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Partial protection against classical swine fever virus elicited by dendrimeric vaccine-candidate peptides in domestic pigs

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ABSTRACT

We report the immunogenicity of three dendrimeric peptide vaccine candidates for classical swine fever virus (CSFV). Each dendrimeric construct contained four copies of a B-cell epitope from the E2 glycoprotein of CSFV [construct 1: E2 (694–712); 2: E2 (712–727); 3: E2 (829–842)] joined to a T-cell epitope from the NS3 protein (residues 1446–1460). Intramuscular immunization of domestic pigs with the different constructs significantly reduced the clinical score after lethal challenge with CSFV. In contrast, control pigs developed severe clinical signs of the disease. All pigs vaccinated with construct 1, containing a B-cell epitope from the E2 B–C domain, developed an antibody response that recognized not only the original dendrimeric immunogen but also its constituting E2 epitope in linear form, albeit no neutralizing antibodies were detected prior to viral challenge. Two of these pigs were partially protected, which associated with the induction of IFN-γ producing cells and of neutralizing antibodies upon challenge. Interestingly, the serological response elicited by construct 1 lacked antibodies to E2 A domain, used as infection markers. The dendrimeric approach could therefore provide a basis for the development of CSFV marker (DIVA) vaccines, and contribute to a better understanding of the immune responses against CSFV.

1. Introduction

Classical swine fever (CSF) is one of the most devastating diseases for the pig industry throughout the world (reviewed in [1]). The disease is endemic in Asia, areas of Central and South America and in many East European countries [2,3]. CSF virus (CSFV), the etiological agent of CSF, is an icosahedral and enveloped positive strand RNA virus that belongs to the Pestivirus genus of the Flaviviridae family [4]. Pestivirus RNA contains a single large open reading frame (ORF) flanked by two untranslated regions (UTRs). The ORF encodes a polyprotein of about 3900 amino acids that in infected cells is processed by cellular as well as viral proteases to yield four structural (C, Erns, E1, E2) and eight nonstructural proteins (Npro, P70, NS2, NS3, NS4A, NS4B, NS5A, NS5B) [5].

A progressive CSF eradication program has been implemented in the European Union (EU) since the early 1990s. Oral vaccination of wild boar population is currently enforced for disease control in some EU countries [6]. However, emergency marker vaccines, namely those allowing the differentiation of vaccinated from infected animals (DIVA), are still required (reviewed in [7]) for ethical, welfare and economical reasons, since the modified live vaccines hitherto available do not ensure such differentiation [7]. Despite considerable efforts towards the development of a CSFV vaccine, new strategies are still needed that (i) confer protection even when no neutralizing antibodies are detected, (ii) allow differentiation of infected from vaccinated animals, and (iii) fulfil safety requirements (reviewed in [3,7]).

Neutralizing antibodies specifically recognize the structural E^{rns} and E2 proteins [8,9] and their presence has been associated with protection [10]. Antibodies against the NS3 protein are also induced upon infection but, despite their ability to recognize different pestiviruses, they do not neutralize the virus [11]. E2 envelope glycoprotein is the major target for neutralizing antibo-

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dies during natural CSFV infection [10,12,13] and it has been the main component in the design of CSFV DIVA vaccines [1,3,7]. Co-immunization of pigs with the NS3 protein seems to increase the induction of specific antibodies elicited by an E2 subunit vaccine, even though the role in protection of NS3 protein remains unknown [14].

Synthetic peptides are promising candidates for the control of viral diseases by vaccination, as they pose no risk for pathogen replication and, if appropriately formulated, they may function as DIVA vaccines [15]. Several immunogenic linear peptides corresponding to different regions of the A or BC domains of E2 have been proposed and used as vaccines against CSFV in either mono- or multi-peptide formulations [15–20]. Some mixtures of linear peptides have been reported to induce CSFV-specific neutralizing antibodies as well as protection against CSFV challenge infection. However, in most cases they failed to confer complete protection from clinical signs upon viral challenge [15,16]. On the other hand, little information is available on the effect of these peptide vaccines on the levels of viremia and virus shedding [15,16].

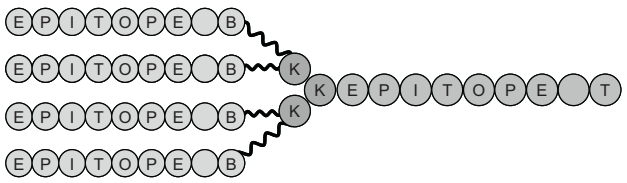
Dendrimers are recognized as having desirable features for multimeric presentation of epitopes in candidate vaccines [21]. Multimerization is a nature-mimicking strategy of antigen presentation that has proven successful in the development of human-made vaccines, particularly by means of dendrimeric (e.g., branching) designs [22]. Numerous reports on the immunogenicity of dendrimers have been published but only a few in vivo therapeutic studies have been reported [23]. Furthermore, this strategy allows the possibility of combining different epitopes (B and T) to potentiate the immune response elicited and it can also be useful for basic investigations of the mechanisms governing the induction and control of immunity (reviewed in [21]).

