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Research Paper

A T-cell epitope on NS3 non-structural protein enhances the B and T cell responses elicited by dendrimeric constructions against CSFV in domestic pigs

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ABSTRACT

It has been recently reported by our group that dendrimeric constructs combining B- and T-cell epitopes from classical swine fever virus (CSFV) provided partial protection against experimental infection. This research evaluated four newly designed constructions while taking into account our previous work, including the direct implication that a T-cell epitope from the NS3 protein contributes to the generation of the immune response against CSFV. To this end, the dendrimeric constructions, including either this NS3 T-cell epitope alone or two different B-cell epitopes without this T-cell epitope, were used to immunise pigs. Thus, construct 1, containing the NS3 T-cell epitope and four copies of a previously described B-cell epitope, significantly reduced the clinical scores and RNA viral loads after challenge relative to the control group. In three out of six animals in this group, vaccination achieved partial protection and was associated with IFN-gamma producing-cells and neutralising antibodies. In contrast, the pigs immunised with construct 2, again with four copies of the B epitope of construct 1 but lacking the T-cell motif, developed more severe clinical signs. Finally, the additional constructs 3 and 4 included four copies of a B epitope that was different from the epitope used in constructs 1 and 2 with or without the abovementioned NS3 T-cell epitope, respectively. Pigs immunised with these latter constructs developed low levels of peptide-specific antibodies that correlated with equally low levels of cellular responses, an absence of neutralising antibodies and a lack of protection. Even so, the clinical scores in the first week after the challenge were less severe for animals vaccinated with construct 3 than for those given construct 4. Our results confirm the relevant role of the B-cell epitope in residues 694–712 of the glycoprotein E2 (which is used in both constructs 1 and 2) for protection against CSFV, as well as the appropriateness of the newly used NS3 peptide as a specific T-cell epitope in domestic pigs.

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1. Introduction

Classical swine fever (CSF) is one of the most devastating diseases for the pig industry worldwide (reviewed in Dong and Chen, 2007). The disease is endemic in Asia, Central and South America, as well as in many Eastern European countries (de Arce et al., 2005; Ganges et al., 2008). Emergency marker vaccines, namely, those that enable the differentiation of vaccinated from infected animals (DIVA), are still required (reviewed in Beer et al., 2007). The E2 envelope glycoprotein is the primary target for neutralising antibodies during natural CSFV infection (van Zijl et al., 1991; Konig et al., 1995), and this target has been the main component in the design of CSFV DIVA vaccines (Beer et al., 2007; Dong and Chen, 2007; Ganges et al., 2008).

Dendrimers are recognised as promising tools for the multimeric presentation of epitopes in candidate vaccines. Multimerisation is a nature-mimicking strategy of antigen presentation that has proven successful in the development of human-made vaccines, particularly by means of dendrimeric (e.g., branching) designs (Tam et al., 2002). This strategy facilitates the interactions between different (B and T) epitopes to potentiate the elicited immune response, and the strategy can also be useful for basic investigations of the mechanisms governing the induction and control of immunity (reviewed in Heegaard et al., 2009).

Previous reports on the application of peptide dendrimer vaccines in the animal health context include a successful prototype that confers solid protection against foot-and-mouth disease virus (FMDV) in pigs (Cubillos et al., 2008). We also recently reported the immunogenicity of three dendrimeric peptides tested as vaccine candidates against classical swine fever virus (CSFV) in domestic pigs (Tarradas et al., 2011; Monsó et al., 2010). These constructs were designed to combine in a single platform a T-cell epitope from the NS3 protein (K^{1446}HKVRNEVMVHWFGD^{1460}) that was able to induce lymphoproliferation, IFN-gamma and effector CTL responses in miniature pigs (Armengol et al., 2002) with four copies each of the three B-cell epitopes from glycoprotein E2 {K^{694}EDFRYAISSTNEIGLLGA^{712}, A^{712}EGLTTTWKDYDHNLQ^{727} (Dong et al., 2006), or T^{829}AVSPTTLRTEVVK^{842} (Lin et al., 2000)}. These B-cell epitopes have been previously reported to induce CSFV-neutralising antibodies when administered in monomeric form (Dong and Chen, 2006a,b; Dong et al., 2002, 2006; Liu et al., 2006). Only pigs immunised with the first construct developed high and uniform antibody responses against the immunogen, as well as achieving partial protection from a CSFV challenge (Tarradas et al., 2011). Additionally, the sera from pigs immunised with this dendrimer recognised the linear NS3 peptide, suggesting the possibility that it may also contribute to the protective response (Tarradas et al., 2011).

To investigate the contribution of the NS3 peptide to the protective response, two types of dendrimeric constructs, specifically, B_4T or B_4 (Fig. 1), with or without the T cell motif, were examined. In both of these cases, the B-cell epitope was the above-mentioned 694–712 region of the glycoprotein E2. Additionally, we have tested the immunogenicity of two B_4T-type constructs that combine another E2 neutralising epitope (E^{706}IGLLGAEGLTTTW^{719}) (van Rijn, 2007), with either the NS3 T-cell motif (construct 3) or another T epitope that is described at the C-terminal end of E2 (N^{992}KYYEPRDSYFQQY^{1006}) (Yu et al., 1996) (construct 4) (Fig. 1). Each of these constructs was used to immunise a group of six pigs, which were subsequently challenged with a lethal dose of CSFV. The results confirmed the contribution of the NS3 T-cell epitope to protection against CSFV, and the findings highlighted E2 (694–712) as an efficacious B-cell epitope that confers protection when incorporated into dendrimeric constructions. Furthermore, our data illustrate the general usefulness of the dendrimeric peptide strategy for studying the immune response to CSFV in domestic pigs.

