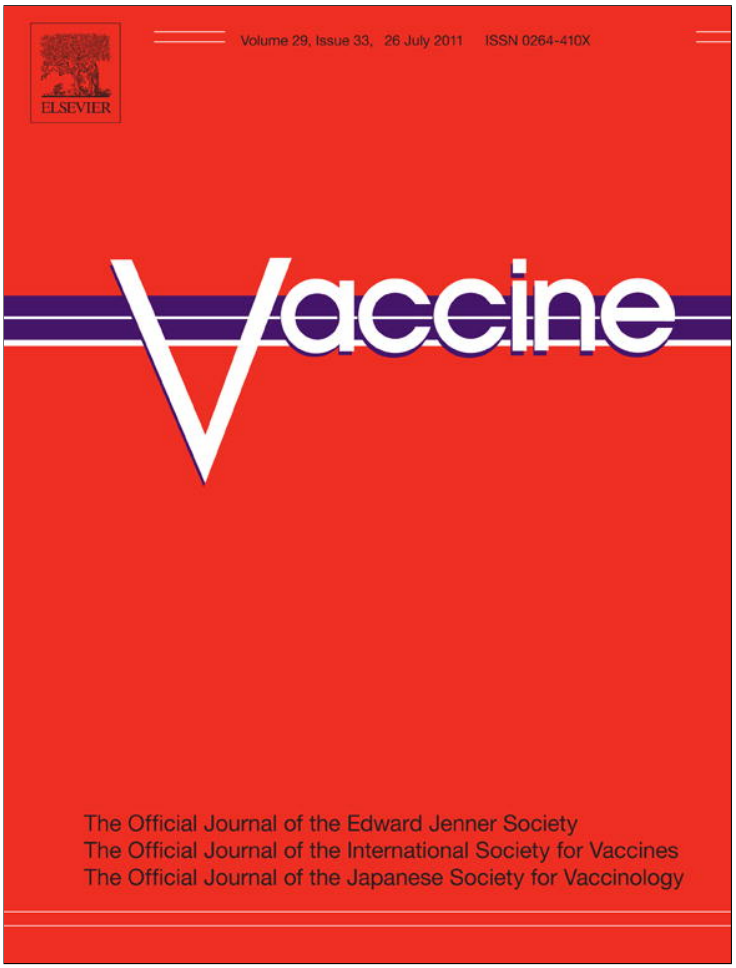

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Enhancing DNA immunization by targeting ASFV antigens to SLA-II bearing cells

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ABSTRACT

One of the main criticisms to DNA vaccines is the poor immunogenicity that they confer on occasions, at least in large animals. Confirming this theory, immunization with plasmid DNA encoding two African swine fever virus genes in frame (pCMV-PQ), failed in inducing detectable immune responses in pigs, while it was successful in mice. Aiming to improve the immune responses induced in swine, a new plasmid was constructed, encoding the viral genes fused in frame with a single chain variable fragment of an antibody specific for a swine leukocyte antigen II (pCMV-APCH1PQ). Our results clearly demonstrate that targeting antigens to antigen professional cells exponentially enhanced the immune response induced in pigs, albeit that the DNA vaccine was not able to confer protection against lethal viral challenge. Indeed, a viremia exacerbation was observed in each of the pigs that received the pCMV-APCH1PQ plasmid, this correlating with the presence of non-neutralizing antibodies and antigen-specific SLA II-restricted T-cells. The implications of our discoveries for the development of future vaccines against African swine fever virus and other swine pathogens are discussed.

1. Introduction

DNA immunization is an extremely simple approach first described in the early nineties [1,2] that has become the technology of choice to obtain vaccines against cancer, infectious diseases and autoimmune disorders, many of them currently under clinical trial. There are three veterinary licensed DNA vaccines and many others will be commercialized in the coming years [3–7].

In spite of the success against several pathogens, one of the limitations on occasions associated with DNA vaccines is the induction of lower immune responses when compared to other methods, especially when immunizing large animals (including humans). Thus, although DNA immunization works, there is still room for improvement. Aiming to enhance the immune responses induced by DNA vaccination, several approaches have been tested in recent years [8–11]. One of the most successfully strategies employed to date is based on targeting the encoded antigens to the sites of the immune induction [12–14], including the use of single-chain variable fragments (scFv) of antibodies [15] that specifically recognize antigens on the surface of antigen presenting cells (APCs) [16,17]. This strategy has been demonstrated to be very efficient in improving the immune responses induced against many different antigens, either using recombinant subunit proteins or DNA vaccination [18–20]. We have recently demonstrated that targeting antigens with a single chain antibody recognizing an invariant epitope of the MHC Class II DR molecule (named APCH1) potentiates the immune response to subunit vaccines, both in mice and rabbits [21]. Here we extend these studies to the field of DNA vaccination in swine. The targeting potential of APCH1 was first demonstrated *in vitro* by transfecting Vero cells with pCMV-APCH1GFP, a plasmid that encodes the scFv fused to the green florescent protein (GFP). Upon transfection, the secreted fusion protein was capable to specifically bind to swine macrophages, while the GFP alone did not. Next, the utility of APCH1 as a genetic adjuvant *in vivo* was definitively demonstrated by immunizing pigs with the pCMV-APCH1PQ plasmid, encoding the APCH1 as a fusion with a chimerical open reading frame (ORF) encoding two immunodominant African swine fever

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Table 1
Primers used for PCR amplification. Each one of the primers used contains a specific restriction site at its 5′-end (underlined and in italic) to facilitate the cloning of the resulting PCR-amplicons in the corresponding plasmids (see Section 2).