Combination of B- and T-cell epitopes in dendrimers has been shown to confer solid protection against challenge with foot-and-mouth disease virus (FMDV) in domestic pigs [24]. In this work, we have extended this approach to explore novel CSFV marker vaccines. To this end, each of three different B-cell epitopes from the B/C (constructs 1 and 2 [17]) and A domains (construct 3 [20]) of E2 glycoprotein was combined with a T cell site from the NS3 protein [25]. Dendrimers were used to immunize pigs that were subsequently challenged with a lethal dose of CSFV. The partial protection and the antibody response elicited point to construct 1 as a CSF DIVA vaccine candidate.

2. Materials and methods

2.1. Synthesis of dendrimeric peptides

The peptide constructs were designed to display on a single platform four copies of B-cell epitopes. The following residues from the Margarita strain (AJ704817) E2 glycoprotein were included: construct 1 (K^{694}EDYRYAISSTNEIGLLGA^{712})$_4$ corresponding to the B-cell epitope in E2 B–C domain [17], construct 2 (A^{712}EGLTTTWKDYDHNLQ^{727})$_4$ corresponding to the B-cell epitope in E2 B–C domain [17], construct 3 (T^{829}AVSPTTLRTEVVK^{842})$_4$ corresponding to the B-cell epitope in E2 A domain [20], and were joined to a T-cell epitope from the NS3 protein (K^{1446}HKVRNEVMVHWFGD^{1460})$_4$ [25] through a Lys tree plus two additional Lys residues defining a putative cleavage site for cathepsin D (Fig. 1) [26]. Synthetic aspects of MAP assembly and purification have been recently reported in detail [26]. A particular feature of the present constructs was the insertion of polyethyleneglycol (PEG)-like amino-3,6-dioxaoctanoic acid (O_2Oc) units between the Lys core and the B epitopes, in order to overcome the poor water solubility of an initial generation of more canonical MAP constructs. This modification turned out to be crucial for easy handling and dosage of the peptide immunogens, which after assembly by standard Fmoc protocols [26,27] were purified to satisfactory purity levels and characterized as the target molecules by MALDI-TOF mass spectrometry.



Construct	B Epitope	T Epitope
1	DEDRYAISSTNEIGLLGA	
2	AEGLTTTWKDYDHNLQ	KHKVRNEVMVHWFGD
3	TAVSPTTLRTEVVK	

Polyethyleneglycol-like amino-3,6-dioxaoctanoic acid

Fig. 1. Schematic representation of the dendrimeric constructs used in this study. The sequences of the peptides included in each construct and polyethyleneglycol-like amino-3,6-dioxaoctanoic acid (link between lysine residues and B cell epitopes) are shown.

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. Cells were infected with 0.1 $TCID_{50}$/cell in 2% FBS and virus was harvested 48 h later. A peroxidase-linked assay (PLA) [28] was used for viral titration, following the statistical method described by Reed and Muench [29].

2.3. Immunization of pigs

To evaluate the immune response induced by the dendrimeric constructs, 18 pigs (Landrace × Large white, 8 weeks old; numbered from 1 to 18) were used. Pigs 1–6 were immunized with construct 1, pigs 7–12 with construct 2 and pigs 13–18 with construct 3. As control, 6 additional pigs were inoculated with saline solution plus adjuvant (numbered 19–24). Two doses of 1.4 mg of the corresponding construct dissolved in 1 mL of saline solution and mixed with 1 mL of Montanide v206 adjuvant (kindly provided by Seppic, France) were administered at days 1 and 21 post-infection (p.i.). Challenge with virus was performed 15 days after the second dose. Intramuscular (i.m.) injection in the neck region was used for all inoculations.

2.4. CSFV challenge and clinical signs score

On day 36 p.i., pigs were challenged with 10^5 $TCID_{50}$ of CSFV (strain Margarita), a viral dose causing severe clinical signs often including death after 10–15 days [30,31]. Both rectal temperature and external clinical signs of disease were recorded daily. Clinical signs were scored from 0 to 7 as follows: 0: no signs; 1: mild pyrexia; 2: pyrexia plus mild clinical signs; 3: mild-moderate clinical signs and absence of nervous disorders; 4: slight nervous disorders and moderate rest of clinical signs; 5: moderate nervous disorders and moderate–severe rest of clinical signs; 6: severe clinical signs (including nervous disorders); 7: death. For ethical reasons, animals were euthanized either when the clinical score reached 5 or higher, or when showing prostration and moderate–severe nervous disorders. After euthanasia, an exhaus-

tive necropsy was conducted in which the presence of pathological signs in different organs and tissues was evaluated.

2.5. Neutralizing assay and E2 specific antibody detection

Serum samples were tested by using a neutralization peroxidase-linked assay (NPLA) [32] and titers were expressed as the reciprocal dilution of serum that neutralized 100 $TCID_{50}$ of strain Margarita in 50% of the two culture replicates. On the other hand, the presence of E2 specific antibodies was detected using a commercial ELISA (CEDITEST; Lelystad); following the manufacturer's recommendations; samples were positive for blocking percentages ≥ 40%.

