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.

Key words: Swine, CSFV, FMDV, immune response, T cell epitope, B cell epitope, dendrimeric peptide, maleimide, humoral response, neutralising antibodies, protection

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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 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,

3

88 elicited similar or higher B and T-cell specific responses in swine than the earlier

89 tetravalent version (Blanco et al., 2016).

90 For CSFV several peptide vaccine strategies have been previously described, although

91 full protection was not achieved in any of these studies. Thus, the peptide vaccine strategy

92 is still in an experimental stage (revised in Blome et al., 2017). By using dendrimeric

93 peptides, a $B_4T(thi)$-type platforms with a B-cell epitope from E2 (residues 694–712) and

94 a T-cell epitope from NS3 (residues 1446–1460) has been described (Monsó et al., 2011;

95 Tarradas et al., 2012, 2011). Despite affording only partial protection, the strategy has

96 allowed characterizing the NS3 peptide as a potent T-helper sequence, capable of

97 enhancing the specific humoral response in domestic pigs, and also proven the usefulness

98 of branched constructs as diagnostic tools (Tarradas et al., 2012).

99 Against this background, we have investigated the immune response elicited by three new

100 versions of the branched constructs (Table 1). One of the constructs (1) is tetravalent, of

101 the $B_4T(mal)$-type, while the other two (2, 3) are bivalent, $B_2T(mal)$-type, differing only

102 in the T-cell epitope: in 2, the aforementioned NS3 sequence is used, as in 1, whereas in

103 3 the [3A(21-35)] T-cell epitope successfully used in FMDV vaccines has been adopted.

104 Given the advantageous performance –both immunological and synthetic– of the

105 maleimide linkage, this connectivity has been chosen in all cases. The constructs have

106 been evaluated in pigs, with a view to compare how bivalent 2 and/or 3 perform relative

107 to tetravalent 1 in terms of CSFV specific responses.

108 Peptides 1-3 were made by thiol-maleimide ligation of pre-purified precursors prepared

109 by solid phase synthesis, as described in detail elsewhere (Blanco et al., 2016; Monsó et

110 al., 2013). The B-cell epitope moiety had an additional C-terminal Cys, while the T-cell

111 epitope sequence was N-terminally elongated with two Lys units followed by either one

112 [$B_2T(mal)$-type] or three [$B_2T(mal)$-type] extra Lys residues in a branched arrangement

113 (see Table 1 for details). All peptides were purified by preparative reverse phase HPLC

114 to near homogeneity (>95% by analytical HPLC) and characterized for identity by

115 MALDI-TOF mass spectrometry.

116 A total of sixteen domestic pigs (Landrace x Large white, 6 week old; numbered 1-16)

117 distributed in four groups of four animals each were used. Animals 1-4 (group 1), 5-8

118 (group 2) and 9-12 (group 3), were immunized with dendrimeric constructs 1-3,

119 respectively. Two doses of 2 mg each of the corresponding construct, dissolved in 1 mL

120 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 a CSFV-specific E2 ELISA (HerdChek CSFV Ab, IDEXX) following the manufacturer's recommendations. Serum samples collected at 13 dpc were also tested by the neutralisation peroxidase-linked assay (NPLA) (Terpstra et al., 1984). For CSFV RNA detection, RNA was extracted from serum and rectal swabs using the viral RNA isolation kit Nucleospin II according to the manufacturer's instructions (Macherey-Nagel). The presence of CSFV RNA in sera was analysed by real time (RT)-PCR (Hoffmann et al., 2005). Positive results were considered for threshold cycle values (CT) equal or less than 42.

Statistical analyses was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). For all the analyses, the pig was used as the experimental unit. The significance level (p) was set at 0.05, with statistical tendencies being reported when $p < 0.10$. A non-parametric test (Wilcoxon) was chosen to compare the clinical parameters and anti-

peptide antibody response between groups throughout the trial. This non-parametric analysis was applied due to the non-normality pattern observed for this parameter and the small number of animals used in each experimental group.