ORF	Sense	Primer sequence
APCH1 *NotI*	Forward	5′-GCGGCCGCCATGGACTTCGGGTTGAGCTTGG-3′
APCH1 *EagI*	Reverse	5′-CGGCCG*AGATCT*CTCGAGCCGTTTGATCTCCACC-3′
PQ *BamHI*	Forward	5′-GGATCCATGGATTCTGAATTTTTTCAACCGG-3′
PQ *BglII*	Reverse	5′-AGATCTTACAAGGAGTTTTCTAGGTC-3′
PQ *NotI*	Forward	5′-GCGGCCGCATGGATTCTGAATTTTTTCAACCGG-3′
PQ *NotI*	Reverse	5′-GCGGCCGCTACAAGGAGTTTTCTAGGTC-3′
GFP *BamHI*	Forward	5′-GGATCCATGGTGAGCAAGGGCGAGG-3′
GFP *BglII*	Reverse	5′-AGATCTACTTGTACAGCTCGTCCATG-3′
GFP *NotI*	Forward	5′-GCGGCCGCATGGTGAGCAAGGGCGAGG-3′
GFP *NotI*	Reverse	5′-GCGGCCGCACTTGTACAGCTCGTCCATG-3′

virus (ASFV) antigens: p54 and p30, in tandem (PQ) [22]. DNA vaccines encoding PQ alone induced very good immune responses in mice but failed in pigs, while pCMV-APCH1PQ induced both specific antibody responses and SLA II-restricted T-cells in pigs, therefore demonstrating the adjuvant effect of the APCH1 molecule. Intriguingly, our DNA vaccine was incapable to protect against the ASFV lethal challenge. In contrast with previously described subunit vaccines based on the same ASFV antigens [22,23], the antibodies induced by pCMV-APCH1PQ were incapable of neutralizing the virus. The implications of these discoveries will be further discussed.

2. Materials and methods

2.1. Plasmid construction

The ORF encoding APCH1 [21,24], originally obtained from an antibody which recognizes the Swine Leukocyte Antigen Class II DR molecule [24], was PCR amplified using specific forward and reverse primers containing the *Not*I and *Eag*I restriction sites, respectively (underlined in Table 1), for direct cloning within the unique *Not*I cloning site of the pCMV plasmid (Clontech). The resulting plasmid, pCMV-APCH1, contained a unique *Bgl*II site (Table 1; written in italics in the APCH1 *Eag*I reverse primer), for in frame cloning of the GFP or PQ ORFs. The ORF encoding PQ [22] and the ORF encoding GFP (Clontech) were PCR amplified using specific primers including the *Bam*HI (sense) and *Bgl*II (antisense) restriction sites (Table 1), to facilitate their cloning in frame with APCH1, therefore obtaining the plasmids pCMV-APCH1PQ and pCMV-APCH1GFP. In order to clone either GFP or PQ alone (with no carrier) within pCMV (Clontech), their ORFs were amplified using specific primers (sense and antisense) including the *Not*I restriction site (Table 1) to obtain pCMV-PQ and pCMV-GFP.

2.2. In vitro plasmid expression and GFP binding experiments

In vitro expression of the encoded proteins in Vero cells (ATCC) was demonstrated by transient transfection using the plasmids: pCMV-GFP, pCMV-APCH1GFP, pCMV-PQ and pCMV-APCH1PQ. For the transfections, lipofectamine-Plus reagent was used following the manufacturer's instructions (GIBCO-BRL). GFP expression was directly followed by fluorescence microscopy, while the expression of the PQ construct was confirmed by indirect immunofluorescence using a mouse monoclonal antibody against p30 [25] or a rabbit polyclonal antibody against p54 [26], both 1:1000 diluted, followed by incubation with a R-Phycoerythrin (PE) conjugated anti-mouse antibody (SIGMA; P-9287) at 1:200 dilution or a fluorescein isothiocyanate (FITC) conjugated anti-rabbit antibody (SIGMA; P-9887) at 1:500 dilution, respectively.

Vero cells transfected with either pCMV-GFP or pCMV-APCH1GFP were incubated in plates with 3 μm pore size polycarbonate membrane inserts (Nunc) to avoid direct contact with co-cultured porcine alveolar macrophages (PAMs). After 24 h of incubation, swine macrophages were fixed with 4% of paraformaldehide for 20 min and observed by fluorescent microscopy to detect GFP attached to the macrophages surface. To confirm the specificity of the assay, macrophages were also stained with the anti-SLAII monoclonal antibody 1F12 [24], followed by incubation with a R-Phycoerythrin-conjugated anti-mouse antibody (Sigma).

