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Evaluation of a viral DNA-protein immunization strategy against African swine fever in domestic pigs

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ABSTRACT

African swine fever virus (ASFV) causes serious disease in domestic pigs for which there is no vaccine currently available. ASFV is a large DNA virus that encodes for more than 150 proteins, thus making the identification of viral antigens that induce a protective immune response difficult. Based on the functional roles of several ASFV proteins found in previous studies, we selected combinations of ASFV recombinant proteins and pcDNAs-expressing ASFV genes, to analyze their ability to induce humoral and cellular immune responses in pigs.

Pigs were immunized using a modified prime-boost approach with combinations of previously selected viral DNA and proteins, resulting in induction of antibodies and specific cell-mediated immune response, measured by IFN-γ ELISpots. The ability of antibodies from pigs immunized with various combinations of ASFV-specific antigens to neutralize infection *in vitro,* and antigen-specific activation of the cellular immune response were analyzed.

1. Introduction

Virulent isolates of African swine fever virus (ASFV), a large, enveloped DNA virus (Tulman et al., 2009), cause an acute hemorrhagic fever in domestic pigs which usually die in fewer than twelve days post-infection. Due to the lack of effective vaccines, African swine fever (ASF) is considered one of the most significant and devastating viral diseases of domestic pigs (Sanchez-Vizcaino et al., 2013).

Recent outbreaks reported in Caucasus countries, Russia and Eastern Europe highlight the urgent need to develop effective vaccines against ASFV. Unfortunately, the development of an effective, safe ASFV vaccine is a complex objective and has so far been unsuccessful. Nevertheless, pigs surviving ASFV infection develop a strong protective immunity, indicating that an effective vaccine against ASFV may be possible.

The use of inactivated viruses and ASFV subunits (viral DNA or proteins) as vaccines represents a safe possibility; however, inactivated preparations of ASFV have not conferred protection so far, even in the presence of adjuvants (Blome et al., 2014). While vaccine strategies based on the expression of ASFV proteins seem to favor the generation of neutralizing antibodies (NAbs) in the vaccinated animals, the combination of recombinant proteins with plasmid DNA expressing ASFV antigens has not been approached. Passive transfer of ASFV antibodies protected pigs against a lethal ASFV challenge (Onisk et al., 1994), demonstrating the potential protective properties for specific antibodies, although there is controversy about the role of antibody-mediated neutralization of ASFV (Escribano et al., 2013). Several ASFV proteins have been reported to induce neutralizing antibodies in immunized pigs, including the proteins p72, p54 and p32 (also called p30) (Gomez-Puertas et al., 1998, 1996). However, pigs immunized with p54 and p32 recombinant baculovirus-expressed proteins were not protected against lethal infection (Gomez-Puertas et al., 1998). In the same line, a vaccine based on the expression of the ASFV proteins p30, p54, p22 and p72 in baculovirus was able to produce neutralizing antibodies but failed to protect pigs from a virulent challenge (Neilan et al., 2004). In contrast, the ASFV hemaglutinine (HA) protein (EP402R, also named CD2v) when functionally expressed in a baculovirus system, showed some degree of protection against a virulent challenge (Ruiz-Gonzalvo

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et al., 1996).

Strategies based on DNA vaccines have been also performed (Argilaguet et al., 2011, 2012) and immunization with a DNA expression library excluding viral CD2v, p30/p32 and p54 conferred partial protection against virulent challenge (Lacasta et al., 2014). Furthermore a very recent work showing the immunization of pigs by DNA-prime and recombinant vaccinia virus-boost to identify ASFV immunogenic and protective proteins has been reported (Jancovich et al., 2018). From these studies several immunogenic ASFV antigens have been identified, but a proper combination of these or still non-described antigens that are able to confer full protection has yet to be elucidated.

In order to contribute to the development of a future subunit vaccine, we have here studied the combination of specific recombinant ASFV proteins and plasmid-expressing viral DNAs, with the aim to enhance both the humoral and cellular immune responses. Pigs were immunized three times using a prime-boost approach with various combinations of viral DNA and proteins, resulting in production of antibodies, some of which were proven to be partially neutralizing *in vitro*. In addition, the specific cell-mediated immune response was measured by IFN-γ ELISpot assays to identify the combination of antigens inducing the best interferon responses.

2. Materials and methods

2.1. Cells and virus

Vero and COS-7 cells (both derived from African green monkey kidneys) were obtained from the American Type Culture Collection (ATCC) to use for virus propagation. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml gentamicin, nonessential amino acids, and 5% fetal bovine serum (FBS; Invitrogen Life Technologies), and maintained at 37 °C under a 5% CO_2 atmosphere saturated with water vapor.

The Vero-adapted ASFV strain Ba71 V and virulent strain E70 were propagated on Vero and COS-7 cells, as described previously (Carrascosa et al., 1982; Enjuanes et al., 1976). In brief, sub-confluent monolayers were cultivated in cell culture treated roller bottles and infected with ASFV at a multiplicity of infection (MOI) of 0.5 in culture medium. At 72 h post infection, cells were pelleted and the supernatant recovered. The supernatant containing viruses was centrifuged at 14,000 rpm for 6 h at 4 °C and the purified infectious virus was resuspended in medium and stored at −80 °C.

2.2. Nascent virus for neutralization assays

Vero cells were infected with ASFV strain Ba71 V at a MOI of 1 pfu/cell. After 24 h, infected cells were softly recovered with a cell scraper and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and cells were washed once with media supplemented with 10% FBS and incubated at 37 °C for 1 h in media with FBS. After this period, cells were centrifuged at 2000 rpm for 10 min and the supernatant was filtered using a filter with a 0.45 μm pore. The virus obtained by this procedure was titrated and used for neutralization assays.

2.3. ASFV infection assays

Vero and COS-7 cells were mock- infected or infected with ASFV strains Ba71 V or E70 at different MOI in DMEM supplemented with 2% FBS. During an adsorption period of 90 min at 37 °C, the virus was removed and cells were incubated at 37 °C. In the case of neutralization for entry assays, the viral infection time was 30 min at 37 °C (without adsorption) and cells were processed.

2.4. Generation of ASFV recombinant proteins

The full-length open reading frame (ORF) of CP530R (coding for the proteins p15 and p35), E183 L (coding for p54), 1-621bp of EP402R (coding for the extracellular domain of CD2v (Goatley and Dixon, 2011)), B646 L (coding for p72) and CP204 L (coding for p32) were synthesized based on ASFV isolate Georgia/2007 (GeneBank: FR682468.1) and cloned into a common plasmid vector (e.g. pUC57, GENEWIZ). ASFV proteins p15, p35, p54 and CD2v-E were expressed in baculoviruses using the BaculoDirect expression system (Invitrogen). ASFV protein p32 was expressed in *Escherichia coli* using the pET101/D-TOPO *E. coli* expression system (Invitrogen).

