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Full protection of swine against foot-and-mouth disease by a bivalent B-cell epitope dendrimer peptide

Esther Blanco [a, *], Beatriz Guerra [a], Beatriz G. de la Torre [b], Sira Defaus [b], Aldo Dekker [c], David Andreu [b, **], Francisco Sobrino [d, ***]

[a] Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, 28130 Madrid, Spain
[b] Departament de Ciències Experimentals i de la Salut, Universitat Pompeu-Fabra, 08003 Barcelona, Spain
[c] Central Veterinary Institute (CVI), Wageningen UR, 8200 AB Lelystad, The Netherlands
[d] Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), 28049 Madrid, Spain

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ABSTRACT

Foot-and-mouth disease virus (FMDV) causes a highly contagious disease of cloven-hoofed animals. We have reported (Cubillos et al., 2008) that a synthetic dendrimeric peptide consisting of four copies of a B-cell epitope [VP1(136–154)] linked through thioether bonds to a T-cell epitope [3A(21–35)] of FMDV [B_4T(thi)] elicits potent B- and T-cell specific responses and confers solid protection in pigs to type C FMDV challenge. Herein we show that downsized versions of this peptide bearing two copies of a B-cell epitope from a type O isolate and using thioether [B_2T(thi)] or maleimide [B_2T(mal)] conjugation chemistries for their synthesis elicited in swine similar or higher B and T-cell specific responses than tetravalent B_4T(thi). Moreover, while partial protection was observed in animals immunized with B_4T(thi) (60%) and B_2T(thi) (80%), B_2T(mal) conferred full (100%) protection against FMDV challenge, associated to high levels of circulating IgG2 and mucosal IgGA, and entirely prevented virus shedding. Interestingly, B_2T(mal) is also the most advantageous option in terms of synthetic practicality. Taken together, the results reported here point out to B_2T(mal) as a highly valuable, cost-effective FMDV candidate vaccine.

1. Introduction

For over 200 years, vaccines have been decisive in the control and/or eradication of infectious diseases. Classical vaccines, consisting of a dead or attenuated version of the infectious agent (e.g., a virus) entail risks such as accidental release of live pathogens, or the possibility of reverting from attenuated to active forms. In this context, subunit vaccines appear as a practical alternative that solves most such problems by excluding the infectious agent. Among various types of subunit vaccines, peptide-based ones are advantageous by (i) total lack of biological hazard; (ii) possibility of displaying various epitopes on a single scaffold; (iii) easy differentiation of infected from vaccinated animals (DIVA condition); (iv) efficient synthetic production; (v) characterization as pharmaceuticals, and (vi) easy transport and storage (Brun et al., 2011; Purcell et al., 2007). These advantages must be set against the recognized low immunogenicity of peptides, which can be improved in various ways (Rueckert and Guzman, 2012), notably by multiple epitope display on a single molecular platform.

Foot-and-mouth disease (FMD) is a highly transmissible infection of pigs and other cloven-hoofed animals, admittedly the most important worldwide in economic impact (James and Rushton, 2002). In areas (Africa, Asia) where FMD remains endemic, it severely handicaps access to international meat markets (Rweyemamu et al., 2008). FMD is usually controlled by inactivated whole virus vaccines whose production has remained unaltered for decades and poses many of the above biosafety concerns (Cottam et al., 2008; Rodriguez and Gay, 2011). These, along with other commerce-related considerations, have caused FMDV-free countries to forgo vaccination, even when facing FMD episodes. Instead, massive slaughter of infected or suspect animals plus drastic, often controversial measures in farming, mobility, etc. are put into effect

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: blanco@inia.es (E. Blanco), david.andreu@upf.edu (D. Andreu), fsobrino@cbm.csic.es (F. Sobrino).

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(Kitching et al., 2007; Sobrino and Domingo, 2001). In this context, peptide-based vaccines are increasingly viewed as an appealing alternative for FMD control.