2.3. Immunization of animals

Plasmid DNA was purified using the Endo-Free Mega plasmid preparation kit (Qiagen).

Six week old Swiss outbreed mice (Swiss ICR-CD1 from Harlan Laboratories, four animals each group) were intramuscularly immunized three times at 2 week intervals. Each vaccine dose (100 μg of DNA in 100 μl) was injected in the right and the left anterior tibial muscle (50 μg each).

Eight week old Landrace X Large White pigs (four animals per group) were immunized with three doses of 600 μg of DNA (1.5 ml each), administered at 2 week intervals. One third of each vaccine dose was intramuscularly injected in the femoral quadriceps, one-third in the tabloid neck and the last third was subcutaneously injected in the ear.

2.4. Detection of specific antibody responses

ASFV specific antibodies in mouse and pig sera were detected, as previously described, by conventional ELISA assay to detect specific antibodies against the p30 structural protein, and by western blot assay to confirm the positive results using crude protein extracts from ASFV infected monkey stable cells (MS) [27,28].

The detection of neutralizing antibodies was performed as previously described [29]. Briefly, 10 fold serial dilutions of the E75 ASFV strain (starting with 10^5 HAU_{50}), were mixed (1:2) with activated or inactivated serum obtained before ASFV challenge from each immunized pig in a 96-well plate. After a 1 h incubation at 37 °C, 4×10^5 PAMs were cultured to each well, and 24 h later porcine erythrocytes (1/100 diluted) were added. After 12 h and every 24 h the plates were observed to follow the appearance of hemadsorption. As a control for the assay, hyperimmune serum from a pig that had recovered from an ASFV infection was used.

2.5. IFNγ-ELISPOT

Peripheral Blood Mononuclear Cells (PBMCs) were separated from whole blood by density-gradient centrifugation with Histopaque 1077 (Sigma). Trypan blue was used to assess viability.

Frequencies of ASFV-specific IFNγ-secreting cells in PBMC were analysed by an ELISPOT assay using commercial monoclonal antibodies (mAbs) (Swine IFNγ Cytoset; Biosource Europe) according to a previously reported method [30]. Briefly, 96-well plates (Costar 3590; Corning) were coated overnight with 8.3 μg/ml of IFNγ capture antibody and 5×10^5 PBMC were dispensed per well to be specifically stimulated in triplicate with 6 μg/ml of baculovirus-derived p30 or p54 recombinant proteins, produced in *Trichoplusia ni* larvae [31], or with the whole virus (10^5 HAU_{50} E75/ml). After a 20 h incubation, cells were removed, plates were incubated with an anti-IFNγ biotinylated antibody (2.5 μg/ml) followed by streptavidin-peroxidase labelling and finally, the reaction was developed by adding insoluble TMB blue (Calbiochem). As a negative control, triplicates of cells were incubated with the same amount of an irrelevant protein also produced in baculovirus-infected *T. ni* larvae. Phytohaemagglutinin (PHA; 10 μg/ml)-stimulated cells were also included as controls

in the assay. The specific frequencies of IFNγ-secreting cells per million PBMCs were obtained after subtracting the spot-counts obtained with unstimulated cells.

2.6. Lymphoproliferation assay

PBMCs were used to test the specific proliferative responses to ASFV p30 and p54 proteins. Briefly, 2.5×10^5 live PBMCs/well were plated in 96-well round-bottomed microtiter plates in RPMI–10% FCS. Triplicate wells of cells were stimulated with baculovirus-derived recombinant p30 or p54, produced in *T. ni* larvae (at 6 μg/ml) for 4 days at 37 °C in 5% CO_2.

As a negative control, triplicates of cells were incubated with 6 μg/ml of a baculovirus-derived and *T. ni* produced irrelevant protein. Cells were also incubated with Concanavalin A (2.5 μg/well) as positive control for the assay. Four days after stimulation, each well was pulsed for 18 h with 0.5 μCi of [methyl-3H] thymidine, and the radioactivity incorporated in harvested cells was measured by liquid scintillation in a Mycrobeta counter (Pharmacia). The stimulation index (SI) was calculated as the mean counts per minute (cpm) of wells containing antigen-stimulated cells to the average cpm of wells containing cells cultured with medium alone.

In order to determine the main pathway of antigen presentation inducing the lymphoproliferative responses, assays were also performed in the presence of optimal inhibitory concentrations of mAbs 4B7 and 1F12 that respectively blocked porcine MHC (SLA) class I or class II presentation [24,32].

2.7. Virus and challenge

All pigs were challenged intramuscularly with a lethal dose of 10^4 hemadsorbing units (HAU_{50}) of the E75 virulent ASFV isolate.

