Appendix 79

TARGETING FMDV MINIGENES TO SLA II POSITIVE CELLS ENHANCES THE INDUCTION OF CELLULAR RESPONSES IN SWINE AND CONFERS PROTECTION AGAINST VIRAL CHALLENGE

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ABSTRACT

In this report we present some preliminary results obtained with the last generation of DNA vaccines based on FMDV minigenes designed in our laboratory. Using previously characterised FMDV B- and T-cell epitopes, we have tested several targeting signals as carriers for our vaccine antigens. From the different molecules assayed, the signal peptide from the CCL20 porcine chemokine showed to be very effective in vitro, both in terms of antigen expression levels and in its capacity to secrete the peptides to the supernatant of the transfected cells; however when tested in vivo, constructs in which viral antigens were fused to this sp failed in inducing an efficient neutralizing antibody response and pigs were unprotected after viral challenge. In contrast, immunization with a DNA plasmid encoding the same FMDV epitopes fused to a single chain variable fragment (scFv) of an antibody that recognizes the Class II Swine Leukocyte Antigen (SLA II) induced a significant T-cell response that, even in the absence of anti-FMDV antibodies, resulted in total protection of 50% of the animals. Protection correlated with the presence of FMDV specific T-cells prior to challenge and with the development of seroneutralizing activity immediately after FMDV-challenge. Thus, both humoral and cellular responses seemed to play relevant roles in conferring sterile protection against FMDV.

1. INTRODUCTION

Foot-and-mouth disease (FMD) is one of the most devastating diseases for animal health, and development of novel, safer marker vaccines is essential to avoid the problems associated to the inactivated virus vaccines currently in use. DNA vaccination has become a promising alternative because of its several advantages over conventional vaccines, i.e.: it avoids the manipulation of infectious virus, which results risky and expensive. Furthermore, DNA is easy to manipulate to generate DIVA-vaccines as well as "a la card" vaccines including conserved epitopes capable to confer protection against more than one virus isolate. In addition, they do not need of maintaining the cold chain until its distribution and costs are low.

Although promising, previous DNA vaccines formulations against Foot-and-Mouth Disease Virus (FMDV) induced weak immune responses needing to be improved (Cedillo-Baron 2001, Wong 2002). Besides the advantages mentioned above, the feasibility of manipulating the DNA constructs allows application of different strategies for optimization and/ or modulation of the immune responses induced (Rodríguez and Whitton 2000). We have previously shown the protective capability of DNA vaccines encoding FMDV B- and T-cell antigenic determinants ("minigenes") fused to the human prion signal peptide, both in mice and in pigs (Borrego 2006, Ganges 2008). In an attempt to enhance the immune responses induced, in this work we have generated a new panel of DNA constructs encoding different combinations of same viral epitopes fused to new targeting molecules: i) the signal peptides from two porcine molecules, expected to improve the introduction of the antigen into the secretory pathway; and ii) a single chain variable fragment (scFv) of an antibody that recognizes the Class II Swine Leukocyte Antigen (SLA II), in order to target antigens to APCs. The vaccine potential of all the constructs generated was first analyzed in vitro and in a mouse model, and based on the results obtained, two of the constructs were finally selected for a further evaluation in an immunization/challenge experiment in swine, natural host for FMDV. In this report we are showing the results of this vaccination experiment.

2. MATERIALS AND METHODS

PLASMIDS

The previously described pCMV-spBTT plasmid (Borrego 2006) was used as template for PCR amplification of different combinations of FMDV Cs8c1 epitopes B, T3A and TVP4 fused in tandem. They were cloned within the pCMV plasmid fused in frame to: i) the ORF encoding the signal peptide from the porcine chemokine CCL20, also named mip3γγ (Acc. nr: AJ311716); ii) the ORF encoding the signal peptide from the porcine CD163 molecule, a surface antigen of monocytes (Acc. nr: AJ311716, Sanchez 1999); and iii) the ORF encoding the single chain of the variable fragment (scFv) of an antibody with affinity to porcine MHC class II antigens (Bullido 1997, Gil 2003). Sequences were confirmed by automatic sequencing and plasmids were produced in a large scale (Endofree QIAGEN kits) to be used in vivo.

EXPRESSION IN CELL CULTURE.