2.6. Dendrimeric- and linear peptide-specific antibody detection

Dendrimeric and linear peptide-specific antibodies in pig sera were tested by an indirect ELISA. Fifty μL of linear peptide (10 μg/mL) or dendrimeric construct (5 μg/mL) in sodium carbonate–bicarbonate buffer (0.05 M $NaHCO_3$, 0.05 M Na_2CO_3, pH 9.4) were coated overnight at 4 °C on high-binding Costar 3590 plates (Corning). After washing three times (0.05% Tween 20 in PBS), free active sites were blocked using blocking buffer (0.5% bovine serum albumin (BSA) in sodium carbonate bicarbonate buffer) during 1 h. Fifty μL/well from each serum sample (pre-diluted 1:100 in blocking buffer), was incubated at 37 °C for 2 h. After further washing, 50 μL/well of anti-swine IgG peroxidase conjugate (Sigma–Aldrich) diluted 1:20,000 in blocking buffer, was added and plates were incubated at 37 °C for 1 h. The amount of coupled conjugate was determined by incubation with 50 μL/well of soluble 3,3′,5,5′-tetramethylbenzidine (Calbiochem) for 5 min. at room temperature. Finally, the reaction was stopped with 50 μL/well of 1 M sulphuric acid and the absorbance was determined at 450 nm.

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

Pigs were bled on day 36 (pre-challenge) and 13 days post-challenge (p.c.). Blood collected in 5 mM EDTA was used to obtain peripheral blood mononuclear cells (PBMC) by density-gradient centrifugation with Histopaque 1077 (Sigma). The total number of live PBMC recovered was estimated by trypan-blue staining. PBMC were cultured in RPMI, 10% FBS, 20 mM L-glutamine (Sigma). Elispot assay for detection of CSFV-specific gamma IFN producing cells was performed as described [33]. 5×10^5 live PBMC/well was plated in triplicates stimulated at 0.02 multiplicity of infection of CSFV Margarita strain or stimulated with 25 μg/mL of each dendrimeric construct (Fig. 1). As controls, triplicate of cells were incubated in the absence of virus (negative control), or with phytohaemagglutinin (PHA) (2 μg/mL). Frequencies of cytokine-producing cells were expressed as responding cells in 5×10^5 PBMC.

2.8. RT-PCR

The presence of CSFV RNA in serum samples was analyzed by real time (RT)-PCR [34]. 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. CT values from 10 to 22 were considered as high; from 23 to 28 as moderate and 29–42 as low RNA viral load, according to [34].

2.9. Statistical analysis

The Mann–Whitney test was used to compare parameters among the groups of animals immunized with the different dendrimeric constructs and the control animals. This non-parametric analysis was chosen due to the number of animals used. For statistical analyses, the NCSS 2004 program (Kavysville, Utah, USA) was used. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Immunization with dendrimeric peptides reduces the clinical score after lethal challenge with CSFV

Fifteen days after the second dendrimer dose (36 days p.i.) all pigs were challenged with a lethal dose, 10^5 $TCID_{50,}$ of CSFV strain Margarita. As previously described for an infection with this virulent strain, control animals (pigs 19–24) developed pyrexia (rectal temperature above 40 °C), which appeared at day 3 p.c. and showed peaks reaching above 42 °C. 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. 2e). Post-mortem analysis of 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).

In contrast, the clinical score observed in the animals immunized with constructs 1–3 was lower than that of control pigs throughout the experiment. This reduction was statistically significant for all three dendrimers studied ($p < 0.05$) from day 4 to day 7 p.c., with the exception of construct 3 at day 7 ($p = 0.07$) (Fig. 2a). After day 7 p.c., four animals from the group immunized with construct 1 (pigs 2, 3, 4 and 5), five from the group immunized with construct 2 (pigs 7–11) and five from the group immunized with construct 3 (pigs 13, 14, 16, 17 and 18) developed moderate or severe clinical signs (Fig. 2b–d). The clinical scores observed for the peptide-vaccinated groups from day 7 onwards were consistently lower than those for control pigs; however, no statistical analysis could be performed due to the low number of animals available at this time of the experiment. On the other hand, pigs 1 and 6 (immunized with construct 1) showed transient peaks of pyrexia and slight anorexia, with an average clinical score ≤ 1 throughout the experiment, suggesting that these animals were partially protected (Fig. 2a and b). Similarly, pigs 12 (immunized with construct 2) and 15 (immunized with construct 3) showed mild clinical signs during the experiment with score values of 2 at 13 days p.c. (Fig. 2c and d).

3.2. Immunization with dendrimers induces peptide-specific antibodies

To analyze the immunogenicity of the dendimeric constructs used in this study, we developed an indirect ELISA for detecting specific antibodies elicited by each of them (see Section 2). As expected, no detectable levels of peptide-specific antibodies were found in sera of control pigs (19–24) (Fig. 3). In contrast, all animals immunized with construct 1 were positive at 14 days p.i., except pig 1, which became positive only after the peptide boost at 36 days p.i. (Fig. 3a). In pigs immunized with construct 2, only two animals (7 and 11) elicited specific antibodies, starting at 36 days p.i. (Fig. 3b). Three pigs immunized with construct 3 were also found to produce