2. Materials and methods

2.1. Dendrimeric peptide synthesis

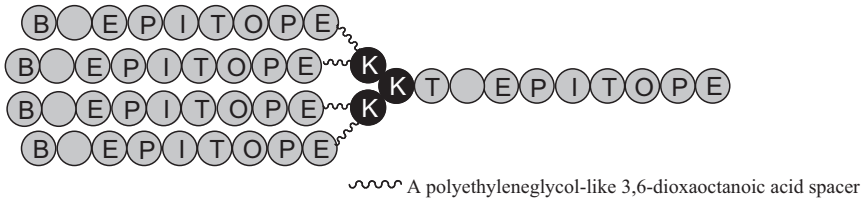
Constructs 1–4 (Fig. 1) were synthesised by Fmoc solid phase methods as previously described (Monsó et al., 2010). The hydrophobicity of several of the constituting epitope sequences, which poses a potential concern for the solubility of the resulting multimeric constructs (Kowalczyk et al., 2011), was compensated for by the introduction of polyethyleneglycol (PEG)-like 8-amino-3,6-dioxaoctanoic acid (O_2Oc) units between the Lys core and the B epitopes. Construct 1 included one O_2Oc unit, whereas constructs 2–4 included two units each. With these modifications, the target dendrimers were obtained in sufficient purity (approximately 90% by HPLC) to allow for their successful characterisation by MALDI-TOF mass spectrometry.

2.2. Cells and viruses

PK-15 cells were cultured in complete DMEM medium, supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. The cells were infected with 0.1 $TCID_{50}$/cell in 2% FBS, and the virus was harvested 48 h later. A peroxidase-linked assay (PLA) (Wensvoort et al., 1986) was used for viral titration following the statistical method described by Reed and Muench (1938).

2.3. Immunisation of pigs

To evaluate the immune response induced by the dendrimeric constructions, 30 pigs (Landrace × large white, 8 weeks old; numbered from 1 to 30) were used. Animals 1–6, 7–12, 13–18 and 19–24 were immunised with constructs 1–4, respectively (Fig. 1). Six additional pigs (25–30) were inoculated with saline solution plus adjuvant as controls. Two 1.5 mg doses of the corresponding construct, dissolved in 1 mL of saline solution and mixed with 1 mL of Montanide v206 adjuvant (Seppic), were administered at days 1 and 21 of the trial.



Construct	Type	B Epitope	T Epitope	Mass (Da)
1	B_4T	KEDFRYAISSTNEIGLLGA B-C domain of E2 (aa 694-712)	KHKVRNEVMVHWFGD non-structural protein NS2-3 (aa1446-1460)	11239.56
2	B_4	KEDFRYAISSTNEIGLLGA B-C domain of E2 (aa 694-712)	none	10316.45
3	B_4T	EIGLLGAEGLTTTW B-C domain of E2 (aa 706-719)	KHKVRNEVMVHWFGD non-structural protein NS2-3 (aa1446-1460)	9345.22
4	B_{4T}	EIGLLGAEGLTTTW B-C domain of E2 (aa 706-719)	NKYYEPRDSYFQQY C terminal end of E2 (aa 902-915)	9352.87

Fig. 1. Schematic representation of the dendrimeric constructs used in this study, including the sequences and the amino-3,6-dioxaoctanoic acid incorporated, for synthetic reasons, as a solubility-enhancing polyethyleneglycol-like spacer.

2.4. CSFV challenge and clinical signs score

An experimental challenge with CSFV was performed 15 days after the second immunisation. Thus, pigs were challenged with 10^5 $TCID_{50}$ of CSFV (strain Margarita) on day 36 of the trial by intramuscular (i.m.) injection in the neck region. This viral dose caused acute CSF and often induced death 10–15 days post-challenge (p.c.) (Tarradas et al., 2011). The rectal temperatures and clinical signs of the disease were recorded daily by a trained veterinarian in a blinded manner. The clinical signs that were compatible with a classical swine fever infection included anorexia, conjunctivitis, diarrhoea, constipation, abdominal petechiae and prostration. Moreover, the nervous symptoms were recorded independently. The clinical status of the animals was scored from 0 to 7, integrating fever, clinical signs (excluding nervous symptoms) and nervous symptoms, as follows: 0 (no signs); 1 (mild pyrexia); 2 (pyrexia + mild clinical signs); 3 (mild-moderate clinical signs, with an absence of nervous disorders); 4 (slight nervous disorders and moderate clinical signs); 5 (moderate nervous disorders and moderate-severe clinical signs); 6 (severe clinical signs [including nervous disorders]); and 7 (death). For ethical reasons, the animals were euthanised either when the clinical score reached a value of 5 or higher or when showing prostration behaviour and moderate-severe nervous disorders. After euthanasia, a necropsy was conducted to evaluate the presence of any pathological signs in various organs and tissues. The experiments were approved by the Ethics Committee for Animal Experiments of the Universitat Autònoma de Barcelona (UAB) according to existing national and European regulations.

2.5. Dendrimeric peptide-specific antibody detection

Dendrimeric peptide-specific antibodies in pig sera (IgG, IgM and IgA isotypes) were tested by indirect ELISA. Throughout the experiment, the antibody IgG response was measured in sera from the immunised and control groups before peptide immunisation, at 21 and 36 days p.i., and at 7 and 13 days p.c. Likewise, the IgM and IgA antibody responses were measured in the sera from immunised and control groups at 36 days p.i. (pre-CSFV challenge). Briefly, 5 μg/mL of the dendrimeric construct in sodium carbonate–bicarbonate buffer (0.05 M $NaHCO_3$, 0.05 M Na_2CO_3, pH 9.4) was coated overnight at 4 °C on high-binding Costar 3590 plates (Corning). After washing three times (0.05% Tween in PBS), free active sites were blocked using blocking buffer (0.5% bovine serum albumin [BSA] in sodium carbonate bicarbonate buffer) for 1 h. Fifty microlitres per well from each serum sample was pre-diluted for IgG detection (1:100 dilution) and for IgM and IgA detection (1:50 dilution) and incubated at 37 °C for 2 h. After further washing, 50 μL/well of anti-swine IgG peroxidase conjugate (1:20,000; Sigma–Aldrich) was added for IgG detection. For IgM or IgA detection, 50 μL/well were added of anti-swine IgM peroxidase conjugate (AbD Serotec, Oxford, UK) or anti-swine IgA peroxidase conjugate (AbD Serotec, Oxford, UK), respectively. Both conjugates were diluted to 1:5000 with blocking buffer, and plates were incubated at 37 °C for 1 h. The antibodies were detected with the HRP substrate 3,3′,5,5′-tetramethylbenzidine (Calbiochem). Finally, the reaction was stopped with 50 μL/well of 1 M sulphuric acid, and the absorbance was determined at 450 nm.