Monolayers of BHK-21 cells were transfected using the lipofectamine-Plus reagent (GIBCO-BRL). At 48 h after transfection, supernatants and cells extracts were collected and analyzed by immuno-dot using monoclonal antibody SD6, recognizing the B FMDV Cs8c1 epitope included in the constructs (Mateu 1987). Expression of the antigen in transfected cells was also analyzed by immunofluorescence and flow cytometry using MAb SD6.

IMMUNIZATION OF MICE.

Groups of 4 Swiss mice received 3 intramuscular (IM) doses of 100 microg each of the different plasmids every two weeks. Specific FMDV antibodies in serum samples (before inoculation and after the last boost) were detected by a neutralization assay (Mateu 1987). One month after the last DNA boost, mice were euthanized and their spleens collected in order to analyze the specific cellular response by intracellular Cytokine Staining (ICCS) (Borrego 2006).

IMMUNIZATION OF PIGS.

Pigs received 400 micrograms per dose of the corresponding plasmid every two weeks. Animals 1 to 4 received 3 shots of the pCMV-spCCL20-BTT plasmid; animals 7 and 8 received 3 shots of the pCMV-scFv-BTT plasmid and another two pigs (animals 11 and 12) received only one shot of the same plasmid (pCMV-ScFvBTT) at the time of the second boost for the rest of the pigs. Pig 5 was inoculated with an irrelevant pCMV plasmid. 15 days after receiving the last DNA dose, all animals were needle-challenged with 10^4 $TCID_{50}$ of FMDV Cs8c1. Pigs were daily monitored for clinical signs of disease for 10 days, when they were euthanized. Blood and swab samples were taken at different time points along the experiment. Samples collected at day 2 and 3 after infection were analysed for viral detection by isolation on IBRS cells monolayers and/or RT-PCR targeted to the 3D protein (Borrego 2006, Saiz 2003); specific antibodies were determined by neutralization assays (Mateu 1987) and 3ABC-ELISA (Blanco 2002). Peripheral blood mononuclear cells (PBMCs) obtained before FMDV challenge and at day 10 after viral infection were used to measured cellular responses by IFN-gamma ELISPOT (Díaz and Mateu 2005).

3. RESULTS

Construction of plasmids and in vitro expression.

Aiming to improve our previous results with the human prion signal peptide (Borrego 2006, Ganges 2008), we generated a new panel of DNA constructs fusing the FMDV BTT epitopes to new targeting signals. We selected on one hand, two signal peptides (sp) from two different porcine molecules: the chemokine CCL20, and CD163, a surface antigen of monocytes. On the other hand, the FMDV peptides were fused to the scFv of an antibody that recognizes the Class II Swine Leukocyte Antigen (SLA II), aiming to target the vaccine encoded antigens to the APCs .

Antigen expression driven by these plasmids was first analyzed by immunodot, both in supernatants and cell extracts from transiently transfected cells. As shown in fig.1a., clear differences in the expression levels of the viral antigen were observed, being the CCL20 sp the most efficient. Interestingly, the expression levels obtained with this new construct resulted 50-fold higher than those obtained with the pCMV-spBTT construct, encoding the FMDV BTT antigens fused to the human PrP signal peptide, previously showed to confer partial protection *in vivo* (Borrego 2006, Ganges 2008). Surprisingly, no specific signal was detected in cells transfected with the

pCMV-scFv-BTT construct (not shown). However, low, albeit detectable expression levels of the antigen in these cells was observed by immunofluorescence (data not shown).
We next generated a new set of plasmids containing different combinations of the viral minigenes fused to the CCL20 sp and evaluated their *in vitro* expression (fig.1b). Surprisingly, the presence or absence of the VP4 FMDV peptide clearly affected not only the expression levels but overall the antigen localization, since its elimination allowed a much more efficient secretion of the antigen.

Immunogenicity in adult mice.