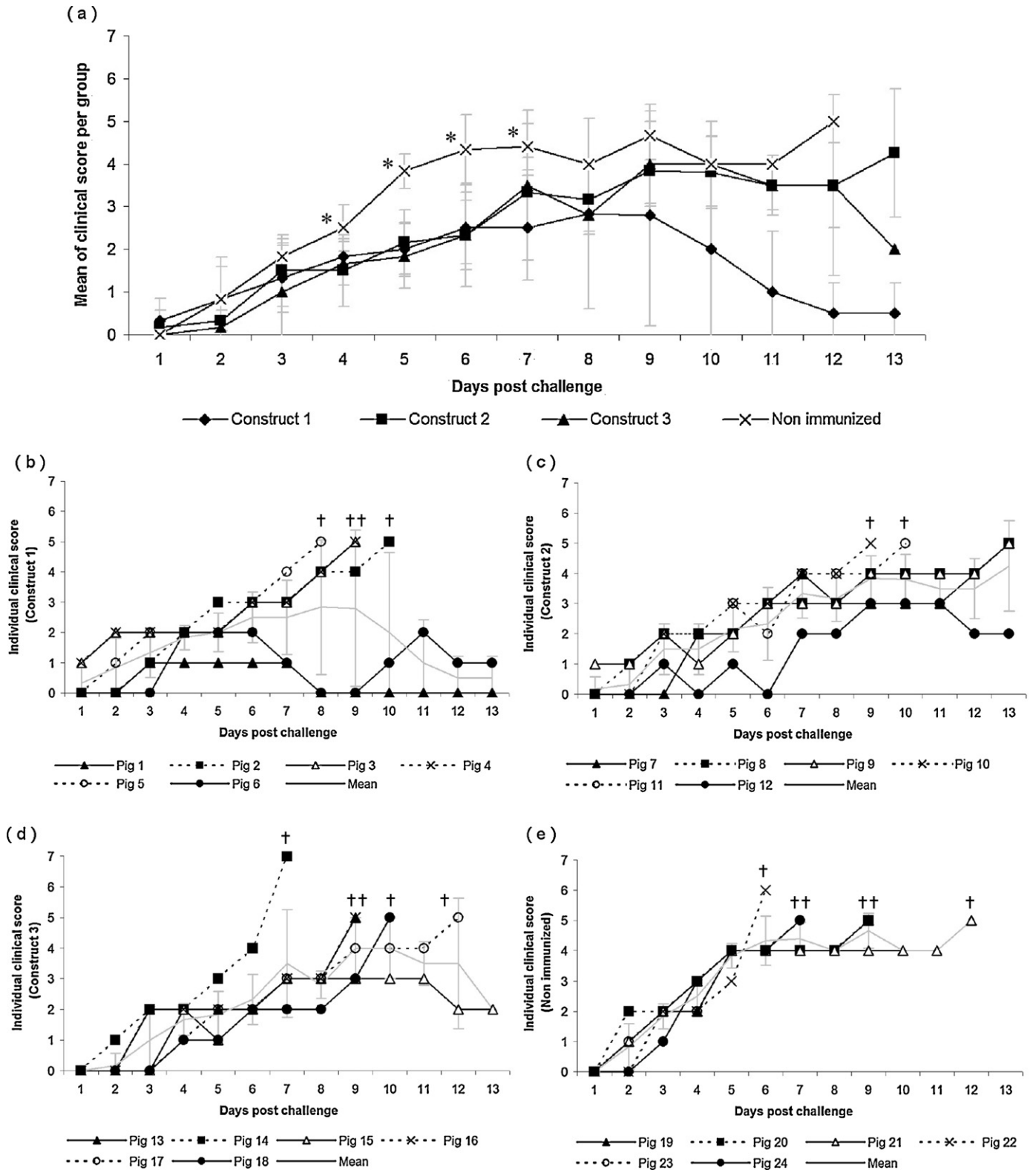


Fig. 2. Clinical scores after CSFV challenge. (a) Mean clinical scores in the dendrimer-vaccinated and control groups after CSFV challenge. Asterisk indicates statistical significance between dendrimer-immunized and control groups ($p < 0.05$) with the exception of construct 3 at day 7. Clinical signs of CSF in pigs inoculated with (b) construct 1 (animals 1–6), (c) construct 2 (7–12), (d) construct 3 (13–18), and (e) clinical signs of CSF in control pigs (19–24) given saline and adjuvant. The symbol † indicates euthanasia for humane reasons or natural death. The mean clinical score for each group is also shown with a gray line in graphs with individual scores. Scores as defined in Section 2.4.

specific antibodies, two of them (pigs 14 and 17) at 21 days p.i. and the third (pig 13) at 36 days p.i. (Fig. 3c).

We then investigated whether antibodies elicited against the dendrimeric constructs retained the capacity to bind the linear peptides displayed on the constructs and reproducing the CSFV epitopes. To this end, indirect ELISAs to detect the corresponding peptide-specific antibodies were developed (see Section 2). Specific antibodies to NS3-epitope were found in four animals immunized