2.6. Neutralising and E2 specific antibodies detection

Serum samples were tested with a neutralisation peroxidase-linked assay (NPLA) (Terpstra et al., 1984), and the titres were expressed as the reciprocal dilution of serum that neutralised 100 $TCID_{50}$ of the strain Margarita in 50% of the two culture replicates. The presence of E2-specific antibodies was detected using a commercial ELISA (CEDITEST; Lelystad) following the manufacturer's recommendations.

2.7. Isolation of porcine PBMC and Elispot assay for the detection of CSFV-specific gamma IFN producing cells

Blood was drawn from pigs on day 36 (pre-challenge). This sample was collected in 5 mM EDTA, which was used to obtain peripheral blood mononuclear cells (PBMC) by density-gradient centrifugation using the Histopaque 1077 system (Sigma) (Tarradas et al., 2011). The Elispot assay for the detection of CSFV-specific gamma IFN producing cells was performed as previously described (Diaz and Mateu, 2005; Tarradas et al., 2011). Next, 5×10^5 live PBMC/well were plated in triplicates, stimulated either at 0.02 multiplicity of infection of CSFV Margarita strain or stimulated with 25 μg/mL of each dendrimeric construct. As controls, triplicates of cells were incubated in the absence of the virus (negative control) or with phytohaemagglutinin (PHA) (2 μg/mL). The frequencies of cytokine-producing cells were expressed as responding cells in 5×10^5 PBMC.

2.8. Lymphoproliferation assay

Purified PBMC were used to test the specific proliferative responses to CSFV. Proliferation assays of swine lymphocytes were performed as previously described (Ganges et al., 2005). Briefly, 2.5×10^5 live PBMC/well were plated in 96-well flat-bottomed plates, in RPMI–10% FBS. The triplicate wells of cells were stimulated with different doses of the CSFV strain Margarita for 5 days at 37 °C in 5% CO_2. As controls, the triplicates of cells were incubated in the absence of the virus (negative control) or with concanavalin A (2.5 μg/well). Five days after stimulation, the cell proliferation was quantified using a colorimetric ELISA that measures the incorporation of 5-bromo-2′-deoxyuridine (BrdU) during DNA synthesis in proliferating cells (Cell Proliferation Biotrak ELISA System, v2 [Amersham]). This ELISA was performed following the manufacturer's recommendations. The results were expressed as Δoptical density (ΔOD), which was calculated as the mean OD (from stimulated culture) – mean OD (from negative control culture, i.e., the medium alone). Positive results were considered with a value of 0.35 ΔOD.

2.9. RT-PCR

The presence of CSFV RNA in serum samples was analysed by real time (RT)-PCR (Hoffmann et al., 2005). This test is used in our laboratory for an inter-laboratory comparison of CSFV diagnosis organized by the EU Reference Laboratory. Positive results were considered for threshold cycle values (CT) equal or less than 42. Samples in which fluorescence was undetectable were considered as negative.

2.10. Statistical analysis

All statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). For all analyses, the pig was used as the experimental unit. The significance level (α) was set at 0.05, with statistical tendencies being reported when $p < 0.10$. A MIXED repeated measures model was used to analyse the effects of day of infection and experimental group (i.e., the control or the different dendrimeric construct groups) on the post-infection clinical scores. The same statistical method was also employed to evaluate the effects of day of immunisation and experimental group (i.e., the control or the different dendrimeric construct groups) on the dendrimeric peptide-specific antibody detection, taking into account the days post-challenge as a time covariate. Moreover, a non-parametric test (Mann–Whitney) was chosen to compare the immunological and clinical parameters between groups throughout the trial. Thus, the different constructs were always compared to the control group, as well as to each other, at each sampling point if possible. This non-parametric analysis was chosen due to the number of animals used in each experimental group.

3. Results

3.1. The presence of an NS3 T-cell peptide epitope in the dendrimeric constructs reduces the clinical score after a lethal challenge with CSFV

Fifteen days after the booster (i.e., 36 days after baseline) immunisation using dendrimer constructs, all pigs were challenged with a lethal dose of 10^5 $DICT_{50}$ CSFV strain Margarita. As previously described, for an infection with this virulent strain, the control animals (pigs 25–30) developed pyrexia (rectal temperature above 40 °C), which appeared at day 3 p.c. and showed peaks reaching above 42 °C (data not shown). Moderate and severe clinical signs of the disease (≥4 points in score value), such as anorexia, conjunctivitis, diarrhoea, constipation, abdominal petechiae, severe nervous signs, and prostration, were observed from day 5 p.c. in these pigs (Fig. 2). A post-mortem analysis of the non-vaccinated, challenged control pigs showed pathological lesions typical of CSFV infection, such as marginal spleen infarcts, hemorrhagic mesenteric and mediastinic lymph nodes, enteritis, kidney petechiae, central nervous system lesions, pulmonary oedema and hydrothorax (data not shown).