The higher levels of expression achieved with the new constructs encouraged us to test them in mice as a first evaluation of their vaccine potential. Therefore, groups of Swiss mice received 3 IM injections of each one of the plasmids. As negative controls one group was inoculated with the empty plasmid pCMV, and a second group received plasmid pCMV-CCL20neg, encoding the CCL20 sp fused to the complementary strain of the BTT ORF. After the last DNA dose, sera were analyzed for detection of FMDV neutralizing antibodies (fig.2a). The only construct found to generate high levels of neutralizing antibodies was pCMV-CCL20sp-BTT, with one only animal showing neutralizing titers (PRN_{50}= 1.7), that well might assure protection against FMDV (Borrego 2006). Surprisingly, all the other constructs failed in inducing good antibody responses, in spite of their high expression levels observed *in vitro*. Surprisingly, mice immunized with pCMV-CCL20sp-BT3A did not improve the humoral responses induced in spite of the efficient secretion of the encoded antigen (fig. 2a).
Cellular responses were evaluated by Intracellular IFN□ Staining of splenocytes after *in vitro* stimulation with a mixture of the 3 FMDV peptides included in the vaccine (Borrego 2006). Interestingly, a strong positive response was exclusively observed in animals inoculated with pCMV-scFv-BTT (fig 2b). In two of the 4 mice of this group it was possible to detect both $CD4^+$ and also $CD8^+$ T-cells that specifically secreted IFN□ in response to *ex vivo* stimulation. The percentages of specific $CD8^+$ T-cells in these animals were surprisingly high (23.2% and 8.7%). As mentioned, no responses were detected for the rest of the animals.

In summary, antigenicity and *in vivo* immunogenicity did not seem to correlate, at least upon DNA vaccination with FMDV minigenes.

DNA immunization and viral challenge in pigs.

Since immune responses had been only detectable in mice inoculated with two of the five plasmids tested: pCMV-CCL20sp-BTT (neutralizing antibodies) and pCMV-scFv-BTT (IFN□-secretion by $CD4^+$ and $CD8^+$ T-cells after specific stimulation), we decided to further analyse the protective capacity of these two plasmids in swine, a natural host for FMDV. Thus, groups of four pigs were immunized and subjected to viral challenge as described under Materials and Methods. Progression of disease in the pigs was evaluated daily using a clinical score based on a semi-quantitative rating of clinical signs (rectal temperature, lameness, vesicle formation on each one of the four feet, on the tongue, mouth and snout, and vesicle size) (Fig.3). All parameters regarding development of disease in control animal, pig 5, were as expected for the virus and dose used for challenge (Borrego, unpublished results). All the animals immunized with the pCMV-CCL20sp-BTT showed clinical signs of disease undistinguishable from those found in the control pig. In clear contrast, two animals inoculated with the pCMV-scFv-BTT construct (pigs 8 and 12) showed no signs of disease during the 10 monitored days. Interestingly, animal 12 had received a single DNA dose before viral challenge. The other two animals within this group (pigs 7 and 11), showed a delay on the disease onset, with a shorter period of acute disease, and milder clinical signs (small-size vesicles appearing only in the feet and tongue) when compared to the control animal (pig 5).
Viremia for all the animals immunized with the pCMV-CCL20sp-BTT peaked at day 2, one day earlier than for pig 5 (Table 1), correlating with clinical signs of disease. In contrast, pigs 8 and 12, inoculated with the pCMV-scFv-BTT construct, showed no detectable virus at any time and sample tested, in agreement with the lack of signs of disease. Curiously enough, the other two animals within this group (pigs 7 and 11) had similar viremia titres than control animal (table 1, first row) in spite showing milder symptoms of disease (fig. 3). Remarkably, viral detection by RT-PCR in swabs from this pCMV-scFv-BTT group resulted mainly negative, while the rest of the animals scored positive both at day 2 and 3 after FMDV infection (Table 1, second row).

Immune response after dna vaccination.

Disease outcome and viral loads suggested that immunization with the pCMV-scFv-BTT construct had induced a protective immune response in the pigs. In an attempt to identify the mechanism(s) responsible for protection, we analysed both humoral and cellular responses in samples collected at day 43 (prior to challenge). No neutralizing antibodies at the time of challenge were detected in

any of the DNA-vaccinated pigs, independently of the plasmid used. Interestingly, a clear positive response was detected by IFN□. ELISPOT, in which animals inoculated with the scFv-construct showed a significant number of IFN□ producing cells that specifically responded to BEI-inactivated virus (fig 4), thus indicating that the protection afforded was mediated by cellular mechanisms. No significant responses (neither humoral nor cellular) were found in the pigs immunized with pCMV-CCL20sp-BTT or the control pig.