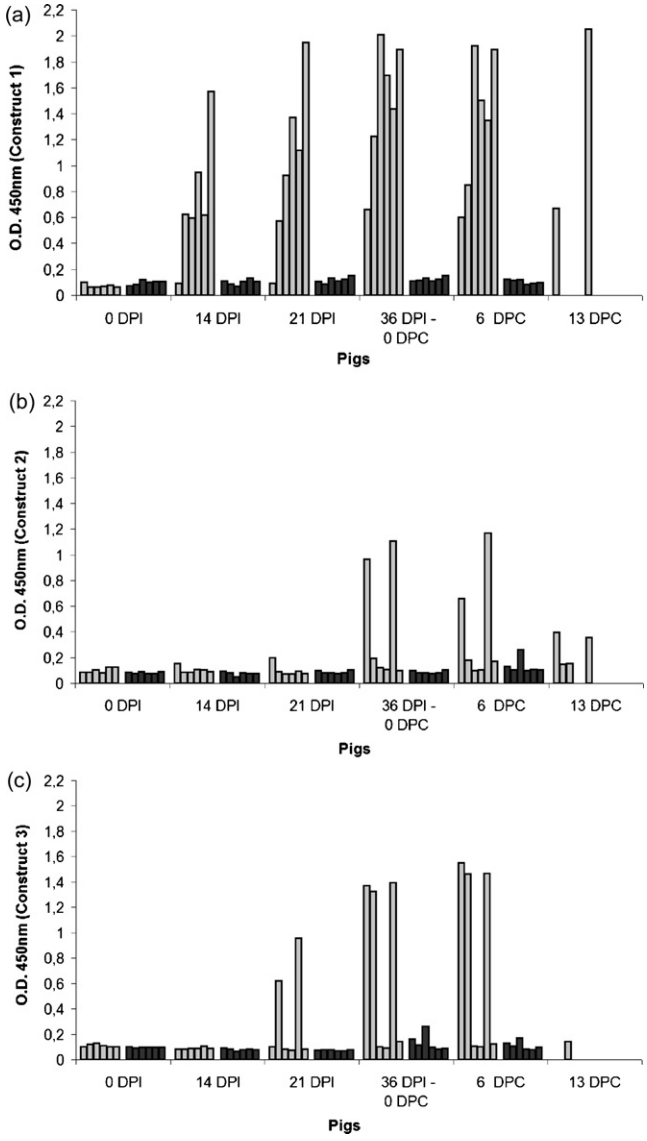


Fig. 3. Specific antibody responses against constructs after vaccination and CSFV challenge. (a) Induction of specific antibodies against construct 1 in animals immunized with construct 1 (pigs 1–6). (b) Induction of specific antibodies against construct 2 in animals immunized with construct 2 (pigs 7–12). (c) Induction of specific antibodies against construct 3 in animals immunized with construct 3 (pigs 13–18). Immunized animals were represented with gray bars and non-immunized pigs (19–24) with black bars. Animals not shown (13 days p.c.) were euthanized earlier.

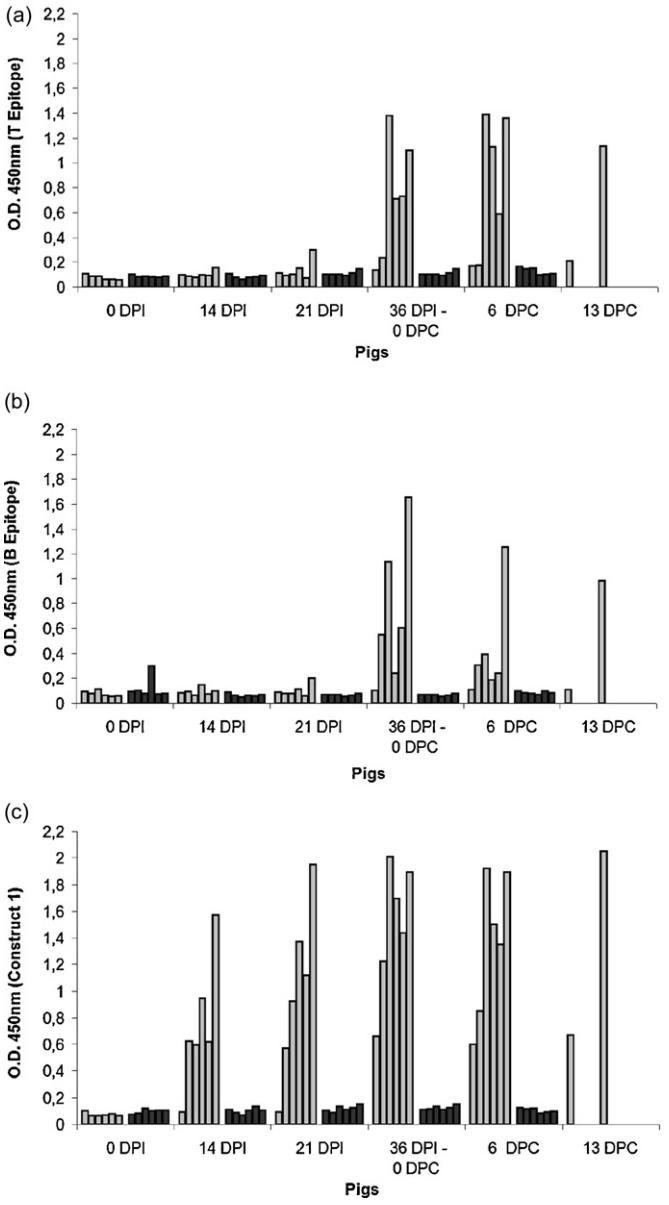


Fig. 4. Specific antibody responses against construct 1 and the linear peptides thereof, after immunization (DPI) and CSFV challenge (DPC). Pigs 1–6 were immunized with construct 1 (gray bars). Animals 19–24 were not immunized (black bars). (a) Level of specific antibodies against NS3 T-epitope. (b) Level of specific antibodies against B-epitope (E2 residues 694–712). (c) Level of specific antibodies against construct 1. Only pigs 1 and 6 are shown after 13 days p.c.; other animals were euthanized earlier.