The clinical score observed after infection (y variable) was significantly affected ($p < 0.05$) by the day of infection, as well as by the experimental group (x variables), after carrying out a MIXED model procedure (see Section 2 for details). In general, the clinical scores in each of the immunised groups were lower than in the control group from day 4 p.c. to the end of the trial. The lowest clinical scores were observed in animals immunised with construct 1 (Figs. 1 and 2) with significant differences ($p < 0.05$) from day 6 to 10 relative to the other groups (Fig. 2). In pigs

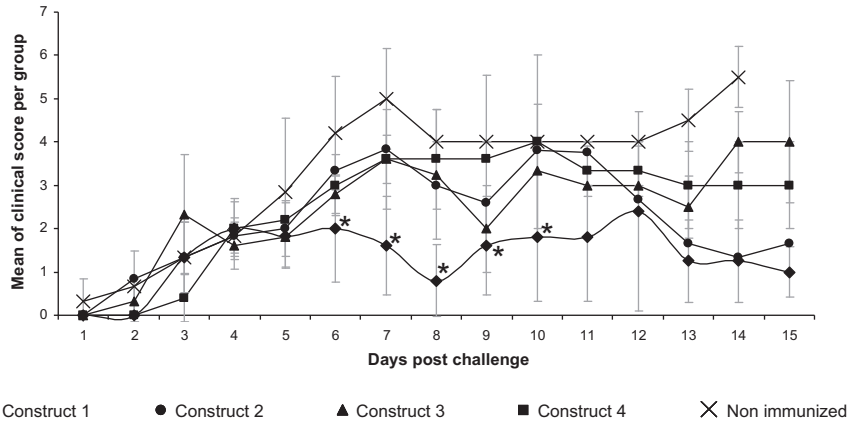


Fig. 2. Clinical scores after CSFV challenge. The mean of the clinical scores in the dendrimer-vaccinated and control groups after CSFV challenge. Asterisk indicates statistically significant differences between animals immunised with construct 1 and control pigs ($p < 0.05$). Scores are defined in Section 2.4. Values are expressed as the mean ± standard deviations.

immunised with constructs 2 (E2[694–712] B epitope, no NS3 T epitope), 3 (E2[706–719] B epitope, NS3 T epitope) or 4 (E2[706–719] B epitope, E2[902–915] T epitope), the clinical scores were also lower than those observed for control pigs; however, the differences were not statistically significant throughout the trial. Only at day 6 p.c. was a statistically significant trend ($p = 0.06$) observed towards lower scores with construct 3 versus the control (Fig. 2). The clinical scores observed in pigs immunised with construct 1 from day 10 p.c. until the end of the experiment were consistently lower than the values observed for the other groups (i.e., the control pigs and immunised with constructs 2–4); however, no statistical analysis could be performed at 13 days p.c. due to the low number of animals available at this time of the experiment (Fig. 2). Thus, pigs 1 and 3 (immunised with construct 1) were euthanised at 6 and 10 days p.c., respectively. Pigs 10, 11 and 12 (immunised with construct 2) were euthanised at 11 days p.c. Pigs 18, 16, 17 and 15 (immunised with construct 3) were euthanised at 6, 9 and 10 days p.c., respectively. Pigs immunised with construct 4, including 20 and 23, were euthanised at 8 days p.c., pig 19 was euthanised at 10 days p.c. and pig 24 died before the 36 days post-immunisation point. Pigs 27 and 29, from the control group, were euthanised at 6 days p.c., and finally, pigs 25 and 26 were euthanised at 11 days p.c.

3.2. The presence of an NS3 T-cell peptide epitope in the dendrimeric constructs enhances the specific antibody response

The IgG antibody response was measured in the sera of the immunised and control groups before peptide immunisation, at 21 and 36 days p.i., and at 7 and 13 days p.c. Likewise, IgM and IgA antibody responses were measured in the sera from immunised and control groups at 36 days p.i. (pre-CSFV challenge). As expected, the control pigs (25–30) did not develop detectable levels of peptide-specific antibodies throughout the trial (Fig. 3). In contrast, all animals immunised with construct 1 (Fig. 1) were positive for an IgG response at 21 days p.i. with the exception of pig 5, which became positive after the second immunisation at 36 days p.i. (Fig. 3a). In pigs immunised with construct 2 only, two animals (8 and 9) elicited peptide-specific IgG antibodies at 21 days p.i. with all animals measuring positive (OD above 0.2) at 36 days p.i. (Fig. 3b). Moreover, statistically significant differences were observed between constructs 1 and 2 versus controls at 21 and 36 days p.i. and at 7 days post-challenge. However, only a trend towards statistical significance was observed between construct 3 and controls at 21 and 36 days p.i. (Fig. 3c).

The IgG antibody titres that were observed after the infection (y variable) were significantly affected ($p < 0.05$) by the days post-immunisation, as well as by the experimental group (x variables), based on a MIXED model procedure (see Section 2 for details). The IgG antibody titres observed for construct 1 at 21 and 36 days p.i., as well as at 7 days p.c., were significantly higher than those detected for pigs in groups 2–4 ($p < 0.05$) (Fig. 3). At 13 days p.c., the titres of IgG for pigs in group 1 were also higher than those in the other groups; however, it was not possible to conduct any statistical analyses due to the low number of surviving animals.

In general, the IgG antibody response following the boost immunisation with construct 2 was more homogeneous and had higher titres compared to constructs 3 and 4; however, these differences were not statistically significant. On the other hand, in group 3, three and four animals out of six elicited specific IgG antibodies at 21 and 36 days p.i., respectively. In contrast, none and two pigs elicited antibodies in group 4 at 21 days p.i. and 36 days p.i., respectively.

