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A bivalent dendrimeric peptide bearing a T-cell epitope from foot-and-mouth disease virus protein 3A improves humoral response against classical swine fever virus

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SUMMARY

Three dendrimeric peptides were synthesized in order to evaluate their immunogenicity and their potential protection against classical swine fever virus (CSFV) in domestic pigs. Construct 1, an optimized version of a previously used dendrimer, had four copies of a B-cell epitope derived from CSFV E2 glycoprotein connected to an also CSFV-derived T-cell epitope through maleimide instead of thioether linkages. Construct 2 was similarly built but included only two copies of the B-cell epitope, and in also bivalent construct 3 the CSFV T-cell epitope was replaced by a previously described one from the 3A protein of foot-and-mouth disease virus (FMDV). Animals were inoculated twice with a 21-day interval and challenged 15 days after the second immunization. Clinical signs were recorded daily and ELISA tests were performed to detect antibodies against specific peptide and E2. The neutralising antibody response was assessed 13 days after challenge. Despite the change to maleimide connectivity, only partial protection against CSFV was again observed. The best clinical protection was observed in group 3. Animals inoculated with constructs 2 and 3 showed higher anti-peptide humoral response, suggesting that two copies of the B-cell epitope are sufficient or even better than four copies for swine immune recognition. In addition, for construct 3 higher neutralizing antibody titres against CSFV were detected. Our results support the immunogenicity of the CSFV B-cell epitope and the cooperative role of the FMDV 3A T-cell epitope in inducing a neutralising response against CSFV in domestic pigs. This is also the first time that the FMDV T-cell epitope shows effectivity in improving swine immune response against a different virus. Our findings highlight the relevance of dendrimeric peptides as a powerful tool for epitope characterization and antiviral strategies development.

Classical swine fever (CSF) is a highly contagious disease causing huge economic losses to the pig industry worldwide. Its etiological agent, classical swine fever virus (CSFV), is a member of the *Pestivirus* genus within the *Flaviviridae* family (Simmonds et al., 2012). The disease remains endemic in Central and South America, Eastern Europe and some regions of Asia, where vaccination with live attenuated vaccines is routinely used, even though such vaccines do not allow the differentiation of vaccinated from infected animals (DIVA concept) (Coronado et al., 2017). It is known that the epidemiological situation generated by CSFV in endemic countries is quite complex in spite of the extensive vaccination programs. Thus, the need for a vaccine that can induce an effective immune response and meets DIVA criteria has become a major goal of CSFV research (Blome et al., 2017; Ganges et al., 2008). In such context, identification of epitopes providing enhanced cellular and humoral immune responses is crucial in the development of both potent DIVA vaccines and diagnostic tools essential for CSFV control.

A well recognised strategy to improve the immunogenicity of peptide antigens is to present them in a clustered dendrimeric (branched) format first introduced by Tam (Tam et al., 2002) as multiple antigenic peptide (MAP) systems. The MAP design is based on

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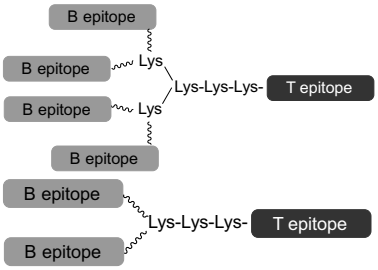
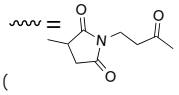
Table 1
Dendrimeric peptides used in this study.

Construct	Type	General structure[a]	B-cell epitope	T-cell epitope
1	B_4T(mal)		E2 glycoprotein of CSFV, residues 694–712: KEDFRYAISSTNEIGLLGA	Non-structural NS3 protein of CSFV, residues 1446–1460: KHKVRNEVMVHWFGD
2	B_2T(mal)			
3	B_2T(mal)			Non-structural protein 3A of FMDV, residues 21–35: AAIEFFEGMVHDSIK

[a] In all constructs, the C-terminal Cys thiol group is linked to the Lys core via a 3-maleimidopropionic acid unit ().

a branched oligolysine core to which various copies of the peptide antigen are attached. MAP-based constructs are effective as candidate vaccines, as well as for identification of new viral epitopes and basic virus-host interactions research (reviewed in (Heegaard et al., 2010)).

Previous work in some of our laboratories has shown the ability of dendrimeric peptide constructs to provide solid protection against foot-and-mouth disease virus (FMDV) in domestic pigs (Cubillos et al., 2012, 2008). FMDV is a picornavirus that produces a highly transmissible and devastating disease of farm animals, mostly cattle and swine (Blanco et al., 2016).