For Baculovirus expression, individual ASFV genes were cloned into the pENTR/D-TOPO vector (Invitrogen). The donor plasmids were incubated with LR Clonase II and BaculoDirect linearized baculovirus DNA. To produce recombinant baculoviruses, generated DNAs were transfected into *Spodoptera frugiperda* 9 (SF9) cells using Cellfectin II reagent (Invitrogen) and then recombinant baculovirus was selected using 100 μM of Ganciclovir. Infected cells were cultured for 72 h and cell pellets were collected for protein purification. The CP204 L gene (p32) was cloned into a pET101/D-TOPO vector (Invitrogen) for *E. coli* expression. The TOPO cloning reactions were transformed into One Shot Top10 chemically competent *E. coli* (Invitrogen). The plasmid p32-pET101 was transformed into BL21 competent *E.coli* and then cultured for protein expression and purification. The integrity and correct orientation of the recombinant gene was confirmed by PCR and DNA sequencing.

Recombinant proteins with histidine tags were purified via affinity chromatography using Ni-NTA superflow resin (Qiagen) after lysis of cells under native condition. The lysis of cells under native condition was performed on cell pellets resuspended in 50 mM sodium phosphate, 500 mM sodium chloride, 10 mM imidazole, 5% glycerol, 400 units benzonase nuclease per 1 g of pellet (pH 8.0), followed by two freeze/thaws and 6 short (10 s) sonications. The lysate was centrifuged at 10,000 rpm, at 4 °C for 20 min. Supernatant was used for Ni-NTA resin purification. Purified proteins were dialyzed against phosphate buffered saline (pH 7.4, 150 mM NaCl, 4 mM EDTA, 10% glycerol). Purification of ASFV proteins were confirmed via Coomassie blue staining of SDS-PAGE gels and western blots. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Thermo Scientific) at an absorbance of 562 nm, using bovine serum albumin (Sigma-Aldrich) as the protein standard.

2.5. Generation of pcDNA 3.1 plasmids expressing ASFV-specific genes

Overlap PCR was used to insert the full-length EP402R gene (coding for CD2v), CP204 L (coding for p32), B646 L (coding for P72), CP312R into pcDNA 3.1 (Invitrogen) adding the following restrictions sites to the amplification primers: a *Bam*HI site to the 5′ end and an *Eco*RI site to the 3′ end of EP402R; a *Bam*HI site to the 5′ end and an *Eco*RI site to the 3′ end of CP204 L; a BamHI site to the 5′ end and an XhoI site to the 3′ end of B646 L; and a *Sma*I site to the 5′ end and an *Eco*RI site to the 3′ end of CP312R. The fragments were amplified by PCR from a lysate of cells infected either by ASFV E70 or Ba71 V, using the following primers: 5′- CGCGCGGGATCCATGATAATAATAGTTATTTTTTTAATGTG -3′ and 5′-CGCGCGGAATTCTTAAATAATTCTATCTACATGAATAAGCG -3′ for EP402R; 5′-GGGGGGGAGTCCGTTATGGATTTTATTTTAAAT -3′ and 5′- CGCCGCCTCGAGCTAAAACATTAAATGTAGGTGA -3′ for CP204 L; 5′-GCGCGGATCCATGGCATCAGGAGGAGCTTTTTGTC-3′ and 5′-CGCGCTCGAGTTAGGTACTGTAACGCAGCACAGCTG-3′ for B646 L; and 5′-GGGCCCGGGGATGTTACTAGTAAAAATGACT -3′ and 5′-GGGG GAATTCTTATTAAGCAATAGCGATCTG-3′ for CP312R. PCR products and pcDNA vector were digested with the corresponding restriction enzymes and ligated with T4 ligase (Roche). Products were transformed into *E. coli* DH5α by heat shock. Correctness of respective plasmid construct was confirmed by DNA sequencing and blast analysis. The ORF D250R (Ba71 V)-g5R (Malawi), that encodes a decapping protein (ASFV-DP) which has a Nudix hydrolase motif and decapping activity *in vitro* was generated following a similar strategy as described previously

Table 1
Groups of pigs in which studies on reactivity to p15, p35, p54 and CD2v-E proteins +/- ASFV pcDNAs were achieved.

Group (n = 5)	Antigens for immunization		Individual number of pig
	Recombinant Protein	Plasmid DNA	
1 A	p15	-	1-01,1-0 2
	p15	p72	1-03, 1-04, 1-05
1B	p35	-	1-07, 1-31
	p35	CD2v	1-08, 1-09, 1-10
1C	p54	-	1-11, 1-12
	p54	p32	1-13, 1-14, 1-15
1D	CD2v-E	-	1-16, 1-17
	CD2v-E	CP312R	1-18, 1-19, 1-20
1E			
	CD2v-E	p72	1-21, 1-23, 1-24
1F			
	p15	p32	1-28, 1-29, 1-30
1G			
	p35	CD2v	1-33, 1-34, 1-35
1H			
	p54	CP312R	1-38, 1-39, 1-40

(Quintas et al., 2017).

2.6. Ethics statement

All animal studies were performed under an Institutional Animal Care and Use Committee-approved protocol of Kansas State University in compliance with the Animal Welfare Act. The animal studies were carried out at the Large Animal Research Center (Biosafety level 2 facility) and the biosecurity research institute (Biosafety level 3 facility) at Kansas State University, Manhattan, KS, USA.

2.7. Animals and experimental design

2.7.1. First experiment

Forty conventional three-week old piglets were acclimated for one week and divided into 8 groups with 5 pigs per group (group 1 A to group 1 H). Pigs were immunized with a combination of different recombinant proteins and pcDNA constructs, depending on the group, as shown in Table 1. Specifically, piglets were inoculated intramuscularly (IM) with 100 µg of the respective recombinant protein in ISA 25 adjuvant (SEPPIC) and/or 100 µg of the respective pcDNA construct. Piglets were vaccinated three times at two-week intervals (days 0, 14 and 28). One week after the last immunization on day 35 post initial immunization (dpi), pigs were humanely euthanized by intravenous injection of appropriate concentration of pentobarbital sodium. Blood was collected on the days of immunization and prior to euthanasia.

2.7.2. Second experiment

Thirty conventional three-week old piglets were randomly divided into 6 groups (group 2 A to group 2 F, Table 2), with 5 pigs per group. Piglets were acclimated and housed the same as described for the first animal study. Piglets were inoculated IM with 100 µg of the respective recombinant protein mixed with ISA25 adjuvant (SEPPIC) and 100 µg of each pcDNA construct, as it is shown in Table 2. Piglets were immunized three times at 0, 21 and 35 dpi. Blood was collected on the days of vaccination and prior to euthanasia, which occured at 49 dpi.

