is determined by the species in which the adjuvant is applied. The decision to use water in oil in water double emulsion Montanide™ ISA 206 adjuvant was based on the immunogenicity results previously shown in pigs [49,50]. Interestingly, no macroscopic lesions were observed after immunisations and animals maintained the healthy status during all the experimental period. The histopathological studies show a local recall 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 immune response by recruiting professional APC to the immunisation site, by increasing the delivery of antigen to APCs or by improving the uptake of the antigen by DCs, enhancing the efficacy of immune responses. Thus, our chimeric VLPs could be considered another important candidate to add to the list of FMDV-VLP-based vaccines, since 3A epitope is shared by different serotypes.

The type of cellular immune response was determined by measuring IFN-γ, a marker for T helper type 1 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 several pathogens, particularly viral infections. IFN-γ stimulates MHC expression in antigen-presenting cells and efficiently inhibits FMDV replication [51]. Chimeric RHDV-VLP immunised 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 T cell response in the anti-viral immunity. For the development of a potent immunisation, a prolonged immune response is required to provide protection against a subsequent infection. Hence, the potential of chimeric RHDV-VLPs immunisation to sustain a memory T cell response was investigated after two weeks. Results show that immunisation with chimeric RHDV-VLPs was sufficient to induce a specific cellular memory response. Although induction of neutralising antibodies is considered to be the most important immune correlate with FMDV protection, specific T cells are also induced in convalescent and conventionally vaccinated animals and are relevant for protection [52]. A previous study with dendrimeric epitope based on 3A peptide and a relevant B-epitope (FMDV site-A) has shown that high stimulation indexes (SI) were present in protected animals against FMDV challenge [31]. Thus, it is conceivable to speculate that chimeric VLP-immunised animals exhibiting similar SI to 3A peptide in Cubillos et al. might exert some degree of response against FMDV infection. An additional study has to be performed to get insight the efficacy of RHDV-3A-VLP immunisation, since the aim of this study was only to investigate the immunogenicity of the vector in pigs.

The 3A epitope has been already described and characterised as a T helper immunodominant epitope [29]. As such, the 3A epitope is SLA-II restricted and therefore, a certain degree of variability in the response was expected when working with an outbred population, as the one used in this study. Thus, some animals will respond better than others, being some of them unresponsive. If we consider dendrimeric peptide-based vaccination, previous studies have shown similar variability in the cellular response than the one shown in our study [31,50]. However, another plausible explanation for our results would be that a comprehensive kinetic on the cellular response against chimeric RHDV-VLPs has not been performed in each animal and therefore the optimal window to detect the T cells responses might have been lost for some animals.

Nonetheless, we analysed the responses in animals from the groups inoculated with RHDV-3A-VLPs plus the adjuvant, since responses in these groups were more homogeneous and they can be evaluated better than in their counterparts. In the adjuvanted groups, 100% of the animals responded to the vector RHDV-VLP by ELISPOT and by lymphoproliferation. Considering the specific response to the 3A peptide, 11/15 animals (73%) responded in the ELISPOT assay and 10/15 (66%) in the lymphoproliferation assay. Generally speaking, pigs giving the greatest response to the vector were the ones that elicited the highest response to the peptide. Taking into account the variability in an outbred population, these data indicated a clear relationship between the responses to RHDV-VLPs and the responses to the 3A peptide.

Earlier results obtained in our lab indicated that in order to induce an efficient cellular response with chimeric RHDV-VLPs, the foreign T-cell epitope should be inserted at the N-terminal end of the VP60 capsid protein, which locates at the internal face of the VLPs [18]. On the other hand, we have recently obtained very promising results concerning the ability of chimeric RHDV-VLPs to induce a potent neutralising humoral response against inserted foreign B-cell epitopes in mice (unpublished results), indicating that the best suited insertion site for such epitopes is located at the predicted exposed loop within the P2 subdomain of the RHDV capsid protein, previously identified by our group [18]. Taken together, our results open the way for generating chimeric RHDV-VLPs designed for co-delivering foreign T and B-cell epitopes (inserted at the N-terminal and at predicted exposed loop of VP60 protein, respectively), which might eventually be used to immunise pigs using the experimental conditions established in this study.

Additionally, when DCs were stimulated *in vitro* by RHDV-VLPs, there was an important degree of immunogenicity elicited by RHDV-VLPs *in vivo*, not only in mice [18] but also in swine (this report). Therefore, if RHDV-VLPs were able to stimulate human DCs it might be reasonable to speculate that they might be a good vaccine vector for human diseases as well. Indeed, RHDV-VLPs were able to stimulate immature human monocyte derived DCs *in vitro*, paving the way for further uses of RHDV-VLPs as vaccine vectors in humans.

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2012.01.069.

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