The original prototype (Cubillos et al., 2008) was a MAP-like construct [B_4T(thi)] containing four copies of a B-cell epitope [residues (136–154) of viral protein VP1] linked through thioether bonds to a T-cell epitope identified in residues (21–35) of non-structural protein 3A of FMDV shown to significantly improve the immune response against FMDV in domestic pigs (Cubillos et al., 2012). Recently, a structurally simplified version of that B_4T(thi) prototype, bearing only two copies of the B-cell epitope and using thioether [B_2T(thi)] or maleimide [B_2T(mal)] linkages to the T-cell epitope sequence, elicited similar or higher B and T-cell specific responses in swine than the earlier tetravalent version (Blanco et al., 2016).

For CSFV several peptide vaccine strategies have been previously described, although full protection was not achieved in any of these studies. Thus, the peptide vaccine strategy is still in an experimental stage (revised in Blome et al., 2017). By using dendrimeric peptides, a B_4T(thi)-type platforms with a B-cell epitope from E2 (residues 694–712) and a T-cell epitope from NS3 (residues 1446–1460) has been described (Monsó et al., 2011; Tarradas et al., 2012, 2011). Despite affording only partial protection, the strategy has allowed characterizing the NS3 peptide as a potent T-helper sequence, capable of enhancing the specific humoral response in domestic pigs, and also proven the usefulness of branched constructs as diagnostic tools (Tarradas et al., 2012).

Against this background, we have investigated the immune response elicited by three new versions of the branched constructs (Table 1). One of the constructs (1) is tetravalent, of the B_4T(mal)-type, while the other two (2, 3) are bivalent, B_2T(mal)-type, differing only in the T-cell epitope: in 2, the aforementioned NS3 sequence is used, as in 1, whereas in 3 the [3A(21–35)] T-cell epitope successfully used in FMDV vaccines has been adopted. Given the advantageous performance – both immunological and synthetic – of the maleimide linkage, this connectivity has been chosen in all cases. The constructs have been evaluated in pigs, with a view to compare how bivalent 2 and/or 3 perform relative to tetravalent 1 in terms of CSFV specific responses.

Peptides 1–3 were made by thiol-maleimide ligation of pre-purified precursors prepared by solid phase synthesis, as described in detail elsewhere (Blanco et al., 2016; Monsó et al., 2013). The B-cell epitope moiety had an additional C-terminal Cys, while the T-cell epitope sequence was N-terminally elongated with two Lys units followed by either one [B_2T(mal)-type] or three [B_2T(mal)-type] extra Lys residues in a branched arrangement (see Table 1 for details). All peptides were purified by preparative reverse phase HPLC to near homogeneity (>95% by analytical HPLC) and characterized for identity by MALDI-TOF mass spectrometry.

A total of sixteen domestic pigs (Landrace x Large white, 6 week old; numbered 1–16) distributed in four groups of four animals each were used. Animals 1–4 (group 1), 5–8 (group 2) and 9–12 (group 3), were immunized with dendrimeric constructs 1–3, respectively. Two doses of 2 mg each of the corresponding construct, dissolved in 1 mL of NaCl 0.9% solution and mixed with 1 mL of Montanide v206 adjuvant (Seppic), were administered at days 1 and 21 of the experiment by intramuscular (i.m.) injection in the neck region. Four additional pigs (13–16, group 4) were also i.m. inoculated with saline solution plus adjuvant as negative controls. Fifteen days after the second immunization (day 36), pigs were challenged with 10^5 $TCID_{50}$ of CSFV (Margarita strain) by i.m. injection in the neck (Tarradas et al., 2012, 2011). Animals remained infected during fifteen days post CSFV challenge (end of the trial) in the BSL3 animal facility at CReSA (Barcelona, Spain). A peroxidase-linked assay (PLA) (Wensvoort et al., 1986) was used for viral titration following the statistical method described by (Reed and Muench, 1938).

The rectal temperatures and clinical signs were recorded daily by a trained veterinarian in a blinded manner. The clinical status of the animals was scored from 0 to 6 as reported for this viral strain (Tarradas et al., 2014). Animals with a clinical score value of 5 or higher or showing prostration behaviour were euthanized for ethical reasons, 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.

Dendrimeric peptide-specific antibodies in pig sera were tested by means of construct-specific ELISAs. Specific anti-peptide IgG was detected at 1,7,14, 21 and 36 days post vaccination (dpv) as well as at the day of CSFV challenge, 5, 8 and 13 days post challenge (dpc), as described (Tarradas et al., 2012, 2011). In all cases, sera from control animals were included as negative controls. Cut-off value was set at 0.5 O.D. Serum samples were also analysed using