2.8. Assessment of immunogenicity

2.8.1. Antibody detection in serum by ELISA tests

ELISAs were performed by using the recombinant ASFV proteins as antigens. Briefly, wells were coated with 200 ng of the respective recombinant ASFV protein in 100 µl of PBS 1X and incubated overnight at 4 °C. Sera from immunized pigs were incubated at dilutions of 1/20 and 1/200 in 100 µl of diluent provide by the kit at 37 °C for 1 h, followed by peroxidase-conjugated anti-pig IgGs (1/5,000; kindly gifted by Dr. E. Tabarés). The optical density (OD) value was measured at 405 nm within 5 min of adding the stop solution. Diluent, washing solution and stop solution are from the INGEZIM PPA DAS kit (Ingenasa).

Table 2
Groups of pigs immunized with combinations of p15, p35, p54, p32 and p72 +/- specific DNA constructs.

Groups (n = 5/G)	Antigens for vaccination		Individual number of pig
	Recombinant protein	Plasmid : pcDNA3	
2 A	p72		2-40
	p72	p72	2-36, 2-37, 2-38, 2-39
2B	p72		2-35
	p72	g5R	2-31, 2-32, 2-33, 2-34
2C	p32		2-30
	p32	p32	2-26, 2-27, 2-28, 2-29
2D	p32		2-20
	p32	g5R	2-21, 2-22, 2-23, 2-24
2E	p15 + p35 + p54		2-19, 2-20
	p15 + p35 + p54	CD2v	2-16, 2-17, 2-18
2 F	p72 + p32		2-10
	p72 + p32	CD2v	2-06, 2-07, 2-08,2-0 9

2.8.2. Western blot

Cells infected with Ba71 V or E70 ASFV were washed at 16 hpi with PBS and lysed in RIPA modified buffer (50 mM Tris-HCl pH 7.5, 1% NP40, 0.25% Na-deoxycolate, 150 mM NaCl, 1 mM EDTA) supplemented with protease and phosphatase inhibitors cocktail tablets (Roche). The protein concentration was determined by a Pierce BCA Protein Assay kit (Thermo Scientific). Infected cell lysates (40 µg of protein) or purified recombinant ASFV proteins were fractionated by SDS-PAGE and transferred to Immobilon membranes (Amersham). The membranes were incubated with sera from pigs immunized with various recombinant proteins: p54 (1/800), CD2v (1/800), p35 (1/400), p15 (1/1000), then exposed to horseradish peroxidase-conjugated pig secondary antibody (dilution 1/80,000), followed by chemiluminescence (ECL, Amersham Biosciences) detection by autoradiography.

2.8.3. Neutralization assays

Sera from non-immunized (pre-immune sera) and immunized (immune sera) animals were incubated at 56 °C for 30 min for inactivation and diluted 1/8 using DMEM supplemented with 20% of FBS and 0.05% Tween-80. ASFV-Ba71 V virus produced as described above was incubated with pre-immune or immune sera at 37 °C overnight. The level of virus neutralization was analyzed by using three different techniques: i) Confocal Laser Scanning Microscopy (CLSM): Vero cells were infected at a MOI of 5 with ASFV previously incubated with 1/8 dilution of either pre-immune or immune sera for 30 min at 37 °C; cells were then prepared for CLSM and analyzed for viral entry by incubation with an anti-p72 monoclonal antibody (17LD3, gifted by Ingenasa). Detailed methods are described in Supplementary Section. ii) Fluorescence Activating Cell Sorting (FACS): Vero cells were infected at a MOI of 1 with ASFV previously incubated with 1/16 dilution of either pre-immune or immune sera for 18 h at 37 °C; the percentage of cells expressing the major viral capsid protein p72 was then determined. iii) Virus titration: Vero cells were infected at a MOI of $4{\cdot}10^{-4}$ fpu/ml for 72 h with ASFV previously incubated with 1/8 dilution of either pre-immune or immune sera for 18 h at 37 °C, and virus titer was analyzed in Vero cells.

2.8.4. IFN-γ secreting PBMCs (ELISPOT) and IFN-γ ELISA

Antigen-specific IFN-γ response was determined by enzyme-linked immunospot (ELISPOT) assay, using anti-pig IFN-γ antibodies (P2G10 RUO or biotinylated anti IFN-γ P2C11 RUO, Becton Dickinson

Pharmingen) following manufacturer's instructions (Mateu de Antonio et al., 1998; Prussin and Metcalfe, 1995). Only PBMCs from the second animal experiment were used for IFN-γ ELISPOT assay. Briefly, blood from immunized pigs was collected into sodium heparin and peripheral blood mononuclear cells (PBMCs) were isolated by 1.077 Ficoll-hypaque separation and suspended in RPMI-1640 (Thermo Fisher Scientific) with 10% FBS (cellgro™) and antibiotic-antimycotic Solution (Corning™). MultiScreenHTS IP 96 well filter plates (Millipore) were coated with 5 µg/ml of anti-IFN-γ antibody (P2G10RUO) by incubating at 4 °C overnight; afterwards plates were washed five times with sterile PBS, and then blocked with 10% FBS for 2 h at 37 °C. PBMCs were added to wells at a density of 2×10^5 cells/well. ASFV antigens were added to the wells at a final concentration of 6 µg/ml for protein antigens and 1 µg/ml for DNA plasmid/antigens, in duplicates. Concanavalin A (Con A) mitogen (6 µg/ml) was used as a positive control and media alone was the negative control. Following 36 h incubation at 37 °C, plates were washed with PBS w/0.05% Tween 20 and 0.5 µg/ml of biotinylated anti-IFN-γ antibody (P2C11 RUO) was added for 2 h at room temperature. Afterwards plates were washed and Streptavidin-HRP (BioLegend) in PBS with 0.5% FBS was added (1:2000 concentration). After incubation for 45 min at room temperature, the plates were washed with PBS and colorimetric staining was performed with the NovaRED solution (Vector labs) according to manufacturer's instructions; then the plates were allowed to dry overnight. The spots were counted by using ImmunoSpot 5.0.3 software (Cellular Technology Limited (CTL)).

For IFN-γ ELISA test, PBMCs from immunized pigs of the second animal experiment were isolated and stimulated with respective purified ASFV antigens or mitogen (Con A) as positive control. After 5 days incubation at 37 °C with 5% CO_2, supernatants were collected and tested using the porcine specific IFN- γ ELISA test (Thermo Scientific) according to the manufacturer's procedures. Briefly, 50 µl of cell culture supernatants were added and incubated at room temperature for 1 h. After washing, biotinylated anti-pig IFN-γ antibody was added and incubated at room temperature, followed by washing and Streptavidin-HRP was added. After washing, TMB substrate solution was added for 30 min and the reaction was stopped using the stop solution. The optical densities (OD) were measured at 450 nm. The concentration of IFN-γ was calculated using a standard provided by the manufacturer.

3. Results

3.1. Expression of recombinant ASFV proteins

Full-length ASFV p15, p35, p54, and p72 were expressed together with His-tags in baculovirus. Expression of the tagged, full-length proteins p15, p35, p54,p72, and the extracellular domain of the CD2v protein (CD2v-E), was confirmed by Western blot (Fig. 1).