The detection of IgM and IgA isotype antibodies at 36 days post-vaccination confirms the results obtained for the IgG isotype (Fig. 4). Thus, IgM and IgA titres were significantly higher for construct 1 than for the control group ($p = 0.005$ and $p = 0.01$ for IgM and IgA, respectively) However, only a statistical trend was observed for both IgM and IgA antibodies between the titres observed for construct

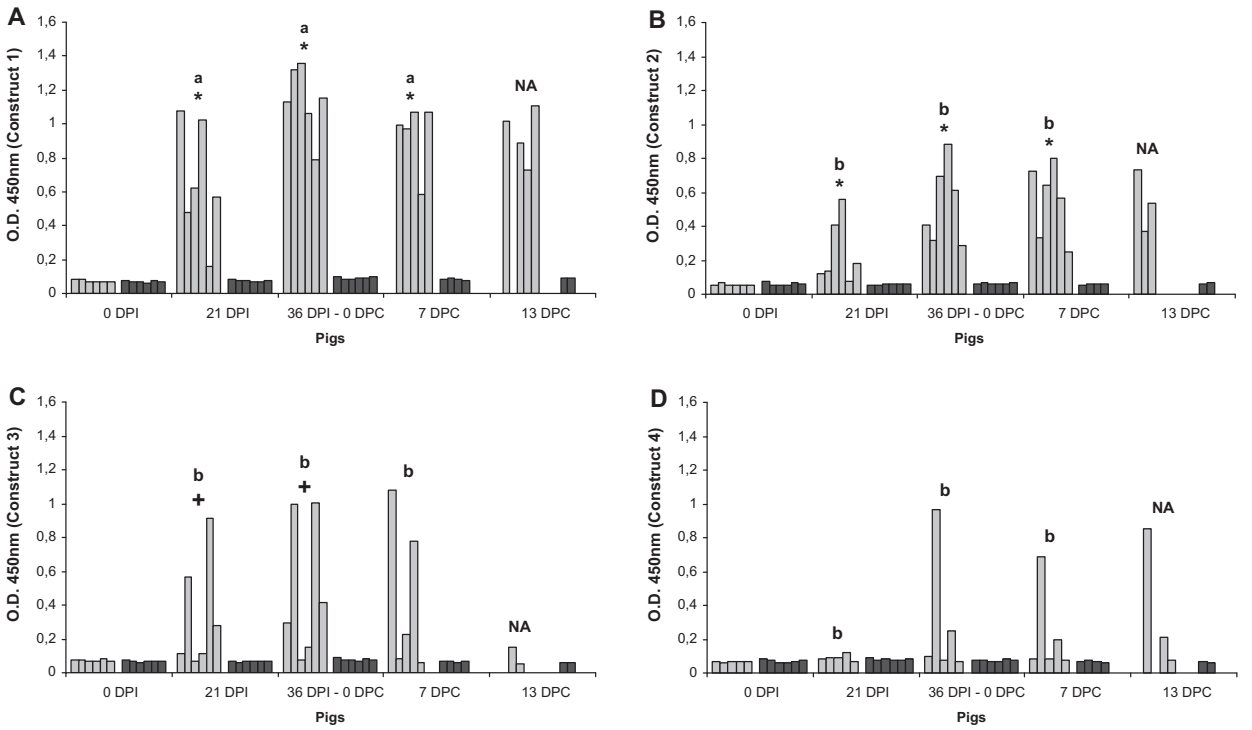


Fig. 3. Specific IgG antibody responses against the constructs following vaccination and CSFV challenge. (A) Induction of specific IgG antibodies against construct 1 in animals immunised with construct 1 (1–6). (B) Induction of specific IgG antibodies against construct 2 in animals immunised with construct 2 (7–12). (C) Induction of specific IgG antibodies against construct 3 in animals immunised with construct 3 (13–18). (D) Induction of specific IgG antibodies against construct 4 in animals immunised with construct 4 (19–24). Immunised animals were represented with grey bars and non-immunised pigs (25–30) with black bars. Animals not shown were euthanised earlier: pigs 1 and 3 (immunised with construct 1) were euthanised at 6 and 10 days p.c., respectively. Pigs 10–12 (immunised with construct 2) were euthanised at 11 days p.c. Pigs 18, 16, 17 and 15 (construct 3) were euthanised at 6, 9 and 10 days p.c., respectively. Pigs treated with construct 4 (20 and 23) were euthanised at 8 days p.c., pig 19 was euthanised at 10 days p.c. and pig 24 died before 36 days after immunisation. Finally, pigs from control group, including 27, 29, 26 and 25, were euthanised at 6 and at 11 days p.c., respectively. Asterisks or + symbols indicates significant differences ($p < 0.05$) or statistical trends ($0.05 < p < 0.10$) between immunised animals and those of the control group, respectively. Different letters at the different sampling times show statistically significant differences ($p < 0.05$) between constructs. NA: Statistical analysis at 13 days p.c. could not be performed due to the low number of animals available at this time of the experiment.

2 versus the control. Finally, IgM titre was significantly higher for construct 3 than for the control group ($p = 0.04$) (Fig. 4).

3.2.1. *Dendrimeric peptides that include a NS3 T epitope induced E2-specific antibodies and a neutralising antibody response at 13 days p.c. in surviving pigs.*

In samples collected at various p.i. and p.c. time points, ELISA detected E2-specific antibodies from 13 days p.c. At that time, animals showing slight clinical signs of CSFV infection were positive for E2-specific antibodies (construct 1: pigs 2, 4–6; construct 2: pigs 7–9; and construct 3: pigs 13 and 14). In contrast, the surviving pigs immunised with construct 4 and those from the control group were negative (Fig. 5).

As expected, the presence of neutralising antibodies correlated well with a decrease in clinical signs. Thus, pigs 4 and 5 (immunised with 1) and 9 (immunised with 2) showed protective levels (>1:32) of neutralising antibodies at 13 days p.c. (Fig. 5). In contrast, pigs 13–14 and 21–22 (immunised with 3 and 4, respectively) were negative. Finally, no neutralising antibodies were detected in the control animals at 13 days p.c. Again, it was not possible to carry out a statistical analysis of the antibody titres observed between the experimental groups due to the low number of animals available at 13 days p.c. (Fig. 5).

3.3. *Immunisation with dendrimeric constructs containing the NS3 epitope induces CSFV-specific IFN-gamma producing cells and lymphoproliferation response*

An Elispot assay was used to determine the number of IFN-gamma-producing cells in the PBMCs of pigs from the five groups in response to CSFV and the corresponding dendrimeric constructions. These analyses were performed after animals had been immunised twice with the constructs (36 days p.i.).