Three of the four pigs immunized with construct 3 showed a potent and early (14 dpv) antibody response against the peptide used for immunization as determined by dendrimeric peptide-specific ELISA. This response increased in all animals after the second immunisation (day of viral challenge) (Figure 1). Three of the four pigs immunized with construct 2 showed peptide-specific antibodies at 21 dpv and at 36 dpv. Finally, the lowest anti-peptide antibody response was found in pigs immunized with construct 1, which showed a detectable response only after boost immunisation (day of challenge) that was maintained until the end of the experiment at 15 dpc. As expected, control animals did not show specific anti-peptide antibodies against any of the dendrimers analysed. Thus, construct 3 evoked the quicker and higher anti-peptide humoral response among the dendrimers analysed with statistical significant difference ($p=0.03$) from the day of viral challenge (at 36 dpv) until 8 dpc.

Regarding the protection conferred by these dendrimers upon viral challenge, control animals developed pyrexia (rectal temperature above 40 °C), which appeared between 4 and 5 dpc. From 7 dpc these pigs also developed severe clinical signs related with CSFV and all were euthanized between 11 and 13 dpc with the highest clinical score values (> 4 points). In contrast, animals from the three vaccinated groups showed delayed onset of CSFV, moderate to severe clinical signs (>3 points in score value). One pig from each immunized group had to be euthanized before the end of the trial, at 11 dpc (groups 2 and 3) and at 13 dpc (group 1). However, all immunized groups exhibited statistically significant lower clinical scores than those of the control pigs ($p<0.05$) during the first 10 dpc (Figure 2). Animals inoculated with peptide 3 showed statistical difference with the control group from day 6 to 10 dpc, whereas the other groups showed statistical difference from day 6 to 9 dpc (group 1) and at days 6 and 10 dpc (group 2). Furthermore, the mean clinical score value was lower for group 3 towards the end of the study (Figure 2).

Control animals failed to develop detectable anti E2 antibodies by the commercial ELISA (HerdChek CSFV Ab, IDEXX). In contrast, four out of the twelve peptide immunized animals developed a specific E2 antibody response at 13 dpc, two pigs from group 1 and the other two from groups 2 and 3, (even this latter having an FMDV epitope), respectively. As previous studies, neutralising antibody response to CSFV after

186 dendrimeric peptide immunization with titres over 1:32 was found only at 13 dpc

187 (Tarradas et al., 2012); in one animal from group 1 (1:40) and two pigs from group 3,

188 (1:160 and 1:40, respectively).

189 CSFV RNA was detectable by qRT-PCR in serum samples from all pigs at 5 dpc with a

190 mean Ct value of 29 in the four experimental groups. At 13 dpc, the Ct values ranged

191 from 22.31 to 24.01 (group 1), 19.86 to 25.39 (group 2) and 23.12 to 28.63 (group 3) in

192 immunized-challenged pigs (Table 2).

193 Despite the change in the conjugation method between B- and T-cell peptides from

194 thioether to maleimide, tetravalent construct 1 conferred levels of protection similar to

195 those described for peptide [B4T(thi)] (Monsó et al., 2011; Tarradas et al., 2011).

196 Interestingly, animals inoculated with constructs 2 and 3 showed a higher anti-peptide

197 humoral response than animals from group 1. Contructs 2 and 3 comprise only two copies

198 of the B cell epitope, suggesting that bivalence is advantageous for dendrimer recognition

199 by the swine immune system, as reported for FMDV analogous constructs (Blanco et al.,

200 2016, 2013). Despite the anti-peptide antibody response elicited by the CSFV

201 dendrimeric constructs was unable to confer complete protection against CSFV, our

202 results support that bivalent dendrimers, in particular construct 3, evoke faster and higher

203 antibody responses that the tetravalent construct 1 (Figure 1).