with construct 1 (pigs 3, 4, 5 and 6) from 36 days p.i. until the end of experiment at 13 days p.c. (Fig. 4a). Also, in the same interval of time, pigs 2, 3, 5 and 6 showed specific antibodies against the B epitope included in this construction (E2 residues 694–712) (Fig. 4b). At day 14, 21 and 36 p.i., the antibody levels to the dendrimeric construct were significantly higher ($p < 0.05$) than those found for the monomeric B- and T-epitopes (Fig. 4c). However, at day 6 p.c., the antibody titers against the dendrimeric construct were similar to those against the NS3 epitope. No significant differences were observed between antibody levels determined using either B- or T-linear lineal peptides as plate antigen. On the contrary, the presence of specific antibodies against the linear epitopes in the groups of animals immunized with construct 2 or 3 was negative during the trial (data not shown).

3.3. Dendrimeric peptides induce E2-specific antibodies and neutralizing activity after challenge

Determining the specific antibody response against E2 glycoprotein and the neutralizing antibodies elicited in the immunized pigs is essential for understanding the mechanisms involved in protection against CSFV infection. To this end, samples from immunized animals collected before challenge at 0 and 21 days p.i. and at 36 days p.i., and at 6 and 13 days p.c. were analyzed for the presence of either E2-specific antibodies in ELISA or for neutralizing antibodies by NPLA (see Section 2). None of these antibodies were detected before 13 days p.c. (data not shown). At that time, the animals showing no or small clinical signs of CSFV were positive for E2-specific antibodies in ELISA (pigs 1, 6, 12 and 15) (Fig. 5). Likewise, detection of neutralizing antibodies correlated with decreased clinical

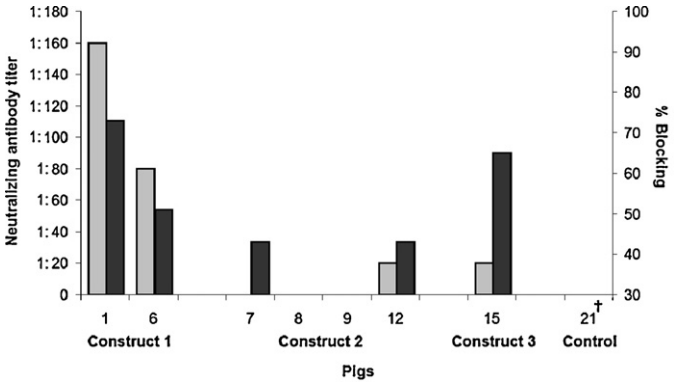


Fig. 5. Specific antibody response against E2 glycoprotein and neutralizing antibody titers at 13 days post challenge. Pigs 1 and 6 were immunized with construct 1, pigs 7, 8, 9 and 12 with construct 2, and pig 15 with construct 3. Control pig 21 inoculated with saline. † Serum from pig 21 collected at 12 days p.c. Gray bars indicate neutralizing antibody titers and black bars % blocking. Animals not shown were euthanized earlier.

signs. Thus, pigs 1 and 6 (immunized with construct 1), showed levels (>1:32) of neutralizing antibodies at day 13 p.c. higher than those observed for pigs 12 and 15 (immunized with constructs 2 and 3, respectively) (1:20). On the contrary, the only animal in the control group that survived until day 12 p.c. was negative for both E2-specific and neutralizing antibodies (Fig. 5).

3.4. Peptide immunization induces CSFV-specific IFN-gamma producing cells

To assess whether dendrimer immunization elicited specific T cell responses, an Elispot assay was used to determine the number of IFN-gamma-producing cells in PBMC stimulated with either CSFV or the dendrimeric constructs (Fig. 6). When average values from each of the immunized groups were compared before challenge, the only statistically significant difference, relative to control group, was found for pigs immunized with constructs 2 and 3 stimulated with whole virus ($p = 0.004$ for both). In the group of pigs immunized with construct 1, and despite two partially protected animals (pigs 1 and 6) showing high titers in response to virus and dendrimer, differences were not significant ($p = 0.06$), probably due to the high variation in response observed between animals (three negatives out of six and two pigs with high values) After CSFV challenge (13 days p.c.) pigs 1 and 6 showed a higher frequency of CSFV-specific IFN gamma-producing cells. At this time, the small number of animals prevented statistical analysis between groups (Fig. 6).

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

Inoculum	CT[b]		
	Pigs	6 days p.c.	13 days p.c.[a]
Construct 1	1	27.4	Negative
	2	24.6	
	3	23.7	
	4	24.1	
	5	23.3	
	6	23.6	28.0
Construct 2	7	27.3	22.4
	8	27.3	21.3
	9	23.8	20.5
	10	23.0	
	11	28.5	
	12	27.3	27.4
Construct 3	13	24.2	
	14	23.2	
	15	23.3	24.2
	16	25.9	
	17	29.3	
	18	28.1	
PBS	19	23.4	
	20	27.9	
	21	23.3	19.1[c]
	22	24.7	
	23	27.2	
	24	25.9	

[a] The absence of CT values corresponds to pigs euthanized before 13 days p.c.
[b] CT values from 10 to 22 was considered as high; from 23 to 28 moderate and 29–42 as low RNA viral load (Section 2).
[c] Serum sample from pig 21 was collected at 12 days p.c.