In FMDV, the main B-cell antigenic determinant is a continuous site located around positions 140 to 160 of capsid protein VP1 (Acharya et al., 1989; Mateu, 2004). However, the protection conferred by peptides spanning this region is limited (Bittle et al., 1982; Taboga et al., 1997), it being now generally accepted that, for effective protection against FMDV, a T-cell response is required (McCullough et al., 1992). We have reported that inclusion of a T-cell epitope located in residues 21–35 of FMDV non-structural protein 3A (Blanco et al., 2001), juxtaposed to the aforementioned B-cell epitope, significantly improves the immune response (Cubillos et al., 2012), although it does not afford full protection in challenged pigs.

In order to enhance the effectiveness of B- and T-epitope presentation, we designed and tested as candidate vaccine a dendrimeric peptide consisting of the T-cell epitope N-terminally elongated with a Lys tree branching out into four copies of the B-cell epitope. This construct, henceforth denominated B_4T(thi), elicited high titers of FMDV-neutralizing and IgA antibodies in both pigs and outbred mice (Swiss CD1 strain), activated T-cells and induced IFN-γ release, hence performing as an effective vaccine conferring solid protection in swine against type C FMDV challenge (Cubillos et al., 2008).

Following up on these promising results, we have prepared B_4T(thi) prototypes displaying type O (presently the most prevalent FMDV serotype) sequences (Sumption et al., 2008) as epidemiologically more consequential B-cell epitopes. We have also re-evaluated B-cell epitope multivalency by comparing the immune response in Swiss CD1 mice of constructs with two or four copies of the B-cell epitope, showing that multivalency not only improves over colinear B-T display but also that bivalent constructs outperform tetravalent ones in terms of humoral (neutralizing antibodies) and cellular (IFN-γ) responses, particularly when the B- and T-cell epitopes are connected by maleimide linkages (Monso et al., 2013).

Herein a thorough comparison of these features (i.e., tetra-vs. bivalency, maleimide vs. thioether linkage) and how they influence the immune response —including protection from challenge —is reported for the swine, an FMDV natural host. We confirm that bivalent presentation of B-cell epitopes, and their connection to the T-cell epitope via maleimide linkages results in a vaccine candidate with optimal properties, hence reasonable prospects of clinical application.

2. Materials and methods

2.1. Peptides

Peptides reproducing the B and T epitopes of FMDV O-UKG 11/01 in different arrangements are shown in Table 1. All peptides were made from precursors prepared by solid phase synthesis protocols and purified prior to conjugation. One precursor corresponded to the B epitope with an additional C-terminal Cys (free thiol form); the other to the T epitope, elongated N-terminally with two Lys units followed by either three [B_4T(thi)] or one [B_2T(thi) and B_2T(mal)] additional Lys in a branched arrangement. Additional details on the synthesis are available in refs (Cubillos et al., 2008; Monso et al., 2013). The final products were purified to near homogeneity by HPLC (see, Supplementary Fig. S1) and characterized by MS.

2.2. Virus

A virus stock derived from FMDV isolate O-UKG 11/01 (The Pirbright Institute, UK) by two amplifications in swine kidney cells (IB-RS-2 cells) was used. The resulting virus maintained the consensus sequences at the capsid region (Nuñez et al., 2007).

2.3. Animals and experimental design

The immune response to peptides on Table 1 was assessed in eighteen 9–12 weeks-old male pigs (TOPIGS 20TM breed), free of antibodies to FMDV and to endemic respiratory pig diseases (PRRSV, *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, and *Pasteurella multocida*). The study was approved (no. 2013121) by the Central Veterinary Institute (CVI) animal experiment ethical review committee in compliance with Dutch law. Pigs (numbering as in Table 2) were randomly assigned to 3 groups of 6 animals each and immunized twice (days 0 and 21) by intramuscular injection with 2 mL of Montanide ISA 50V2 emulsion (Seppic, France) containing 2 mg of either B_2T(thi), B_2T(mal) or B_4T(thi). Two additional non-vaccinated pigs were kept as infection controls. Animals were housed under standard CVI conditions until day 34, when they were placed in separate units of the high-containment facility and challenged at day 39 with 1.6×10^4 plaque forming units (pfu) of FMDV O-UKG 11/01, by inoculation at two sites of both main claws of the left-hindfoot pad (0.1 mL/site). Animals were monitored for clinical signs of disease during 10 days, and then euthanized at day 49. Those exhibiting lesions on all 4 feet were euthanized before the end of the experiment.