204 On the other hand, higher neutralizing antibody titres (>1:32) at 13 dpc, which have been

205 previously related with CSFV protection (Terpstra and Wensvoort, 1988), were elicited

206 by construct 3 immunized pigs at 13 dpc. This response combined with the reduction and

207 delayed onset of moderate-severe CSFV clinical signs; supports the role of the CSFV B-

208 cell epitope in the E2 glycoprotein (694–712). Likewise, suggests the cooperative

209 capacity of the FMDV 3A [3A (21-35)] T cell epitope in the induction of an effective

210 neutralising antibody response against CSFV in domestic pigs. These findings correlate

211 with previous studies which suggests that the FMDV 3A T cell epitope may facilitate the

212 antigen presentation and generate a boost effect against FMDV in the swine immune

213 system (Blanco et al., 2016; Cubillos et al., 2012). In this regard, it is worth mentioning

214 that the FMDV 3A T cell epitope included into the construct 3 fails to detect FMDV

215 specific antibodies in infected swine. Thus, the use of this epitope would not generate

216 cross-reactions in the serological response of FMDV. Considering that, our results

217 provide valuable information in the development of new CSFV diagnostic strategies.

218 Further optimization of dendrimeric construct 3, could generate a more potent protection

219 against CSFV. These findings highlight the relevance in the use of dendrimeric peptides

220 for epitope characterization as powerful tools in the development of antiviral strategies

221 in animal health.

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229 **Competing interests**

230 The authors declare that they have no competing interests.

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306 **Figure legends**

307

308 **Figure 1.** Anti-peptide antibody response detected by dendrimeric peptide-specific

309   ELISA in animals inoculated with construct 1 (A), construct 2 (B) and construct

310   3 (C). Black bars represent inoculated animals at different time post immunization

311   and viral challenge. In the graphic, 0 dpc corresponds with the day of CSFV

312   challenge. Animals not shown at 8 and 13 dpc were euthanized before day of

313   sampling. * Symbol indicates a euthanized pig after sampling. Construct 3 elicited

314   higher anti-peptide humoral response among the dendrimers analysed with

315   statistical significant difference ($p < 0.05$) from the day of viral challenge until 8

316   dpc.

317

318 **Figure 2.** Mean clinical score per group after CSFV challenge. Symbol *, indicates
319   statistical difference between control group and all peptide-inoculated groups
320   (p<0.05). Symbol ±, indicates statistical difference between control group and
321   groups 1 and 3 (p<0.05). Symbol #, indicates statistical difference between control
322   group and group 3 (p<0.05). One pig from each immunized group had to be
323   euthanized before the end of the trial, at 11 dpc (groups 2 and 3) and at 13 dpc
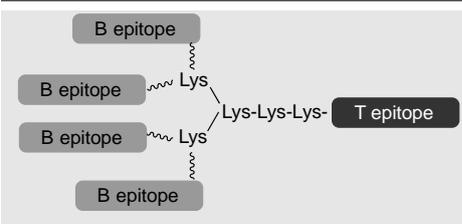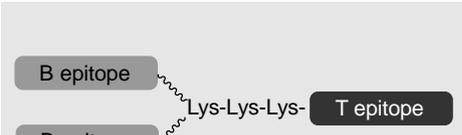324   (group 1)

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329 **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
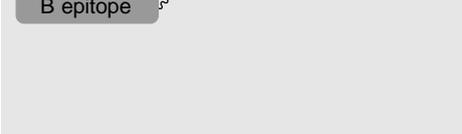
330 [a] In all constructs, the C-terminal Cys thiol group is linked to the Lys core via a 3-
331 maleimidopropionic acid unit ($\sim\!\!\sim\,=$)

332

Table 2. Detection of CSFV RNA for real time RT-PCR in serum samples collected after CSFV challenge (13 dpc).

Group	Animal	Ct value	Mean Ct value per group
Construct 1	1	24.01	23.92
	2	22.31	
	3	26.15	
	4*	23.23	
Construct 2	5	19.86	22.72
	6	22.91	
	8	25.39	
Construct 3	9	28.63	25.34
	10	23.12	
	12	24.27	
Control	16	22.99	22.99

* Pig euthanized after sample collection