3.5. Determination of viral RNA after challenge

A real time RT-PCR was performed in serum samples collected at different days p.c. to assess whether RNA viral load correlated with the severity of the clinical symptoms developed upon infection (Table 1 and Fig. 2). At day 6 p.c. sera from all dendrimer-immunized

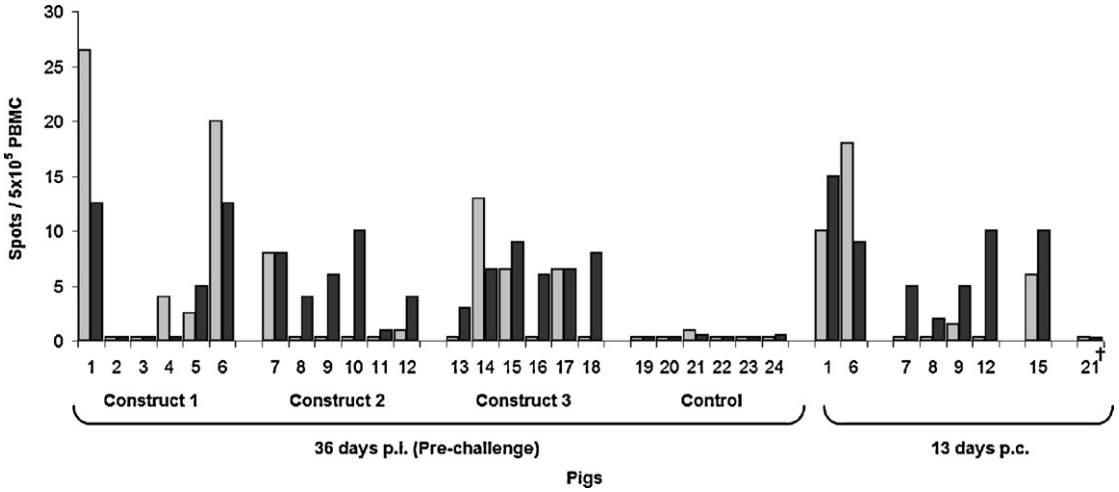


Fig. 6. Induction of CSFV-specific IFN-gamma producing cells in vaccinated and non-vaccinated animals. Pigs 1–6 were immunized with construct 1, pigs 7–12 with construct 2 and pigs 13–18 with construct 3. Control pigs (19–24) were inoculated with saline. PBMC were stimulated with either CSFV (black bars) or the respective construct (gray bars). Values for control pigs (19–24) are similar for any dendrimer tested (1–3). † PBMC sample from pig 21 was collected at 12 days p.c. Animals that are not shown at 13 days p.c. were euthanized earlier.

animals were positive for RT-PCR detection and showed CT values corresponding to moderate RNA viral loads. Among the survival animals at day 13 p.c., serum from pig 1 (immunized with construct 1), was negative for viral RNA detection at 13 days p.c., and that of pig 6 showed a moderate RNA viral load (CT value 28.0). Of the surviving animals from groups immunized with constructs 2 and 3, only pig 12, which showed the lowest clinical score inside these groups, had a moderate RNA viral load (CT value 27.4) (see Fig. 2 and Table 1). At this time, the survival control animal (pig 21) showed CT values corresponding to a high RNA viral dose.

4. Discussion

In this work we have studied the immune response elicited and the protection conferred in pigs by dendrimeric constructs displaying three different B-cell epitopes from the CSFV E2 glycoprotein in combination with a T-cell epitope identified in the NS3 protein of this virus. The B-cell epitopes selected had been previously reported to induce CSFV neutralizing antibodies when administered as monomeric peptides [17,18,20,25], while the T-cell epitope in NS3 [25] was reported to be a potent T cell antigen in vitro capable of inducing lymphoproliferation, IFN-gamma and effector CTL responses [25]. This NS3 T cell epitope, identified in "in-bred" homozygous MHC-d/d miniature pigs, is to our knowledge one of the few well characterized CSFV T cell epitopes [25].

As reported for infections with the same doses of CSFV strain Margarita [30], the six non-vaccinated control pigs developed typical clinical signs of disease upon CSFV challenge, reaching severe levels (scores > 5) that advised euthanasia at days 6–12 p.c. In contrast, the clinical scores for animals immunized with constructs 1–3 were significantly lower than those of control animals.

The partial protection observed in the three groups of peptide-immunized animals correlated with the induction of peptide-specific antibodies. Thus, pigs immunized with construct 1 developed the highest levels of antibody response against the dendrimeric peptide, which were uniform in the six animals (Fig. 3). In addition, only sera from four of the six pigs immunized with construct 1 were able to recognize the linear peptides T- and B-cell epitopes thereof (Fig. 4). The fact that serum samples from pigs immunized with construct 1 recognize the linear NS3 peptide reported to be a CSFV-specific helper T-cell [25], suggests the possibility that this epitope may have a positive effect on the antibody response generated by construct 1. However, antibody levels against linear peptides were significantly lower than those induced by dendrimer 1 (Fig. 4). These results might reflect an increased sensitivity of the ELISA using multimeric constructs as compared to linear peptides. It is known that small molecules exhibit low immunogenic and antigenic properties [35]. Recently, dendrimeric peptides have been recognized as an effective tool to enhance peptide antigenicity in immunoenzymatic assays for hepatitis G diagnosis [36].