Clinical signs of ASF (fever, anorexia, lethargy, shivering, skin cyanosis and recumbency) were monitored daily. Blood samples were collected before and at different times after virus challenge for determination of viremia. The virus was titrated in PAMs by a hemadsorption assay. Briefly, serial dilutions of sera were incubated with 5×10^5 PAMs/well. After 24 h porcine erythrocytes (1/100 diluted) were added to each well. After 12 h and every 24 h the plates were observed to follow the appearance of hemadsorption. Titres were calculated by the Reed and Muench method [33] and expressed as HAU_{50}/ml.

3. Results

3.1. DNA immunization with pCMV-PQ works in mice but fails in pigs.

Transient expression experiments in Vero cells with pCMV-PQ allowed the optimal detection of both p30 and p54 ASFV determinants at 48 h post-transfection by indirect immunofluorescence using specific antibodies against either p54 or p30 (Fig. 1A, left and right panels, respectively). Once *in vitro* expression had been confirmed, non-syngenic mice were immunized with either pCMV-PQ or pCMV as a negative control group. As expected, control animals did not develop specific responses, while all four animals immunized with pCMV-PQ developed specific antibodies against p30, detectable by ELISA after the first boost (data not shown) and reaching their maximum titre after the third immunization (Fig. 1B). Surprisingly, a similar immunization schedule totally failed at inducing detectable antibody responses when immunizing pigs (Fig. 1C).

3.2. In vitro targeting of GFP to APCs using the APCH1 molecule

Aiming to improve the immune responses induced by our DNA vaccine, a new plasmid was generated encoding PQ fused to the APCH1 molecule (pCMV-APCH1PQ). To first confirm the potential of the APCH1 molecule to target antigens to APCs, Vero cells were transfected *in vitro* either with pCMV-GFP or with pCMV-APCH1GFP, encoding the GFP alone or fused to APCH1 molecule, respectively and 24 h later, PAMs were added to the trans-well plate, therefore facilitating the transfer of secreted proteins but avoiding direct contact between both cell types. No specific GFP-fluorescence was detectable in $SLAII^+$ macrophages co-incubated with pCMV-GFP transfected cells, while specific signal was found in those incubated with Vero cells transfected with pCMV-APCH1GFP (Fig. 2), thus demonstrating the capability for APCH1 to target GFP to SLA II-bearing cells *in vitro* and confirming previous results obtained with the soluble APCH1-2L21 protein, a fusion of APCH1 with a linear antigenic peptide derived from theVP2 capsid protein of canine parvovirus [21].

3.3. APCH1 fusion enhances the humoral response induced in pigs after DNA immunization

Once the *in vitro* targeting potential of APCH1 had been demonstrated, an *in vivo* immunization experiment was performed to characterize the immune responses induced by pCMV-APCH1PQ both in mice and pigs. As controls for the assay, groups of four animals were either immunized with pCMV-PQ or with pCMV. In contrast with the lack of responses obtained in pCMV and pCMV-PQ immunized mice after a single shot, three of the four mice immunized with pCMV-APCH1PQ showed detectable antibody responses after the first vaccine dose (Fig. 3A). However, the level of specific-antibodies reached after DNA boosting with either pCMV-PQ or pCMV-APCH1PQ was very similar (Fig. 3A).

A very different picture was observed after pig immunization. Every single pig immunized with pCMV-APCH1PQ showed detectable antibody titres against p30 after three DNA injections, in clear contrast to the absence of detectable responses in pigs immunized with the pCMV-PQ vaccine (Fig. 3B). Confirming the ELISA results, only sera from pCMV-APCH1PQ immunized pigs showed the presence of both p30- and p54-specific antibodies in a western blot assay (Fig. 3C). As expected, the anti-p54 antibodies detected a multiple band pattern ranging from 24 to 30 kDa of molecular weight, corresponding to the diverse viral subpopulations differing in the highly immunogenic protein p54 [34,35], present in the cell culture propagated-ASFV used to develop the assay [27].

Finally, the sera from pCMV-APCH1PQ pigs were used to test their capacity to inhibit the ASFV infection in PAMs. In our assay, none of the DNA-immunized pigs showed any detectable inhibitory capability, while a very clear dose-dependent reduction of virus infection was observed when using a hyperimmune serum as a positive control. Conversely, sera from pCMV-APCH1PQ immunized animals seemed to exacerbate the *in vitro* infection when using a very low multiplicity of ASFV. A clear hemadsorption effect was observed in the presence of these sera even when using 10 HAU_{50} per well, below the detection limit of our assay in the absence of sera (data not shown).

3.4. pCMV-APCH1PQ also induces specific cellular response in pigs

In order to characterize the cellular response induced by the DNA vaccines in pigs, PBMCs collected 15 days after the last immunization were used to analyse both the specific secretion of IFNγ by ELISPOT (Fig. 4A) and the proliferative response (Fig. 4B) after *in vitro* stimulation with the recombinant proteins p30 and p54.