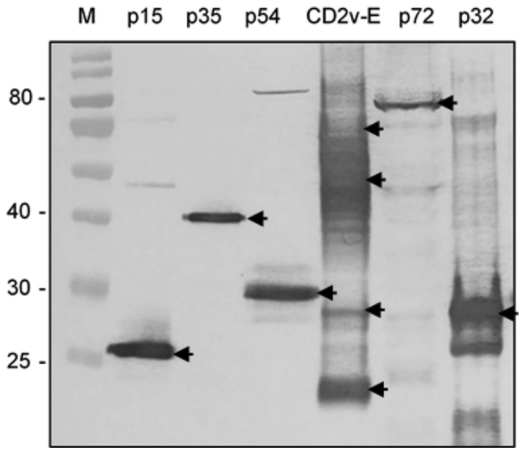

Fig. 1. Expression of recombinant proteins p15, p35, p54, CD2v-E, p72 and p32. A. Western blots were performed to confirm the expression of ASFV proteins using anti-Histidine for p15, p35, p54, CD2v-E, p72 and p32 protein. The respective protein was expressed 26 kDa for p15, 43 kDa for p35, 31 kDa for p54, various isoform for CD2v-E, 79 kDa for p72, and 28 kDa for p32. Black arrows denote the expressed recombinant proteins of interest. M = Protein ladder.

3.2. Evaluation of humoral immune responses after vaccination with ASFV-recombinant proteins and pcDNAs (ELISA test)

The presence of specific antibodies against p15, p35, p54, CD2v-E, p32 and p72 were analyzed in sera from animals immunized with these proteins alone or in combination with specific pcDNAs, by ELISA and Western blot. As shown in ELISA tests depicted in Fig. 2, specific antibodies against the respective ASFV antigens were present in pig sera after three immunizations, either using single-protein immunization (Fig. 2A), or multiple protein immunization (Fig. 2B), as performed during the second animal experiment.

Especially high responses were detected for proteins p15, p32 and p72. Interestingly, pcDNA-CP312R appeared to slightly increase the antibody response generated against CD2v-E and p54 ASFV proteins.

To assess whether the combination of several of the most antigenic proteins was able to increase the level of specific antibodies, we developed the next animal experiment (Experiment 2), in which pigs were immunized with either a mixture of p72 + p32, or with a mixture of p15 + p35 + p54 (Fig. 2B). Furthermore, pcDNA-CD2v was included in combination with each of the protein cocktails, since DNA encoding the N-terminal domain of CD2v has been previously shown to enhance the immune response, when injected together with specific ASFV antigens (Argilaguet et al., 2012). The results showed that p32 was strongly recognized by sera from immunized animals, whereas p72 induced only a moderate antibody response, (independently of containing pcDNA-CD2v). These data reveal that p32 could be an important ASFV target for immune responses *in vivo*. Finally, the sera from pigs immunized with p15 + p35 + p54 reacted similarly against p35 or p54, with slightly higher reactivity observed against p15 (Fig. 2B, lower panel).

3.3. Recognition of viral proteins in ASFV-infected cells by sera from immunized pigs (Western blot)

Western blot was performed in order to study whether the antibodies present in the sera of immunized pigs were able to recognize ASFV-specific proteins in infected cells. As shown in Fig. 3, sera from immunized pigs were tested against extracts from either Mock-infected COS cells or Ba71Vor E70- COS infected cells during 16 h, as well as the respective recombinant ASFV protein used for immunization of pigs. As Fig. 3A shows, sera from pigs immunized with recombinant CD2v-E protein recognized very well a pattern of bands ranking from about 25 to 75 kDa, most likely corresponding to various glycosylated isoforms of CD2v (75 kDa), in agreement with previous reports (Perez-Nunez et al., 2015). Furthermore, a band of approximately 100 kDa in ASFV-infected cells, which is not present in extracts from mock-infected cells. This 100 kDa band corresponds to the expected size of the full-length CD2v glycosylated protein (Perez-Nunez et al., 2015).

Sera from immunized pigs recognize recombinant p54 and p35 proteins in infected cells, (Figs. 3B and C) with molecular weights of around 31 and 42 kDa respectively. However, antibodies present in the sera of p35 immunized pigs were not able to recognize any specific band in ASFV-infected COS cells (Fig. 3C). Sera from animals immunized with p54 protein strongly recognized a band of about 25 kDa in cells infected with both ASFV isolates (Fig. 3B), as expected from the size of the orf encoding for this viral protein. The pig sera against p15 recognized the recombinant protein, but did not react against ASFV-infected cells (Fig. 3D). Sera from pigs immunized with p32 resulted in a very strong and specific recognition of the recombinant protein