Immune response after challenge.

Prior to viral challenge (day 43) and at days 3, 6, 8 and 10 post challenge, serum samples were collected and their neutralizing activity was analyzed. The kinetics of the development of seroneutralizing activity (fig 5) was very similar between animals within group CCL20sp and control animal, pig 5, with high titers of neutralization (PRN50 between 3.0-4.0) detected at first at day 6. In group scFv, in contrast, first detection of neutralization occurred earlier, with significant titers (PRN50 between 1.0-2.0) already detected at day 3 in 75% animals (pigs 8, 11 and 12). Curiously, in pigs 8 and 12 (the two animals protected against disease) this neutralization was not detected at day 6 but reappeared again at later times post-challenge.
Analysis of antibodies to the 3ABC protein, indicative of viral replication, revealed that at day 10 post-infection no seroconversion had occurred in pigs 8 and 12, while the rest of the pigs showed high antibody titers against these non structural FMDV antigens (Table 1, third row), thus supporting the total protection achieved by vaccination with pCMV-scFv-BTT .

4. DISCUSSION

Vaccination against FMDV is still an unsolved question and development of novel and safer effective vaccines circumventing the problems associated to conventional vaccination is a must. In addition to other strategies such as subunit vaccines or recombinant viruses expressing the main antigenic proteins of FMDV (SanzParra 1999, Sobrino 1999, Pacheco 2005, Wang 2002), DNA vaccination has become a promising alternative, as evidenced by results from several works (Beard 1999, Cedillo-Baron 2001, Wong 2002). Besides safety and economical advantages related to their production, the feasibility of manipulating the DNA constructs allows application of different strategies for optimization and/ or modulation of the immune responses induced (Rodriguez and Whitton 2000).
In an attempt to design effective DNA vaccines we decided to target FMDV epitopes to pathways expected to play a key role in protection against FMDV. In previous works we have shown the vaccine potential of a DNA vaccine encoding the FMDV BTT minigenes fused to a strong signal peptide from the human Prion protein (pCMV-spBTT) (Borrego 2006, Ganges 2008). We decided to further explore this strategy, and selected some new signal peptides aiming to improve the introduction of the vaccine encoded antigens into the porcine secretory pathway, increasing so the chances to induce FMDV neutralizing antibodies. It is known for long that neutralizing antibodies are one of the main arms capable to confer protection against FMDV (van Bekkum 1969, McCullough & Sobrino 2004). The signal peptides (sp) selected belong to two different porcine molecules: the chemokine CCL20, and the CD163, a surface antigen of monocytes (Sanchez 1999). When fused to these sp, the levels of expression in vitro of viral antigens were greatly enhanced, and the antigen was efficiently exposed on the cell membrane or even secreted to the milieu, depending on the minigene combination. Unfortunately, our plasmids based on the CCL20sp, even those with most of the antigen being secreted, failed in inducing detectable immune responses in adult mice. Neutralizing antibodies were only detected in one of the animals receiving the pCMV-CCL20sp-BTT construct, and no cellular response was either detected. When tested in swine, this construct failed again in inducing antibodies and animals were indeed unprotected. For this same FMDV Cs8c1-B epitope, this lack of correlation between antigenicity and antigen secretion with immunogenicity has been already observed (Sobrino et al, unpublished results), probably indicating an extreme sensitivity of this epitope to structural conformation, so that changes from its original conformation within the FMDV capsid might strongly affect its immunogenicity .
On the other hand, some works have demonstrated the important role that other mechanisms different from neutralizing antibodies play in protection against FMDV (SanzParra 1999, Takamatsu 2006), although the precise contribution of cellular responses against FMDV is still to be defined. Because of their minimalism, our DNA constructs may be a good tool to get an insight into it. The fusion of antigens to targeting molecules expected to drive them to the sites of the immune induction has been successfully assayed, both in animal models and in livestock species (Leifert 2004). Targeting the antigen to dendritic cells by single-chain Fv antibodies (scFv) specific for APC surface molecules has resulted in an enhancement of immunogenicity (Demangel 2005). Following this strategy, we fused our FMDV BTT antigens to an scFv that specifically recognizes porcine SLAII