None of the control pigs (pigs 25–30) showed an induction of CSFV-specific IFN-gamma-secreting cells against the stimulus used (virus or peptide). On the contrary, the statistical analysis showed that the number of CSFV-specific IFN-gamma-producing cells in pigs immunised with construct 1 (1–6) was higher ($p < 0.05$) than in pigs from the

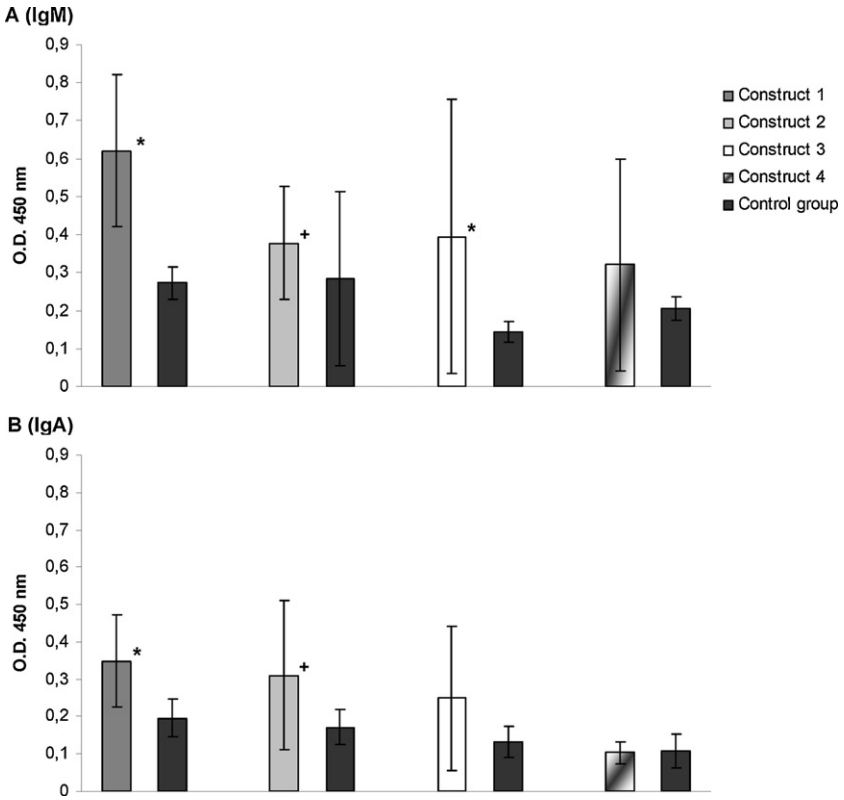


Fig. 4. Specific IgM and IgA antibody responses at 36 days post-vaccination. (A) IgM specific antibody induction at 36 days post-peptide immunisation. (B) IgA specific antibody induction at 36 days post-peptide immunisation. Values are expressed as the mean ± standard deviations, and different symbols have been used to clearly identify the experimental groups. Asterisks or + symbols indicate significant differences ($p < 0.05$) or statistical trends ($0.05 < p < 0.10$) between the immunised animals and those of the control group, respectively.

control group. Similarly, a statistical trend ($p = 0.06$) was identified between constructs 1, 2 and 4 (Fig. 6). Interestingly, the pigs immunised with construct 3 (T-cell NS3 epitope) showed a better response specific to CSFV compared to the pigs immunised with construct 4, although no significant differences were observed.

Similar results were obtained in the lymphoproliferation assay at 36 days p.i. after CSFV stimulation. Thus, the lymphocytes from pigs immunised with construct 1 generally showed a higher ΔOD in response to CSFV compared to the rest of the immunised animals (Fig. 7). In contrast, the non-vaccinated controls pigs showed ΔOD below 0.35

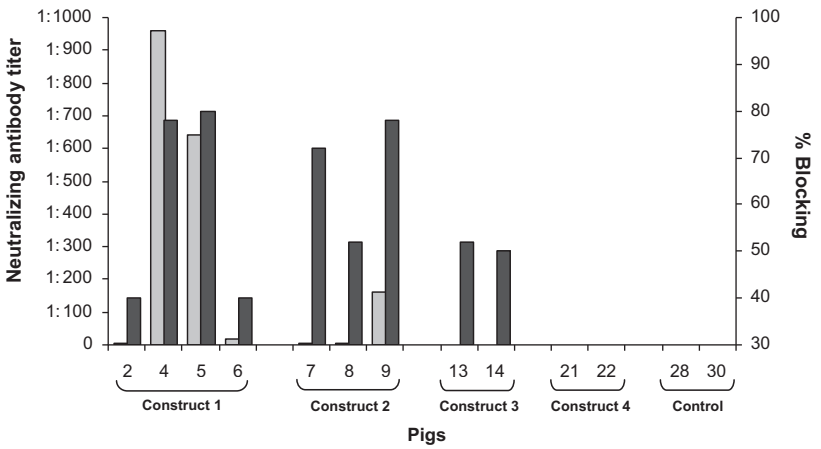


Fig. 5. Specific antibody response at 13 days post-challenge, against the E2 glycoprotein and neutralising antibody titres. Grey bars indicate neutralising antibody titres and black bars represent % blocking, which indicated the detection of E2 specific antibodies. Animals not shown were euthanised earlier, as detailed in Fig. 3.