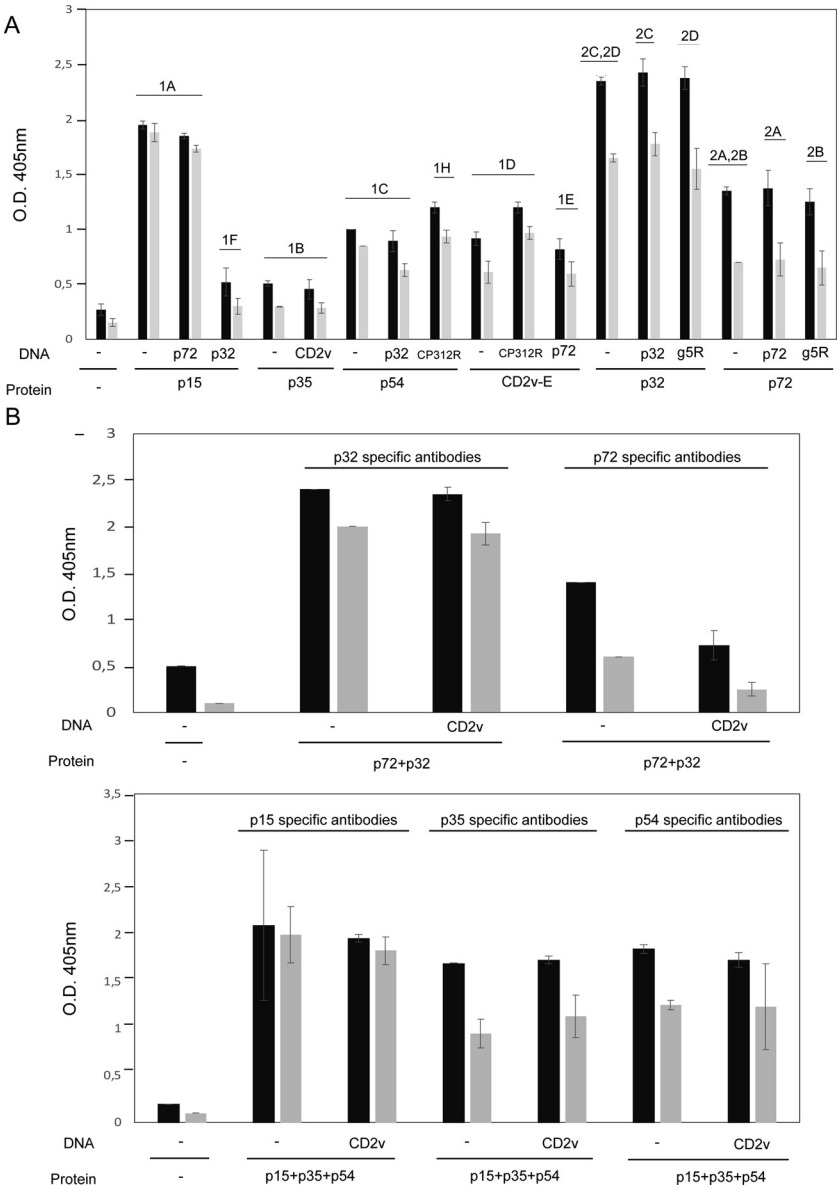

Fig. 2. Antibody detection in sera of pigs immunized with the p15, p35, CD2v-E, p54, p32 and p72 ASFV proteins by ELISA tests. Sera from immunized pigs with (A) one single viral protein or (B) multiple viral proteins in combination with viral DNA, were tested by ELISA using respective single antigens. Two dilutions of sera were tested in each case: 1/20 (black bars) or 1/200 (grey bars). Sera from non-immunized pigs were used as controls. Values of optical density at 405 nm were measured. Experimental group of each combination of protein-DNA is shown. Average of three or two animal sera from each group (according to Tables 1 and 2) with standard error is shown.

(Fig. 3E), revealing bands from 30, 32 and 34 kDa, putatively due to phosphorylation as previously described (Prados et al., 1993), together with a higher band that could represent a dimer of the p32 viral protein. Importantly, the serum against p32 strongly recognizes a specific band in the extracts corresponding to both Ba71 V and E70-infected cells, which is not present in mock cells. Furthermore, the molecular weight of this band exactly corresponds to 32 kDa, in concordance with the Western blot profile seen with an in house p32-specific serum.

The sera from p72-immunized pigs were able to recognize the recombinant protein with a molecular weight corresponding to 72 kDa (Fig. 3F), in concordance with Fig. 1. A weak band was detected in cellular extracts infected with Ba71 V, most likely indicating that p72 recombinant protein did not induce a strong specific antibody response.

3.4. Serum from immunized pigs neutralize ASFV infection in vitro

To analyze the presence of ASFV neutralizing antibodies in the sera of immunized pigs, we used three different methods: (i) inhibition of ASFV entry after 30 min of viral adsorption, (ii) percentage of inhibition of p72 expression after 16 hpi of ASF infection, determined by FACS and (iii) inhibition of the viral production by plaque titration at 48 hpi. A detailed description of these methods is provided in the Materials and Methods section and in the Supplementary Materials.

First, (i) we chose the sera from pigs immunized with the combination p15 + pcDNA-p72, since both of these products are abundantly present in the virion (Simon-Mateo et al., 1997), thus specific antibodies against these proteins could neutralize the virus entry. As shown in Fig. 4, the specific immune serum against p15 + pcDNA-p72 partially impaired the viral entry in Vero cells. This impairment was further quantified showing that virion particles, identified with a specific monoclonal antibody anti p72, were found only in about 45% of the cells incubated with immune serum, compared to 100% cells infected after incubation with pre-immune sera. Next, (ii) we further investigated the neutralization ability of several sera by measuring the expression of the ASFV p72 protein by FACS analysis after 16 hpi.

To achieve this, we first incubated the selected sera together with the virus and then we added this mix to Vero cells. To set up the method, a hyperimmune serum from an ASFV-infected pig (previously developed in our lab), induced a reduction of ASFV infection in Vero cells of around 80% when compared to the pre-immune serum (Supplementary Fig. 1). Neutralization results using sera from immunized pigs by FACS analysis are shown in Supplementary Fig. 2, and