2.4. Virus detection after challenge

Pharyngeal and nasal swabs were examined for the presence of infectious virus by plaque titration on secondary lamb-kidney (LK-2) cells. Ten-fold dilutions of the samples (200 μL, tested in duplicate) were adsorbed for 1 h on cell monolayers in collagen-coated plates (Biocoat1, BD), then maintained in medium containing 1% methylcellulose. After 2 days at 37°C–5% CO_2, plates were stained with 0.1% amido-black in 1 M acetic acid, 0.09 M sodium acetate, 10% glycerol and virus titers expressed as the $\log_{10}$ of the number of pfu/mL.

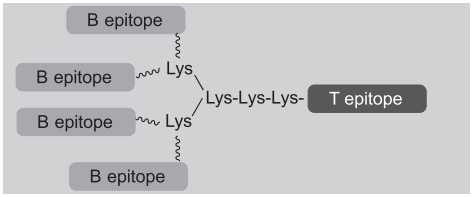
2.5. Virus neutralization test (VNT)

Serial 2-fold dilutions of each serum sample were incubated with 100 infection units - 50% tissue culture infective doses (TCID50) – of FMDV O-UKG 11/01, for 1 h at 37 °C. End-point titers were calculated as the reciprocal of the final serum dilution that neutralized 100 $TCID_{50}$ of homologous FMDV in 50% of the wells.

2.6. Detection of specific anti-FMDV antibodies by ELISA

Total anti-FMDV antibodies were determined by ELISA. Briefly, 96-well plates (Nunc) were coated with sucrose gradient-purified 140s FMDV O-UKG 11/01 in PBS overnight at 4 °C. Duplicate 3-fold dilution series of each serum sample were prepared in 50 μL, starting at 1/100. Pre-immune sera from peptide-immunized pigs and sera from non-immunized animals were used as negative controls. Specific antibodies were detected with HRP-conjugated protein A (Thermo Fisher), diluted 1/4000. Color development was obtained after addition of 100 μL/well of TMB (Sigma Aldrich) and stopped by an equal volume of 1 M H_2SO_4. Plates were read at 450 nm, titers expressed as the reciprocal of the last dilution giving the absorbance recorded in the control wells (serum at day 0) plus 2 SD.

Table 1
Dendrimeric peptides used in this study.

Valency	General structure[a]	Name	MW[d]	HPLC[e]
4		B_4T(thio)[b]	11,236.15	7.1 min (95%)
2		B_2T(thio)[b] B_2T(mal)[c]	6536.66 6742.82	6.7 min (98%) 6.9 min (97%)

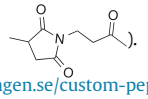
[a] For all three constructs, epitope B = acetyl-PVTNVRGDLQVLAQKAARTC and epitope T = AAIEFFEGMVHDSIK (both in C-terminal carboxamide form).
[b] A thioether linkage (〰 = –CH_2–CO–) connects the C-terminal Cys side chain thiol to Lys core.
[c] The C-terminal Cys side chain thiol is linked to Lys via a 3-maleimidopropionic acid unit (〰 = ).
[d] Determined by LC–MS. Theoretical mass, in parentheses, from http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-propertycalculator.asp).
[e] Retention time on a C18 column (Luna, 4.6 mm × 50 mm, 3 μm; Phenomenex) eluted with a 20–60% linear gradient of solvent B (0.036% TFA in MeCN) into solvent A (0.045% TFA in H_2O) over 15 min. In parenthesis, homogeneity of purified material (see Fig. S1).

Table 2
Evidence for protection in animals immunized with dendrimeric constructions.