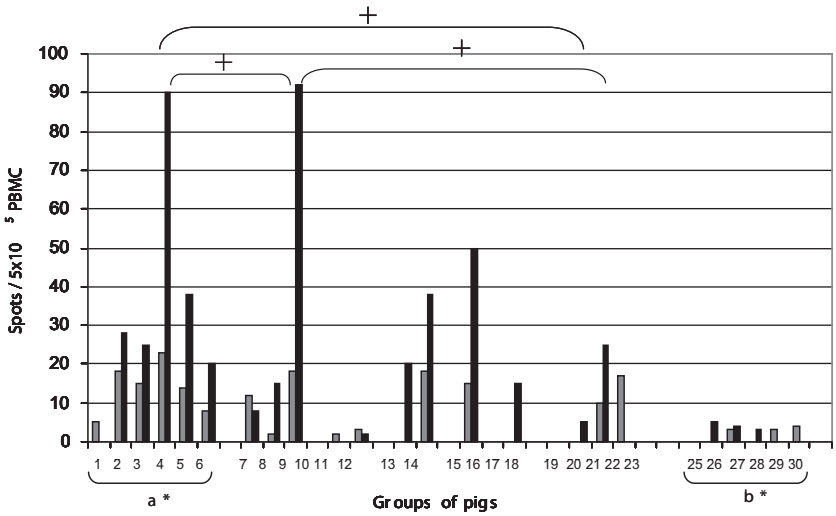


Fig. 6. Induction of CSFV-specific IFN-gamma producing cells at 36 days post-immunisation. Pigs 1–6 were immunised with construct 1, pigs 7–12 with construct 2, pigs 13–18 with construct 3, pigs 19–24 with construct 4 and pigs 25–30 were control animals inoculated with saline. PBMC were stimulated with either CSFV (grey bars) or the respective constructs (black bars). Values for the control pigs (25–30) were similar for all dendrimers tested (1–4). Asterisks indicate significant differences ($p < 0.05$) between construct 1 and control group, and + symbols indicate statistical trends ($0.05 < p < 0.10$) between immunised animals with different constructs.

(Fig. 7). Interestingly, statistically significant differences were only observed between the groups immunised with constructs 1, 2 and 4 compared with the control group. Moreover, a statistical trend was observed between the lymphocyte results from constructs 1 and 3.

3.4. Determination of RNA viral load after a challenge in survivor pigs

RT-PCR was performed on samples collected at different days p.c. (Table 1). Pigs immunised with construct 1 showed the lowest RNA viral load at 7 days p.c. (CT values from 23.5). Interestingly, these groups of pigs showed the lowest clinical score upon infection (Fig. 2). Thus, pig 4, which was immunised with construct 1 (Fig. 1) was negative for viral RNA detection at 13 days p.c., and pigs 5 and 6 showed moderate viral RNA loads (Table 1 and Fig. 2). In group 2, one pig had CT values above 30 at 13 days post-challenge, and this animal showed the lowest clinical score within this experimental group p.c. The other surviving pigs had CT values below 23, which correlated with high RNA viral load. For pigs immunised with constructs 3 or 4 and that survived to 13 days post-challenge, as well as for the non-immunised pigs, the CT values were in a range consistent with high RNA viral loads, and the highest clinical scores observed during the trial were in this group (score values from 3 to 6) (Table 1 and Fig. 2).

4. Discussion

The multiple antigen peptide strategy stands out for its ability to increase the immunogenicity of peptides. In

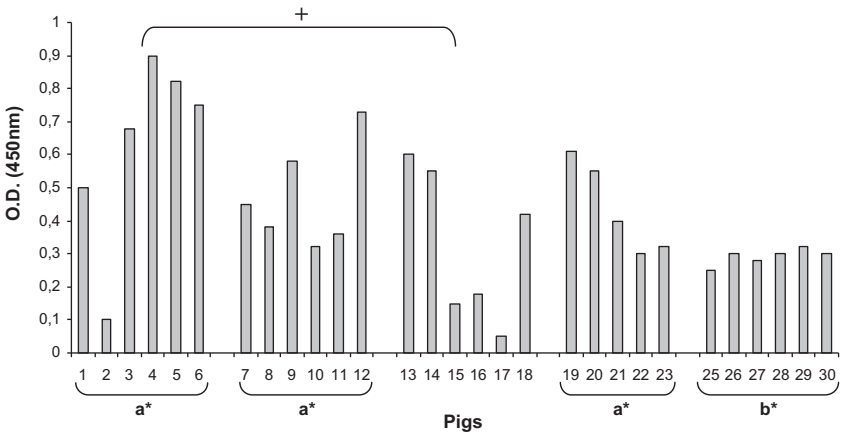


Fig. 7. Lymphoproliferation response against CSFV at 36 days post-immunisation in vaccinated and non-vaccinated animals. The results were expressed as Δoptical density (ΔOD), which was calculated as the mean OD from stimulated culture – mean OD from negative control culture (medium alone). Asterisks indicate significant differences ($p < 0.05$) between the immunised animals and those of the control group, and + symbol indicates statistical trending ($0.05 < p < 0.10$) between immunised animals across different constructs.

Table 1
Detection of CSFV RNA for RT-PCR in serum samples collected after CSFV challenge.

Inoculum	CT[b]		
	Pigs	7 days p.c.[a]	13 days p.c.[a]
Construct 1	1	–	–
	2	27.90	22.93
	3	25.93	–
	4	21.24	Negative
	5	28.28	31.40
	6	23.79	23.08
Construct 2	7	21.46	21.34
	8	25.26	22.77
	9	24.99	30.09
	10	22.18	–
	11	23.10	–
	12	23.07	–
Construct 3	13	24.08	22.29
	14	25.93	21.52
	15	24.20	–
	16	22.19	–
	17	22.86	–
	18	–	–
Construct 4	19	24.67	–
	20	23.01	
	21	21.11	22.5
	22	25.13	20.08
	23	23.24	–
PBS	25	23.04	–
	26	–	–
	27	–	–
	28	23.11	22.29
	29	–	–
	30	24.83	20.53

[a] The absence of CT values corresponds to pigs euthanised before sampling.
[b] CT values from 10 to 22 was considered as high, from 23 to 28 moderate and 29–42 as low RNA viral load.

fact, this strategy has been recognised as an effective tool to enhance peptide immunogenicity in the swine immune system (Cubillos et al., 2008; Li et al., 2011; Tarradas et al., 2011). Likewise, the possibility of combining different B and T epitopes in the same molecule facilitates the study of the mechanisms involved in the immune response (reviewed in Heegaard et al., 2009). Moreover, multiple antigen peptide configurations may protect certain residues from enzymatic cleavage, causing the epitopes to be processed and presented by antigen-presenting cells more efficiently (Sadler and Tam, 2002; Fischer et al., 2003; Li et al., 2011).