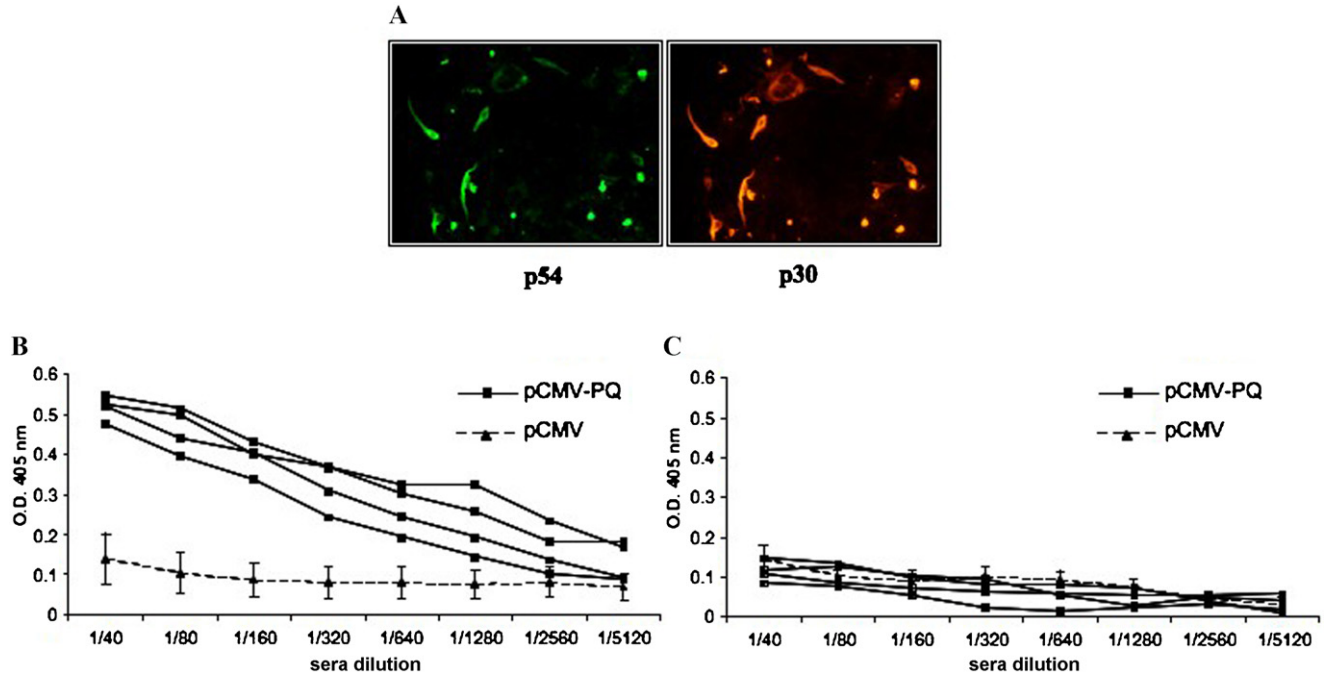

Fig. 1. Detection of p54 and p30 expression in Vero cells transfected with pCMV-PQ by indirect immunofluorescence using rabbit monsospecific serum against p54 and an anti-p30 monoclonal antibody (A). Detection of specific antibodies against p30 by ELISA in sera from pCMV (average of four animals) and pCMV-PQ immunized mice (B) and pigs (C). ELISA OD-values correspond to two-fold serial dilutions of individual sera obtained 15 days after the third immunization. Baculovirus-derived recombinant p30 was used to coat the ELISA plates.

Animals inoculated with pCMV-PQ showed neither specific proliferation nor specific IFNγ-secreting cells, confirming the failure of this construct to induce an immune response in pigs. In clear contrast, all animals inoculated with the pCMV-APCH1PQ showed specific IFNγ-secreting T-cells upon PBMC stimulation with both p30 and p54 proteins (Fig. 4A), also recognizing the whole virus (data not shown). Furthermore, most of the T-cell proliferation corresponded to Class II restricted $CD4^+$ T-cells, since a significant proliferation inhibition was observed when the assay was carried out in the presence of the anti-SLAII antibody (Fig. 4B). Taken together, our data clearly demonstrate that pCMV-APCH1PQ is capable of inducing a broad immunological response in pigs, therefore overcoming the lack of response obtained with pCMV-PQ.

3.5. Immunization with pCMV-APCH1PQ does not protect against ASFV lethal challenge

To determine the protective potential of pCMV-APCH1PQ, all pigs were challenged with a lethal dose of the homologous virulent ASFV strain, E75. As expected, animals inoculated with pCMV-PQ developed ASF clinical signs similar to control pigs immunized with the empty plasmid pCMV. Despite the broad immunological response induced by the vaccine, the pCMV-APCH1PQ immunized animals succumbed to infection with no delay compared to control pigs (Fig. 5A), showing clinical signs and post-mortem macroscopic and microscopic findings similar to the pigs from the other two groups (data not shown). More strikingly, virus titres found in sera from these animals at all times tested were between one and two logs higher on average than those observed in the control group (Fig. 5B), showing statistically significant differences ($p < 0.05$ in a Student t-test) by day 3 post-infection.