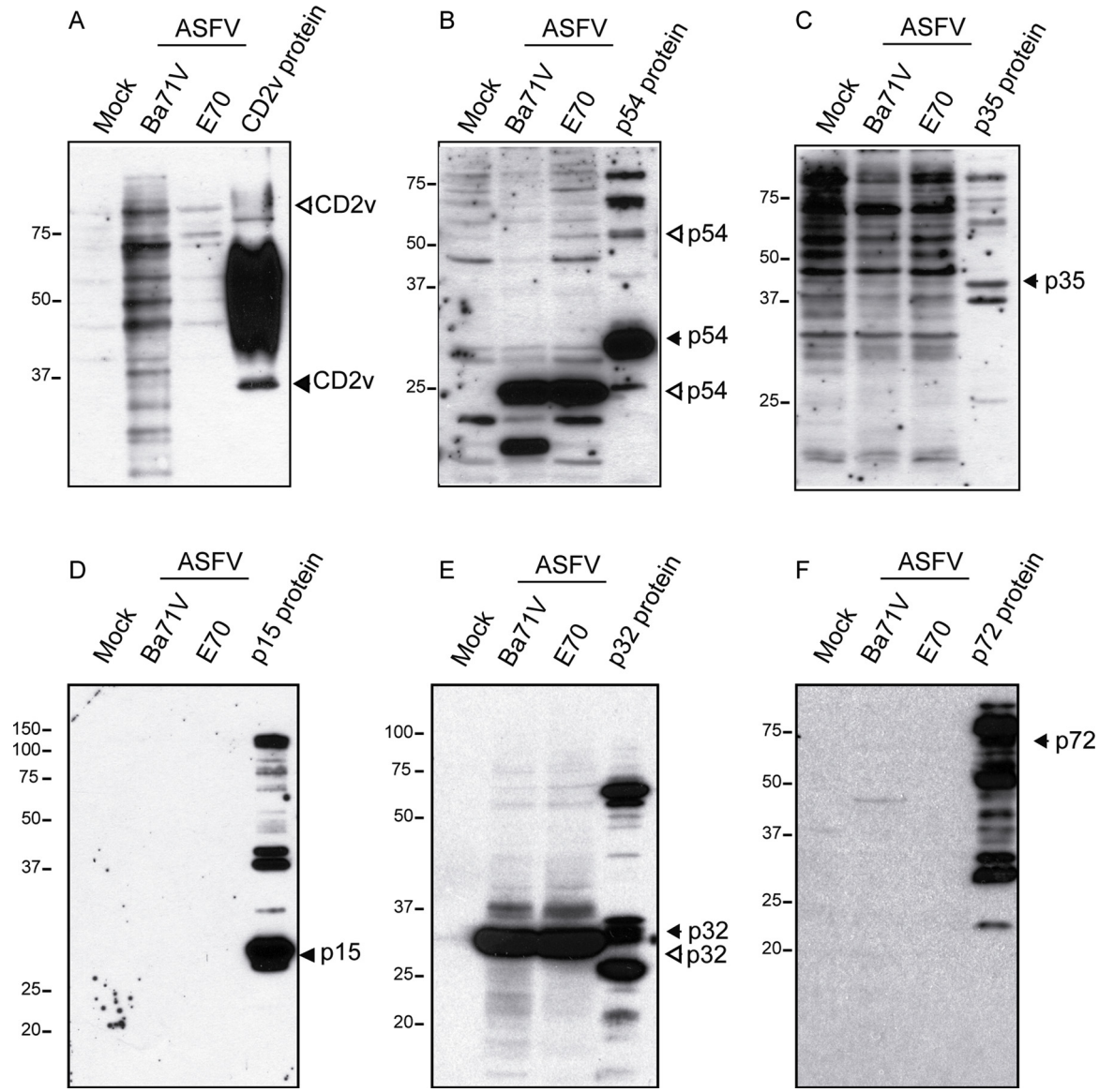

Fig. 3. Recognition of viral proteins expressed during ASFV infection by sera from immunized animals. Sera from immunized animals with A) CD2vE protein + pcDNACP312R (pig 1–18), B) p54 protein + pcDNACP312R (pig 1–39), C) p35 protein + pcDNACD2v-E (pig 1-08), D) p15 protein (pig 1-02), E) p32 protein + pcDNA p32 (pig 2–27) and F) p72 protein + pcDNAp72 (pig 2–36) were used to detect viral proteins in COS-7 cells extracts infected for 16 h with either Ba71 V or E70 ASFV strains by Western blot analysis(open arrows). The specific recombinant proteins used to immunized animals were also analyzed (close arrows).

in Fig. 5A. Specifically, sera from pigs immunized with p15 protein in combination with pcDNA-p32, slightly neutralized ASFV (Fig. 5A), whereas sera from pigs immunized with p35 in combination with pcDNA-CD2v showed a more effective ASFV neutralizing ability of about 20%. Furthermore, we observed that sera from animals immunized with CD2v-E protein and pcDNA-CP312R induced a reproducible, although slight, neutralization compared with the pre-immune sera (Fig. 5A).

In order to verify the neutralization results showed above, we tested the ability of the hyperimmune sera to neutralize ASFV infection *in vitro* by analyzing viral plaque formation (Fig. 5B). The sera from pigs immunized with combinations of protein and pcDNAs (p15 + pcDNA-p32, p35 + pcDNA-CD2v, and CD2v-E + pcDNA-CP312R) were found to induce the higher levels of neutralization, showing a decrease of viral plaque formation compared to pre-immune sera, thus confirming the data shown in Fig. 5A. Taken together, these results show that selected combinations of viral proteins and pcDNAs synergistically induce antibodies that can partially neutralize ASFV infectivity in vitro.

3.5. IFN-γ secreting PBMCs

IFN-γ secreting cells in PBMCs from vaccine groups of the second animal experiment were analyzed by ELISPOT (Fig. 6A). PBMCs were isolated from blood collected from immunized pigs at day 49 dpi. The PBMCs were stimulated with the same antigens as used for *in vivo* immunization. Fig. 6A shows that IFN-γ secreting cells specific for the respective ASFV antigens were barely present in the blood of immunized pigs. The best proteins or protein combinations for the induction of IFN-γ were p72 (34.9 per 200,000 cells) and p15 (35.3 per 200,000 cells), whereas neither combination of p72 + p32 nor p15 + p35 + p54 proteins was able to increase the number of IFN-γ secreting PBMCs.

Fig. 6B shows the concentration of IFN-γ after *in vitro* re-stimulation of PBMCs from immunized pigs using the respective single and combinations of proteins, as measured by a commercial ELISA kit. IFN-γ was detected in supernatants of PBMCs from pigs immunized and re-estimulated with p72 (73.4 pg/ml) and p72 + p32 proteins mixture, (62.6 pg/ml), whereas combination of p15 + p35 + p54 proteins did not

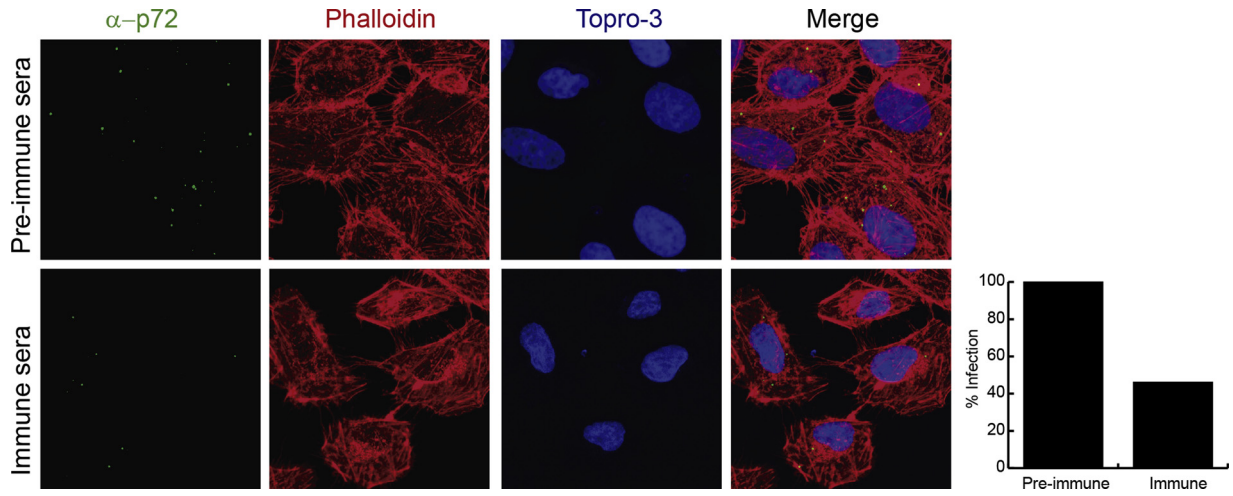

Fig. 4. Blockage of viral entry by pig 1-03 serum. After virus incubation (60 min at 37 ºC) with pre-immune or immune sera from a pig immunized with p15 + pcDNA-p72 (pig number 1-03). 100.000 Vero cells per coverslip were infected with Ba71V 5UFP/cell (500.000 virions/coverslip) for 30 min at 37 ºC, and then washed and fixed for immunostaining. Anti p72 antibody (17LD3) was added to detect virus particles and cell and nuclei were stained with phalloidin and Topro-3, respectively. A number of 100 cells/sample (non-immune or immune sera), were analyzed for p72 immune fluorescence after infection with $5x10^2$ virions. Finally, the enclosed graphic represent the percentage of viral particles visualized into the cells, based on the data shown in CLSM Image.