Our previous work (Tarradas et al., 2011) used three dendrimeric peptides as candidate vaccines against the classical swine fever virus, and this work demonstrated how these types of constructions can significantly reduce the clinical score after a lethal challenge with the virus. Conversely, the fact that the sera samples from pigs immunised with construct 1 (Tarradas et al., 2011) recognised the linear NS3 peptide by ELISA has supported the important role of the NS3 peptide in the immune response of domestic pigs. In the present work, we explore the immunogenicity of four dendrimeric constructions (Fig. 1) against CSFV in domestic pigs to evaluate the generation of the immune response against this virus. We focused our attention on the direct implication that the T-cell epitope, located on NS3 non-structural protein, confers cellular responses in miniature pigs (Armengol et al., 2002). Thus, four dendrimeric constructions have been designed and synthesised, which, at times, can include the NS3 T-cell epitope combined with different B epitopes in the same structure; all of these constructions have been previously reported to be in E2 and NS3 proteins (Yu et al., 1996; Armengol et al., 2002; Dong et al., 2006; van Rijn, 2007) (Fig. 1).

The six non-vaccinated control pigs developed typical clinical signs of the disease upon a CSFV challenge, which reached severe clinical scores (>5), and the afflicted animals were euthanised from day 6 to 12 p.c. In contrast, the clinical scores in each of the immunised groups were lower than in the control group, from day 4 p.c. to the end of the trial. The lowest clinical scores were observed for animals immunised with construct 1 (Figs. 1 and 2) with significant differences ($p < 0.05$) identified from day 6 to 10 relative to the other groups. In parallel, the results obtained through RT-PCR showed an association between the RNA viral load and the disease severity. Primarily, pigs immunised with construct 1 exhibited the lowest RNA viral load and the lowest clinical score values (Table 1 and Fig. 2). Previous work has suggested that the RNA CSFV load detected in the sera of infected pigs has a direct association with the clinical signs that are developed (Uttenthal et al., 2003; Weesendorp et al., 2009; Donahue et al., 2012).

The partial protection observed in pigs immunised with constructs 1–3 (Fig. 2) could be associated with the induction of peptide-specific antibodies after immunisation (Figs. 3 and 4). This inference is based on the group of pigs immunised with construct 1, which developed the highest antibody responses (IgG, IgM and IgA isotypes) against the dendrimeric peptide. These results corroborate our previous work (Tarradas et al., 2011) by demonstrating the ability of the dendrimer 1 to generate a humoural response. Likewise, upon CSFV challenge, pigs immunised with construct 1 were protected in terms of clinical signs (Fig. 2) and elicited higher levels of neutralising antibodies (1:950 and 1:600, respectively) (Fig. 5). These results also confirm the significance of generating a potent and specific memory B cell response after immunisation, upon which a CSFV challenge can render higher levels of neutralising antibodies that, in turn, confer viral protection (Ganges et al., 2005; Busquets et al., 2010).

In comparison to construct 1 (B_4T), the construct 2 (B_4 lacking "T" NS3 epitope) (Fig. 1) induced lower levels of dendrimeric-specific antibodies (IgG, IgM and IgA isotypes) (Figs. 3–5). These lower antibody levels may reflect the higher clinical scores observed in these groups after challenge (Fig. 2), as well as the lower levels of neutralising antibodies and cellular responses detected in these animals at 13 days p.c. (Figs. 5–7 and Table 1). Similarly, the superiority of the B_4T over the B_4 structure (which lacks the NS3 epitope) could confirm the role of the NS3 T cell epitope in enhancing the immune response against CSFV in domestic pigs, as had been previously suspected (Tarradas et al., 2011). Furthermore, when comparing the constructs 4 and 3 (the compositions of which differ only on the "T" epitope of B_4T structure [Fig. 1]), construct 3 induced higher

levels of antibodies against the dendrimeric construct and provided improved levels of protection (Figs. 5–7).

We previously showed that the immunisation of pigs with a plasmid-expressing E2 glycoprotein induced IFN-gamma-secreting cells and specific lymphoproliferation in response to post-vaccination CSFV. These responses correlated with protection against CSFV challenges (Ganges et al., 2005; Tarradas et al., 2010). Such a response and the partial protection it confers was also observed in pigs immunised with construct 1 and, to a lesser extent, in pigs immunised with constructs 2 and 3. Therefore, upon the in vitro stimulation with its corresponding peptide or with CSFV, the primed T cells from pigs in groups 1, 2 and 3, respectively, released IFN-gamma and exhibited a lymphoproliferative response at 36 days p.i. (Figs. 6 and 7).

The co-immunisation of pigs with NS3 protein seems to increase the induction of specific antibodies elicited by an E2 subunit vaccine; however, the specific role in protection played by the NS3 protein remains unknown (Dong and Chen, 2006a). Moreover, our results suggested that the incorporation of the T cell epitope NS3 (Armengol et al., 2002) is beneficial. This benefit may reflect the potentiated induction of IFN-gamma in animals immunised with the dendrimeric constructions tested in this study when including this epitope in its composition. Thus, in synergy with the induction of neutralising antibodies, the T cell epitope NS3 seems to be beneficial for the recovery of diseased animals.

In summary, our results confirm that the T cell epitope in the NS3 peptide can be recognised as a CSFV-specific T-cell epitope in domestic pigs. Therefore, the inclusion of this NS3 epitope in immunisation strategies, such as the dendrimers used in this study, may potentiate the immune response elicited against CSFV. This augmented response may subsequently facilitate a higher induction of peptide-specific antibodies in vivo, as well as an improved detection of peptide-specific antibodies by ELISA. This information might also be critical for developing future serological diagnoses against CSFV. In addition, these results raise the possibility that the dendrimeric peptide strategy may be used to study the immune response provided by different epitopes of CSFV, as well as other pathogens, in domestic pigs.

Conflict of interest

None declared.

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