molecules (Bullido 1997), previously used as recombinant fusion protein to improve the immune responses induced against the fused antigens (Gil 2003). In spite of the low expression levels obtained *in vitro*, the construct pCMV-scFv-BTT showed to be highly immunogenic in mice, and also in swine. Immunization with pCMV-scFv-BTT, even after one single shot, resulted in full protection for 50% of the animals, in which no signs of disease or viral replication were observed, and in partial protection for the rest of the pigs receiving the same construct. Total protection seemed to correlate with the strong induction of specific T-cells prior to FMDV-challenge that secrete IFN□ in response to BEI-FMDV stimulation. The potent antiviral activity of IFN□ on FMDV, both in vitro and in vivo, has already been reported (Moraes 2007, Zhang 2002) as well as a positive correlation with protection (Parida 2006). On the other hand, the capability of pigs 8 and 12 to produce a very early seroneutralizing response seems to play an important role in the protection achieved. Further work is currently going on in our lab in order to better characterize the responses induced both before and after viral challenge.

In summary, vaccination with pCMV-scFv-BTT, expected to drive FMDV antigens to SLA II positive cells, can protect pigs from FMDV challenge. Both induction of specific T-cells before FMDV challenge and a fast neutralizing activity after viral challenge seemed necessary to confer protection. Despite much work remains to be done in order to elucidate immune mechanisms mediating protection, our results open the possibility of using these vaccines to confer protection, even perhaps against heterologous FMDV strains.

5. CONCLUSIONS

1. Both humoral and cellular responses seemed to play relevant roles in conferring sterile protection against FMDV
2. Targeting of antigens to APCs seems to be a very effective approach to induce protective cellular responses
3. Immune responses elicited against highly conserved FMDV T-cell epitopes could overcome the variability of FMDV strains

6. ACKNOWLEDGEMENTS

We thank M. G. Esguevillas, N. delaLosa and people at the animal facilities at CISA for technical assistance, and D. Andreu for the generous supply of synthetic peptides. Work supported by Ramón y Cajal, BIO2005-07592-C02-01, AGL2004-07857-C03-01, TRT2006-00035-C02-00, CONSOLIDER-INGENIO 2010 and EPIZONE.

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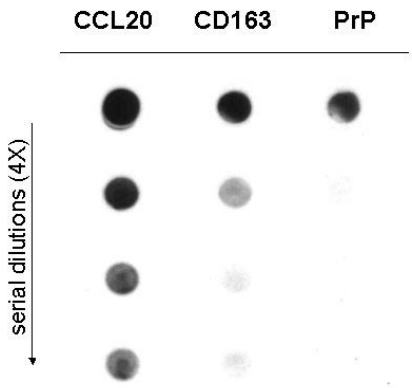

Fig 1 a: Detection of the FMDV B epitope in serial dilutions of protein extracts obtained from cells transfected with plasmids encoding the FMDV BTT minigenes fused to different signal peptides.

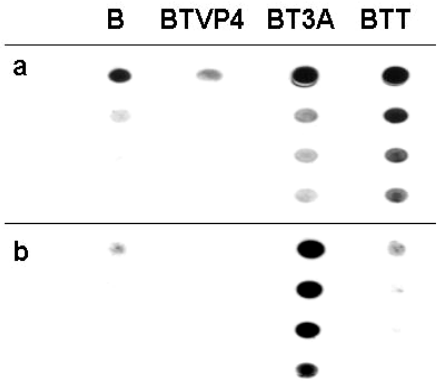

Fig 1 b: Detection of the FMDV B epitope in serial dilutions of a) cell-protein extracts and b) cell-supernatants from cells transfected with plasmids encoding combinations of the FMDV BTT minigenes fused to the CCL20 signal peptide