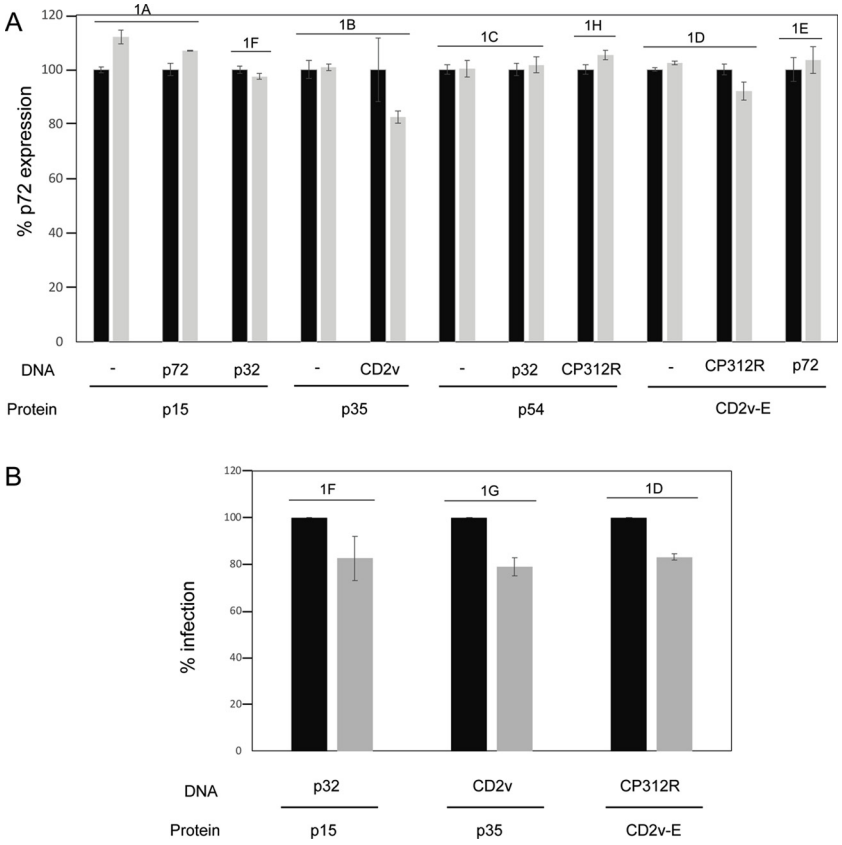

Fig. 5. Neutralization of ASFV infection by sera. A) Ba71 V was pre-incubated with either pre-immune sera (black bars) or immune sera (grey bars) from the different groups of immunization, and used to infect Vero cells. After 16 hpi the percentage of p72 expression was determined by FACS using a p72-specific MoAb antibody (17LD3). B) Ba71 V was pre-incubated with either pre-immune sera (black bars) or immune sera (grey bars) and used to infect Vero cells. Viral production was measured at 5 days after infection by plaque assay and represented as a percentage relative to the virus titer in immune sera samples corresponding to pre-immune sera samples. Experimental group of each combination of protein-DNA is shown according to Table 1. Average of two animal sera from each DNA-protein combination with standard error is shown. According to Table 1: 1 A: animals 1, 2, 4 and 5; 1B: animals 7, 8, 9 and 31; 1C: animals 11, 12, 13 and 14; 1D: animals 16, 17, 18 and 19; 1E: animals 21 and 23; 1F: animals 28 and 29; 1H: animals 38 and 39.

increase secreted IFN-γ, consistent with the results obtained in Fig. 6A.

4. Discussion

Several reports have pointed out that both humoral and cellular responses are involved in ASFV protection (Gomez-Puertas et al., 1996; King et al., 2011; Leitao et al., 2001; Oura et al., 2005; Ruiz Gonzalvo et al., 1986b; Stone and Hess, 1967; Zsak et al., 1993). Since ASFV encodes for more than 150 viral proteins, the identification of individual or combinations of viral antigens that could elicit a protective immune response against infection is rather challenging. Experimental immunization protocols are key to analyze specific immune mechanisms, and for the development of an effective ASF vaccine. Based on previous studies on the function of several ASFV proteins (Andres et al., 2002; Argilaguet et al., 2012; Neilan et al., 2004; Perez-Nunez et al., 2015; Quintas et al., 2017; Rodriguez et al., 1993; Sanchez et al., 2013) we designed an immunization format using combinations of selected ASFV recombinant proteins and plasmids encoding ASFV-specific pcDNAs. The rationale behind combining genotype I and II antigens would provide a potential means to expand cross-protection, which

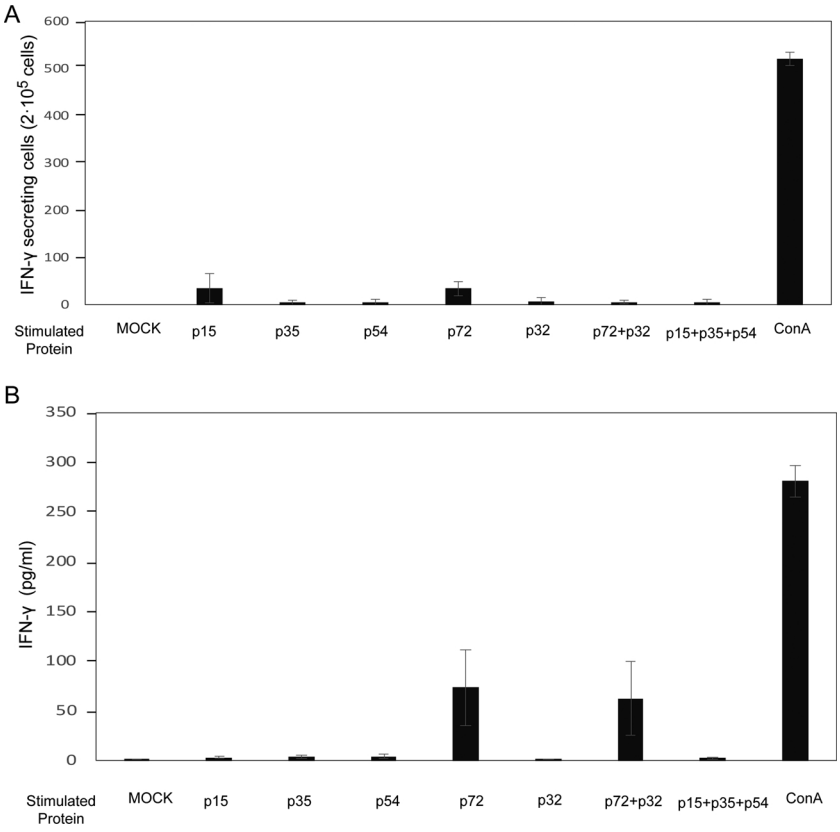

Fig. 6. ELISPOT showing IFN-γ-secreting PBMC after proteins/pcDNAs re-stimulation. A) Number of responding PBMCs derived from immunized piglets on 49 dpi and re-stimulated with indicated viral proteins and pcDNAs. B) IFN-γ concentration measured by a commercial ELISA kit as described in the Materials and Methods section. ConA: concanavalin A. Standard error is presented as bars.

could then be tested in subsequent challenge experiments.