Dendrimeric vaccines of the type described here would allow differentiation of infected from vaccinated animals (DIVA), since all pigs immunized with construct 1 were seropositive after peptide immunization by ELISA, yet these sera did not elicit antibodies recognized by the commercial blocking ELISA detecting antibodies against the A domain of the E2 glycoprotein of CSFV [37,38]. The constructs used in this work do not include the epitope from domain A recognized by this ELISA, which is consistent with the lack of detection. Development of DIVA vaccines is of great interest for CSFV control as well as to avoid trade restrictions on animals and animal-derived products from countries applying vaccination to non-vaccinating countries [39,40].

Despite the reported ability of linear peptides analogous to those displayed on our constructs to induce CSFV neutralizing antibodies [17], none of the dendrimers analyzed elicited detectable CSFV neutralizing antibodies before challenge. In any case, even when the protective results are similar to those previously reported using monomeric peptides [17], a direct comparison cannot be established since: (i) several parameters were different between the studies, such as vaccine dosage, adjuvant, time between vaccination and challenge, and (ii) the viral strains used for challenge, the dose and route of administration and the criteria used to estimate the success of vaccination were different.

Due to the specific pathogenicity of CSFV, particularly its immunosuppressive effects, animals infected with CSFV normally die without eliciting a detectable antibody response (reviewed in [3]). Interestingly, upon CSFV challenge, pigs 1 and 6, immunized with construct 1, were protected from clinical signs and elicited neutralizing antibody levels (>1:32) at 13 days p.c., which have been previously associated with protection [41]. These data suggest that construct 1 can elicit an initial, small population of plasma B cells able to secrete neutralizing antibodies. Current techniques, however, are not sensitive enough to detect such responses. After CSFV infection, these specific memory B cells would be boosted to render higher levels of neutralizing antibodies (1:160) (see Fig. 5). Indeed, a similar observation has also been reported when a consistent and specific MHC class II restricted memory T cell response was generated in the absence of detectable anti-CSFV antibodies after E2-DNA vaccine immunization of domestic pigs [30]. Upon challenge, specific T cell responses were boosted and a rapid rise in the titers of CSFV neutralizing antibodies was noticed which correlated with total protection. These findings confirm the role of B cellular response in the protective immunity against CSFV, and pave the way to understanding how antigenic presentation may influence efficient stimulation of CSFV-specific B cells. Alternatively, some other protective mechanism may be operating that affords these pigs the ability to develop a primary neutralizing B cell response upon challenge infection.

Neutralizing antibodies were also found in two animals vaccinated with construct 2 (pig 12) and construct 3 (pig 15), with titers (<1:32) lower than those observed for pigs 1 and 6 (Fig. 5). These lower neutralizing titers correlated with the development of clinical scores higher that those shown by construct 1 immunized pigs.

Dendrimeric immunization elicited an increase in IFN gamma producing cells. However, the average IFN gamma response was low in the different immunization groups, which is probably related to the limited protection observed. At 36 days p.i., the IFN gamma responses, significantly higher than that in the control group, were restricted to groups immunized with construct 2 and 3 stimulated with virus. However, these responses were lower than those reported to correlate with protection [42]. At the individual level, higher IFN gamma responses were observed for pigs 1 and 6 immunized with construct 1 and, to a lesser extent, in pigs 12 and 15 immunized with construct 2 and construct 3, both surviving CSFV infection. Upon in vitro stimulation with either the corresponding peptide or with CSFV, primed T cells from these animals released IFN-gamma at 36 days p.i. and after virus challenge. One possible explanation for the animal to animal variability on the IFN gamma response is the differential ability of the dendrimeric peptides to bind to the MHC repertoire in the 'out-bred' pigs used in this experiment. In any case, the results obtained here point towards a positive effect in the induction of IFN-gamma in the three constructions tested. In synergy with the induction of neutralizing antibodies, cell-mediated immune response elicited by the dendrimeric constructions seems to be beneficial, particularly for the recovery of diseased animals [43].

The immune response induced upon dendrimeric vaccination is not priming anamnesic responses at the humoral level since a clear increase in antibody titers upon CSFV challenge is not observed. Therefore, a main immunomodulatory effect of the dendrimeric constructs seems to be the induction of T cell responses that may be responsible for the partial protection afforded. Our results suggest that priming of porcine T cells by dendrimeric constructs induces T-cell activation. The IFN gamma response generated might mediate in the early control of CSFV replication before a neutralizing antibody response builds up CSFV protection.

The complexity of the experiment, under BSL3 conditions, impaired further characterization of IFN-gamma induction of by monomeric peptides. New experiments in progress will aim to characterize and to study in detail the immunogenicity of these constructions and individual peptides (NS3 peptides) as well as T cell reactivity against E2. The results in this work point the way to new constructs including new CSFV B- and T-cell epitopes, efforts towards which are currently under way.

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