4. Discussion

Due to its many advantages, DNA immunization is currently being used not only to dissect basic mechanisms of the immune response in research laboratories but also as a promising strategy in many clinical trials against several human diseases. One of

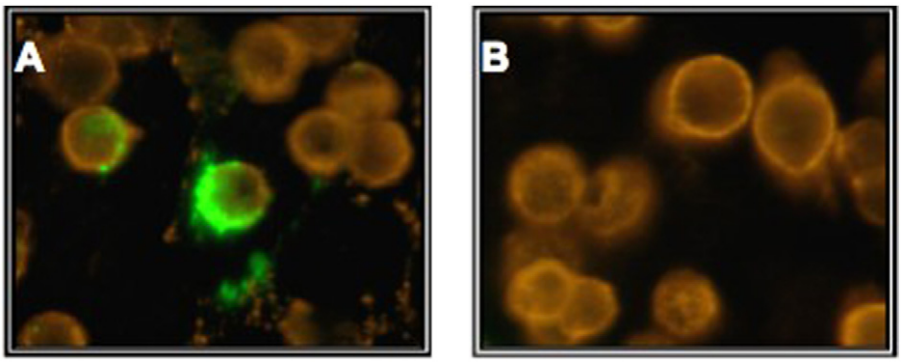

Fig. 2. GFP binding to APCs was tested in SLAII stained macrophages co-cultured with Vero cells transfected with either pCMV-APCH1GFP (A) or pCMV-GFP (B) and separated by polycarbonate membranes in transwell plates.

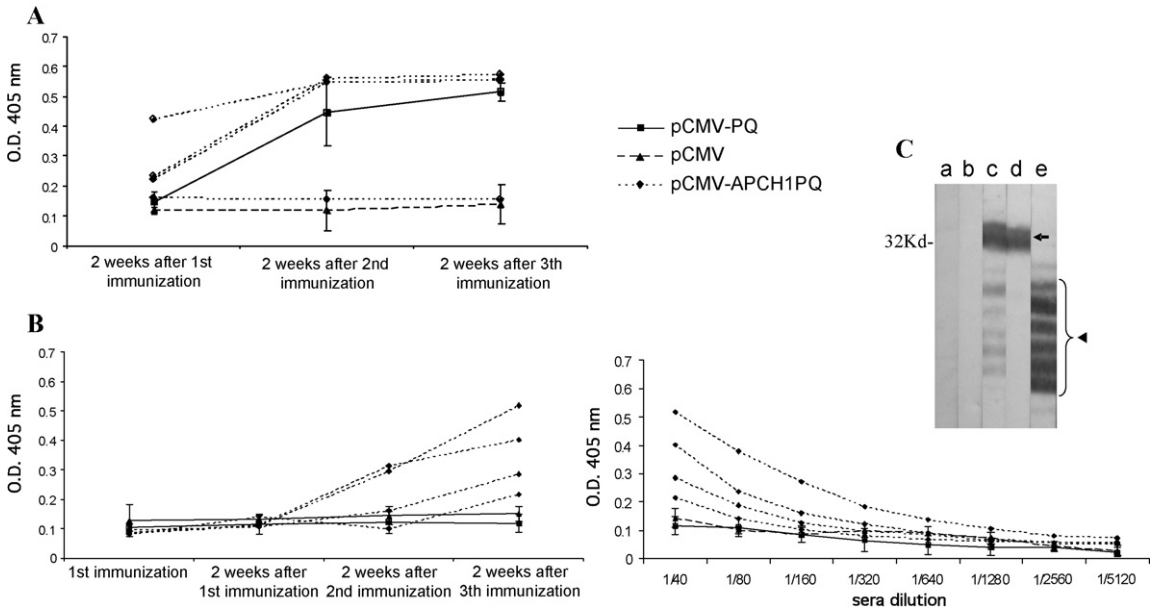

Fig. 3. (A) Time course of anti-p30 antibodies induction in mice after DNA vaccination (ELISA values obtained with sera at 1:40 dilution). (B) Induction of anti-p30 antibodies in pigs after DNA immunization (left panel). Time course of anti-p30 antibody induction (left panel, sera at 1:40 dilution) and antibody titters using sera obtained 15 days after the last immunization (right panel). Inset shows the detection of p30 (arrow) and p54 isoforms [34,35] (arrow heads) by Western-blot using sera (1:40 dilution) from pigs vaccinated three times either with pCMV (a), pCMV-PQ (b), or pCMV-APCH1PQ (c). Data shown corresponds to one representative animal per each group. As controls for the assay an anti-p30 monoclonal antibody (d) and a rabbit monsospecific serum against p54 (e) were used.

the main criticisms originally applied to DNA vaccines, namely the low immune responses they induce on occasions in large animals, including humans, is disappearing mainly thanks to the new strategies developed to increase their efficacy. In this study, our effort was focussed on characterizing the adjuvant effect of a single chain variable fragment of an antibody specific for a swine leukocyte antigen II (APCH1) in DNA immunization protocols in pigs. As a result of these efforts, here we present the capability of APCH1 to target the DNA encoded fused antigens to the SLA-II bearing cells, and its