Inoculum	Pig	Vesicular lesions[a]		Protection[b]	FMDV detection in pharyngeal and nasal swabs at the indicated day[c]							
		Primary	Secondary		0[d]	1	2	3[d]	4	5	7[d]	10[d]
B_2T-thioether	1	1	5	Unprotected	–/–	–	–	2.1/–	2.8	4.2	x	x
	2	1	1	Protected	–/–	–	–	–/–	–	–	–/–	–/–
	3	0	0	Protected	–/–	–	–	–/–	–	–	–/–	–/–
	5	0	0	Protected	–/–	–	–	–/–	2.2	–	–/–	–/–
	6	1	1	Protected	–/–	–	–	–/–	1.5	1.4	2.6/-	–/–
B_2T-maleimide	7	0	0	Protected	–/–	–	–	–/–	–	–	–/–	–/–
	8	1	0	Protected	–/–	–	–	–/–	–	–	–/–	–/–
	9	0	0	Protected	–/–	–	–	–/–	–	–	–/–	–/–
	10	0	0	Protected	–/–	–	–	–/–	–	–	–/–	–/–
	11	0	0	Protected	–/–	–	–	–/–	–	–	–/–	–/–
	12	1	0	Protected	–/–	–	–	–/–	–	1.0	–/–	–/–
B_4T-thioether	13	1	5	Unprotected	–/–	–	–	1.2/-	4.8	3.5	X	x
	14	1	5	Unprotected	–/–	–	5.0	3.3/2.5	x	x	X	x
	15	1	1	Protected	–/–	–	–	–/1.4	1.3	2.0	3.9/-	–/–
	16	1	0	Protected	–/–	–	–	–/–	–	2.0	–/–	–/–
	17	1	0	Protected	–/–	–	–	–/–	–	–	–/–	–/–
	18	1	1	Protected	–/–	–	–	2.0/-	2.2	2.4	–/–	–/–
Non-immunized	19	1	5	Unprotected	–/–	–	–	3.8/1.4	3.8	4.2	x	x
	20	1	5	Unprotected	–/–	–	–	5.0/2.2	x	x	x	x

[a] Vesicular lesions at the injection sites (primary vesicles, at left hind foot) and vesicular lesions at additional sites (secondary vesicles: right hind foot, left front foot, right front foot, tongue, mouth, nose).
[b] Needle-challenge pigs were classified as protected if, at most, lesions were found at the injection sites and one additional site on the body.
[c] FMDV detection in pharyngeal (collected daily post-challenge) and nasal swabs (collected at days 0, 3, 7 and 10 post-challenge), measured by virus isolation and expressed as $\log_{10}$ pfu/mL; –: no virus was detected; x:indicates a pig slaughtered due to extended clinical signs.
[d] FMDV detection/titers in pharyngeal/nasal swabs.

2.7. Detection of isotype-specific anti-FMDV antibodies by ELISA

FMDV-specific IgG1, IgG2 (in sera) and IgA (in sera and nasal swabs) were measured using a modification of the above ELISA. Isotype-specific mAbs were from Serotec. Duplicate 3-fold dilutions of each serum sample were prepared in 50 μL, starting at 1/50 (1/5 for nasal swabs). For nasal swab samples, and to increase assay sensitivity, sample was incubated twice before anti-IgA mAb was added. Antibodies were detected with HRP-labeled anti-mouse (Thermo Fisher) diluted 1/1000 and incubated for 45 min at 37 °C, followed by TMB color development as above. Titers were expressed as the reciprocal $\log_{10}$ of serum dilutions giving the absorbance of control wells (serum at day 0) plus 2 SD.

2.8. PBMC isolation and IFN-γ detection by ELISPOT

Porcine peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque-1077 (Sigma) and cryopreserved prior to assay. Vials were defrosted at 37 °C, washed, resuspended in complete RPMI 1640 and incubated overnight at 37°C–5% CO_2. Cell counting and viability were tested by Trypan blue staining. For the IFN-γ ELISPOT assay 5×10^5 PBMCs were shed in triplicate wells of Immobilon-P plates (Merck Millipore) coated with 5 μg/mL of anti-pig IFN-γ antibody (clone P2G10, BD). For the in vitro antigen recall, PBMCs from peptide-immunized pigs were stimulated with 20 μg/mL of their respective inmmunogenic peptides (Saiz et al., 1992). As positive control, cells were