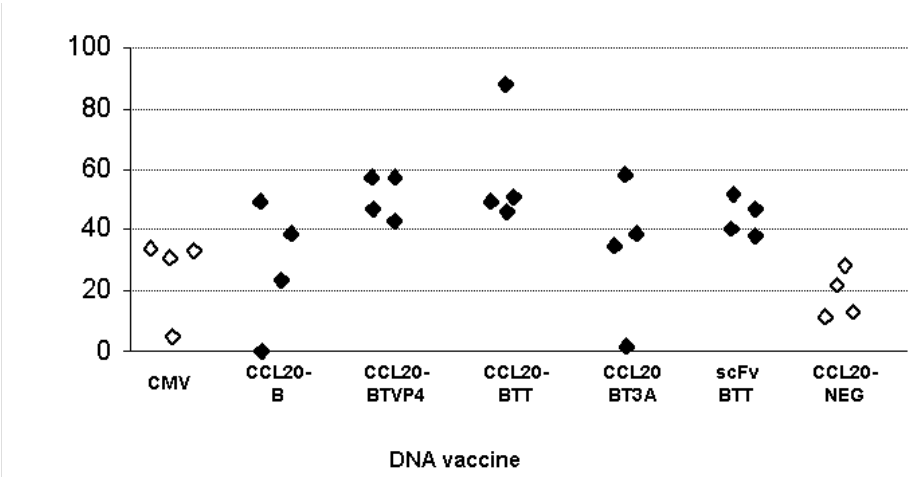

Fig. 2a: Detection of neutralizing antibodies in mouse sera. Each rhomboid represents a single animal.

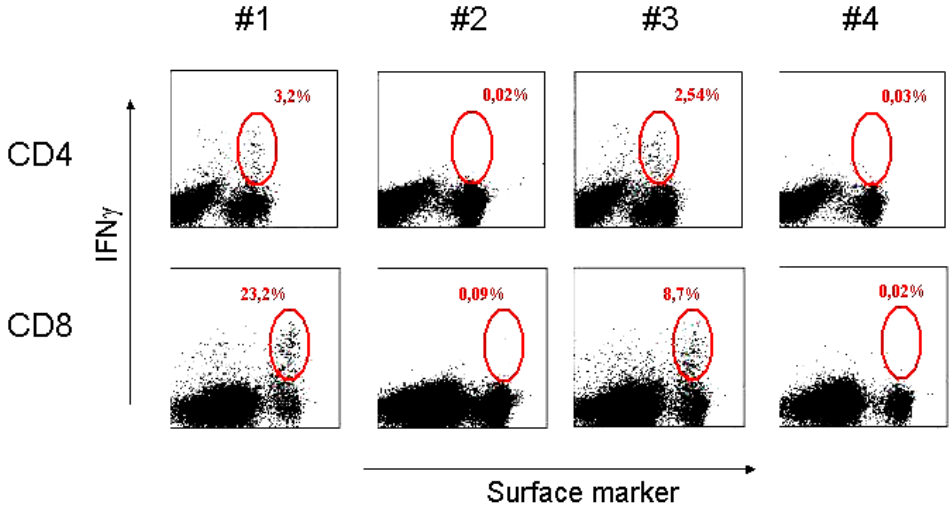

Fig. 2b: Intracellular IFNg detection in splenocytes from mice (#1 to 4) immunized with pCMV-scFv-BTT after *in vitro* stimulation with a mix of the three specific FMDV synthetic peptides encoded in the vaccine.

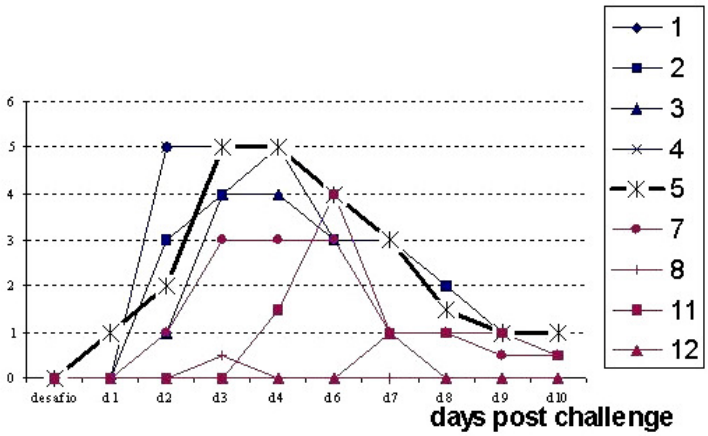

Fig. 3: Clinical score after viral challenge in the DNA-immunized pigs.