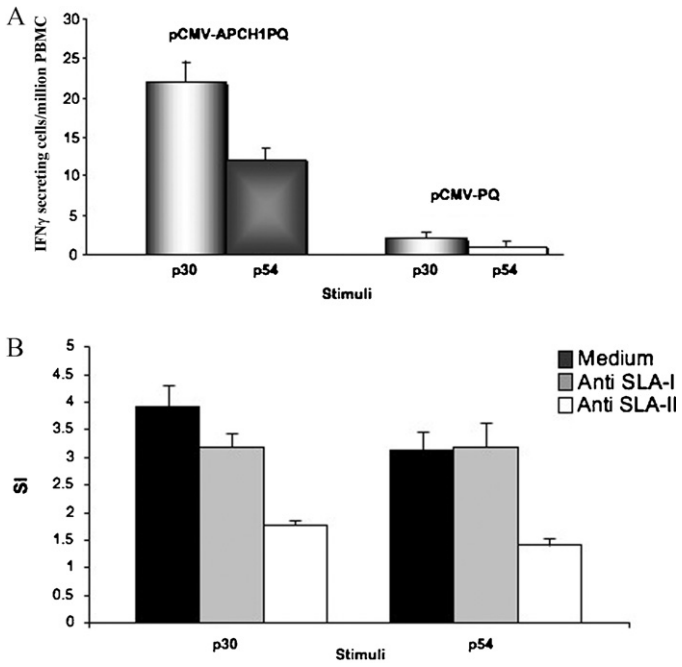

Fig. 4. Cellular responses induced in vaccinated pigs against p30 and p54 proteins. (A) Detection of specific IFNγ secreting cells by ELISPOT after PBMC stimulation with p30 or p54. Results are expressed as the number of IFNγ secreting cells (mean and standard deviation obtained from each group) per million of PBMC. (B) Detection of specific proliferation in pCMV-APCH1PQ immunized pigs after PBMC stimulation with p30 and p54 proteins, in the absence or presence of anti-SLAI or anti-SLAII monoclonal antibodies.

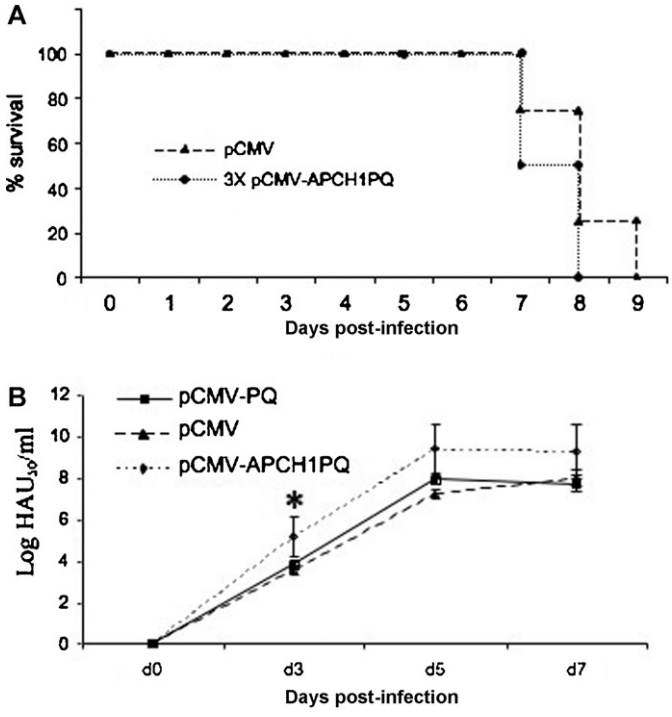

Fig. 5. (A) Percentage of survivors in control (pCMV-immunized) and pCMV-APCH1PQ vaccinated pigs after ASFV lethal challenge (10^4 HAU_{50} of the E75 ASFV virulent strain). (B) Viremia detected in sera from immunized pigs at different days after infection with 10^4 HAU_{50} of the E75 ASFV virulent strain. Results are represented as the logarithm of the HAU_{50}/ml serum (mean and standard deviation from each group are shown). (*) The increase on the viremia titre found at day 3 post-ASFV challenge was statistically significant for pigs immunized with pCMV-APCH1PQ.

potential to improve the specific humoral and cellular responses induced in pigs.