The immune responses induced in pigs were tested by analyzing the ability of the sera to recognize the proteins used as antigens, the ability of immune sera to neutralize ASFV infection *in vitro*, and by determining the ASFV-specific IFN-γ induction from immune PBMCs.

The results indicate that most of the pcDNAs used in combination with proteins in vaccination experiences, did not importantly affect the antibody response against recombinant proteins. There are few exceptions, such as pcDNA-p32, which combined with p15 protein, reduced the level of antibodies compared to the immunization with p15 protein alone; and pcDNA-CP312R, which appeared to slightly increase the antibody response generated against CD2v-E and p54 ASFV proteins. These last results indicate either that pcDNA-CP312R encodes a product efficiently recognized by the humoral immune response, or that the response against both proteins and the plasmid-encoded product acts synergistically. Finally, it is clear that the values obtained against either p35 alone or in combination with pcDNA-CD2v were low in general.

The ELISA tests demonstrated that sera from immunized pigs were able to recognize the recombinant protein (s) used as antigens in each vaccination., Recombinant protein p35 was identified as the weaker immunogen, whereas pigs immunized with the ASFV p15 or p32 recombinant proteins developed the highest antibody response (OD > 2.0); pigs immunized with p72, p54 and CD2v recombinant proteins also developed good antibody measures. These data agree with reports pointing to these proteins as important antigens eliciting strong immune responses (Argilaguet et al., 2012; Gomez-Puertas et al., 1998; Neilan et al., 2004; Reis et al., 2007; Ruiz-Gonzalvo et al., 1996), further targeting CP312R as a new interesting antigenic factor.

Furthermore, we found that serum antibodies from animals immunized with recombinant pCD2-E + pcDNACP312R, recombinant p54 and recombinant p32 + pcDNAp32, were able to recognize by Western blot extracts both of the respective recombinant protein and the specific proteins expressed during the infection with either Ba71 V or E70 ASFV strains. However, sera from animals immunized with recombinant p15, with recombinant p35 + pcDNACD2v-E or with recombinant p72 recombinant + pcDNAp72, were only able to recognize the specific recombinant protein. This last result suggests that conformational modifications of these antigens could occur during the infection and/or that the viral proteins epitopes are not well recognized by the antibodies generated in the sera.

Relatively few reports have described the mechanism of ASFV neutralization, most likely because the most accepted hypothesis is that ASFV induces antibodies that are not fully neutralizing *in vitro* or protective *in vivo* (Escribano et al., 2013; Hess, 1981; Vinuela, 1985). However, several laboratories have demonstrated that ASFV can be partially neutralized by both monoclonal antibodies (Sanz et al., 1985) and immune sera from convalescent swine, infected with homologous or heterologous ASFV strains (Borca et al., 1994; Ruiz Gonzalvo et al., 1986a, b; Zsak et al., 1993). Using a neutralization assay previously established in our laboratory, we show here that serum against p35 in combination with pcDNA-CD2v partially neutralized ASFV *in vitro* (about 20% reduction in plaques). In a similar way, when CD2v was used as protein in combination with pCDNA-CP312R to immunize pigs, the sera neutralized virus infection *in vitro* with about 10% reduction. These results were confirmed by FACS and by titration of viral lysis plates. In contrast, pig antibodies produced against various pcDNAs alone, such as pcDNA-p72, pcDNA-p32, pcDNA-CD2v or cDNA-CP312R, did not neutralize or only slightly neutralized ASFV infection (data not shown). Our data suggest a putative role for antibodies against structural p35 and CD2v proteins, and for pcDNAs encoding p32, CD2v and CP312R, in ASFV neutralization.

It is well known that plasmid DNA can possess its own adjuvant activity due to the presence of unmethylated CpG motifs in certain base contents (Bode et al., 2011). Furthermore, work from (Argilaguet et al., 2012), showed that fusion of the extracellular domain of the ASFV Hemagglutinin (sHA) to p54 and p30, exponentially improved both the humoral and the cellular responses induced in pigs after DNA immunization, but did not confer protection against lethal challenge with

the virulent E75 ASFV-strain. However, these viral antigens (p54, p30, and the CD2v extracellular domain) fused to ubiquitin correlated with the presence of a large number of hemagglutinin-specific CD8(+) T cells in blood and partial protection against virulent challenge.

Nevertheless, the mechanism of the observed synergism between specific ASFV proteins and pcDNAs-encoding ASFV factors is not clear at this time, but one hypothesis is that the pcDNA triggers some non-specific immune responses, which in term boost specific response to the proteins.

Regarding IFN-γ production, it is noticeable that p72 protein was found the main inducer of this cytokine, both in the number of PBMC able to produce it and in the amount of IFN-γ produced by responsive cells. However, we found that p15-specific PBMCs produced relatively low amounts of IFN-γ. Taken together, these results suggest that p72, a strong antibody-inducing viral factor, was found to be able to stimulate IFN-γ-producing PBMC populations. Furthermore, p72 in combination with p32, another potent viral antigen, induced a relatively low population of specific PBMCs, which however produced measurable amounts of IFN-γ.

In conclusion, our work presents data of the immunogenicity of several ASFV antigens, by immunizing pigs with both recombinant proteins alone or in combination with pcDNAs-encoding viral genes. In absence of efficient ASFV vaccines, and LAVs being the only protecting alternative against this pathogen, our data add information about viral antigen-specific responses and would contribute to the generation of future and safer ASFV subunits vaccines, providing information to assess the potential of the analyzed combinations in *in vivo* experiences of challenge against ASFV virulent strains in the near future.

5. Conclusions

- Immunization of groups of pigs with combinations of selected ASFV viral DNA and recombinant proteins resulted in induction of specific antibodies.
- Ability of antibodies present in sera from some of the immunized-pigs to neutralize infection in vitro confers relevance to specific viral antigens used in the immunization.
- Results from IFN-γ ELISpots demonstrated activation of the cell-mediated immune response by immunization of pigs with combination of several viral DNAs + proteins.
- The information presented here can help to the development of future subunit vaccines against ASFV.

Author contributions

JAR and YR designed the overall study and heterologous DNA-protein ASFV vaccine strategy. DPN and NH cloned the ASFV genes in pcDNA vectors. SYS and DM expressed proteins. SYS and NH led two animal experiments and prepared immunogens (recombinant proteins and pcDNA constructs) and made vaccine for immunization. SYS, NH, JAR, IM, DM, LM and vs performed the animal sample collection and clinical monitoring. Laboratory data analysis was performed by SYS, NNG in KSU and RGB, MN, EGS and DPN in CBMSO. The manuscript was written by DPN, SYS, NNG, EGS, YR and JAR, and reviewed by the co-authors.

Conflict of interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetimm.2018.11.018.

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