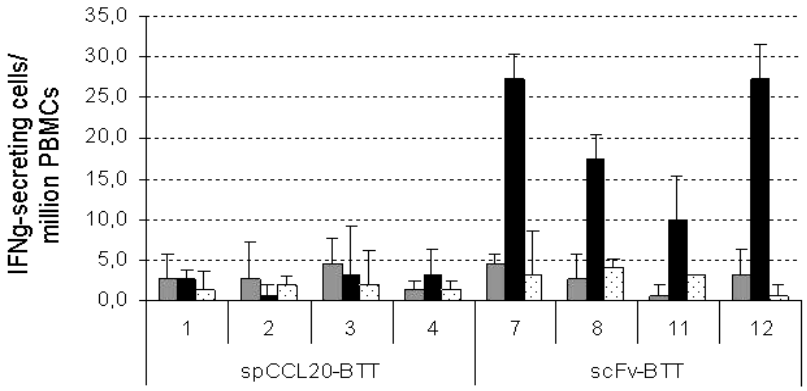

Fig 4: IFNg ELISPOT in DNA-immunized pigs. Average number (of three replicates) of IFN-gamma producing cells per 10^6 PBMCs upon *in vitro* stimulation with: Infectious FMDV Cs8c1 (gray bars); BEI-inactivated FMDV Cs8c1 (black bars) or media alone (white bars).

		Viral isolation and/or RT-PCR on serum samples[a]		RT-PCR on swab samples[b]		Seroconversion to NSP[c]
		Day 2	Day 3	Day 2	Day 3	Day 10
spCCL20-BTT	1	4.0	0	P/P	N/P	1.786
	2	4.5	0	P/P	N/P	1.682
	3	4.0	0	P/P	P/P	0.742
	4	4.0	0	P/P	P/P	1.665
control	5	PCR POS	2.5	P/P	P/P	1.52
scFv-BTT	7	1.0	3.0	N/N	N/P	1.069
	8	PCR NEG	PCR NEG	N/N	N/N	0.035
	11	0	2.0	N/N	N/N	1.234
	12	PCR NEG	PCR NEG	N/N	N/N	0.234

Table 1: Viral load after FMDV-challenge in DNA-immunized pigs. (a) For those samples in which cytopathic effect was detected, results are shown as $TCID_{50}$/10 microl (log10). Some of the negative samples were also assayed by RT-PCR. (b) Results expressed as P (positive) or N (negative) for each one of the two swab (nasal / pharyngeal) samples. (c) Results expressed as OD450nm at day 10 - OD450nm at day 0 in a 3ABC-ELISA.

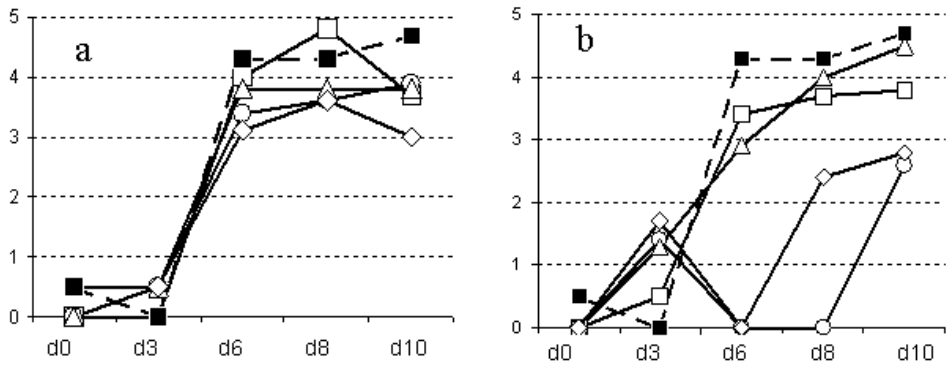

Fig 5: Seroneutralization after viral challenge in DNA-immunized pigs. Results are represented as PRN_{50}, i.e., dilution of serum (log10) causing a reduction of 50% in the number of pfu in a plaque-reduction assay (Mateu). A: pigs immunized with the pCMV-spCCL20-BTT construct (□:nr.1, O: nr.2, □:nr.3, □:nr. 4); B: pigs immunized with the pCMV-scFv-BTT construct (□:nr.7, O: nr.8, □:nr.11, □:nr. 12). Animal 5, negative control, discontinuous line and black squares, is included in both figures for comparison. Results are the mean of at least two independent experiments.