The starting point of our work was based on the lack of induction of an immune response in pigs after pCMV-PQ vaccination. The failure of the plasmid in inducing detectable immune responses was not due to defects in the antigen presentation of the PQ chimera, since immunization with the individual plasmids (pCMV-p30 and pCMV-p54) yielded the same results as pCMV-PQ, working nicely in mice and failing in pigs (not shown). The almost identical pattern of detection observed by IF with the non-crossreactive and specific anti-p30 and anti-p54 antibodies reflects the integrity of the chimerical proteins in cells transfected with either pCMV-PQ or pCMV-APCH1PQ, also confirmed by western-blot (not shown). Therefore, the exponential improvement observed in the immune responses induced in pigs after vaccination with pCMV-APCH1PQ could be totally ascribed to the adjuvant effect of the APCH1 molecule. Such an effect was not that evident in mice after three injections, most probably due to the optimal responses induced by both plasmids when following this immunization schedule (three shots). However, in accordance with this hypothesis, clear differences in the immune responses induced by both plasmids were observed in mice after a single shot. These results confirm and amplify previous results obtained in our laboratory using subunit vaccines in mice and rabbits [21], thus demonstrating the utility of APCH1 as an adjuvant for different vaccination methodologies. Moreover, the promiscuous potential of the 1F12 antibody (from which the APCH1 sequence was obtained) to bind to different animal species apart from swine was previously described [24], opening new avenues for this molecule to become broadly used as an adjuvant, even in humans.

Despite the success of pCMV-APCH1PQ in overcoming the failure of DNA vaccination in pigs, immunized animals were not protected against an ASFV virulent challenge. Little is known about the mechanisms involved in ASF protection. Transfer experiments demonstrated the partially protective potential of specific immunoglobulins [30,36], but controversy still exists about the role of neutralizing antibodies and ASFV protection [37–41], even after immunization with p30 and p54 [23,42,43]. Concerning the cellular response, an extra-set of *in vivo* depletion experiments clearly showed the relevance of $CD8^+$-T cells in protection [44], and results recently obtained in our laboratory demonstrate the potential of DNA vaccines to confer partial protection against ASFV lethal challenge in the absence of detectable antibody responses, confirming the potential that T-cell responses can play in protection (unpublished results).

Contrary to the antibody responses obtained in pCMV-APCH1PQ vaccinated mice, readily reaching their maximum titre after the second shot, pigs seemed not to reach a plateau, even after the third boost, leaving room for the improvement by consecutive DNA shots or even by boosting with an heterologous system to optimize the specific induction of antibodies [45]. A prime-boost strategy is currently being pursued in our laboratory aiming not only to increase antibody titres but also to improve their quality. Indeed, lack of neutralizing activity in sera from pigs immunized with our DNA vaccines might explain the lack of protection afforded by them. Supporting this theory, exactly the same chimera (PQ) administered as a baculovirus-expressed recombinant protein, induced strong neutralizing antibody responses and partially protected pigs from lethal challenge [22,30]. Moreover, the fact that the presence of sera from pigs immunized with pCMV-APCH1PQ enhances the *in vitro* infection of ASFV in alveolar macrophages, when the infection takes place at a very low multiplicity, might explain the higher virus titres in sera from vaccinated pigs. Antibody-mediated exacerbation has been described already for other experimental vaccines and viral infections, especially in virus with macrophage tropism, such as ASFV [46–50].

The inhibitory effect of IFNγ on *in vitro* ASFV replication has been previously described, albeit its relevance in *in vivo* protection has not been completely elucidated [51]. The analysis of the pCMV-APCH1PQ induced specific T-cells demonstrated their capability to specifically proliferate and produce IFNγ, thus indicating that induction of IFNγ specific T-cells is not a sufficient indicator of protection against ASFV. Moreover, the class II restriction showed in most of proliferating cells indicated a main $CD4^+$ T-cells phenotype, perhaps pointing to an intrinsic limitation of APCH1 adjuvant to induce a CTL response, thus limiting the potential capability of the vaccine to protect the animals against the virus. However, due to the lack of known ASFV immunogenic peptides, the specificity of the cellular response could be confirmed by PBMCs stimulation with recombinant proteins or in occasions, with live virus (data not shown), therefore most probably limiting the detection of specific $CD8^+$ T-cells. We are currently developing new CTL assays to *in vivo* characterize the potential induction of CTL responses by our DNA vaccines, which would help to clarify the role of the different cellular phenotypes in the immunity against ASFV.

In summary, here we have clearly demonstrated the adjuvant effect of APCH1 in DNA vaccination protocols. pCMV-APCH1PQ exponentially improved the humoral and cellular responses induced after DNA vaccination, contrasting with the lack of immune response induced previously by pCMV-PQ, thus demonstrating again that DNA immunization limitations in large animals can be overcome when using the appropriate strategy. These results have been recently confirmed by using the foot and mouth disease virus (FMDV) model. A DNA vaccine encoding FMDV minigenes fused to the APCH1 molecule, dramatically improved the immune responses induced, both in mice and pigs and more importantly, was capable to partially protect against FMDV challenge in swine (Borrego et al., submitted). These results, in contrast with the lack of protection afforded against ASFV using the same adjuvant, highlight the importance in vaccine development not only of the strategy used, but also of the mechanisms implied in protection against a particular pathogen. In this sense, the capability of DNA vaccination to be adapted to different strategies and thus potentiating a desired immune response, make this methodology a valuable tool not only for the development of successful vaccines, but also to better understand the role of different immune components in relation to a particular virus.

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