incubated with 10 μg/mL of phytohaemagglutinin (Sigma) using cells incubated without antigen as negative control. After 48 h at 37°C–5% CO_2, plates were washed and incubated with 2 μg/mL of biotinylated anti-mouse IFN-γ antibody (clone P2C11, BD) followed by HRP-streptavidin (BD). Antibody was visualized with 3-amino-9-ethyl carbazole (BD). The frequency of peptide-specific T cells in the responding population was expressed as the mean number of spot-forming cells/10^6 PBMCs, with background values (number of spots in negative control wells) subtracted from the respective counts of stimulated cells.

2.9. Statistical analyses

Differences among peptide-immunized groups in FMDV-antibody titers and number of IFN-γ producing cells, were analyzed by One-way ANOVA, followed by Tukey's post-hoc comparisons tests. Values are cited in the text as means ± SD. To measure the relationship between IgG1 and IgG2 titers in each group, Pearson's correlation coefficient (r) was calculated, while significant protection was measured by Fisher's exact test. All p values are two sided, and p values < 0.05 were considered significant. In figures, p value criteria are assigned as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Statistical analyses were conducted using GraphPad Prism Software 5.0.

3. Results

3.1. Design and synthesis of dendrimeric peptides

B_4T(thi), B_2T(thi) and B_2T(mal) (Table 1), were chosen on prior design considerations. Thus, B_4T(thi) is equivalent to the tetravalent dendrimer in (Cubillos et al., 2008) but with B-epitope sequences derived from FMDV O-UKG 11/01. B_2T(thi) and B_2T(mal), in turn, are as in (Monso et al., 2013). In all cases, peptides were produced by conjugation of a T-epitope N-terminally elongated with Lys residues providing 2 or 4 levels of branching and functionalized with chloroacetyl [B_4T(thi), B_2T(thi)] or maleimido [B_2T(mal)] groups, and an N-acetylated B-epitope with a C-terminal Cys whose thiol group reacts with either chloroacetyl or maleimide units. While both modules were accessible in highly pure form by solid phase synthesis, thioether-based conjugations were slow, unselective processes requiring –particularly for B_4T(thi) (Cubillos et al., 2008)– extensive purification. In contrast, B_2T(mal) is produced almost quantitatively in a clean thiol-ene reaction. After HPLC purification, all constructs were satisfactorily documented for identity by LC-MS.

3.2. B_2T(mal) confers full protection and prevents virus shedding

Domestic pigs, in four different groups, were immunized twice with B_4T(thi), B_2T(thi), B_2T(mal) or non-immunized, and subsequently challenged with FMDV O-UKG 11/01. Animals were examined daily for clinical signs (see Materials and Methods) and considered protected when lesions were only observed at the infection site and/or at a single site (Francis and Black, 1986). As expected, PBS-inoculated controls (pigs 19 and 20) showed full FMD signs upon challenge, with vesicular lesions on the snout and all four feet by days 3 and 5 p.c., respectively (Table 2). In contrast, only three peptide-immunized animals presented extended FMD clinical signs, namely pigs 1 [given B_2T(thi)] and 13 and 14 [given B_4T(thi)]. Two other animals in the B_2T(thi) (2 and 6) and the B_4T(thi) (15 and 18) groups showed secondary lesions on a single foot. The remaining pigs in these groups did not develop clinical signs (Table 2). Thus, both B_4T(thi) and, even more, B_2T(thi) afforded substantial levels (66% and 80%, respectively) of protection against FMDV challenge. Virus shedding was detected in five and three pigs immunized with B_4T(thi) and B_2T(thi), respectively, although a significant ($p < 0.05$) reduction in the maximum amount ($\log_{10}$ pfu/mL) of released virus in pigs tested positive (average of 2.8 and 2.4, respectively) was observed relative to non-vaccinated controls (average of 4.2).

Interestingly, protection scores were highest in pigs immunized with B_2T(mal), as none of the 6 animals developed any FMD sign ($p = 0.03$, Fisher's exact test), and 4 did not even show lesions at the site of challenge. In addition, practically no virus shedding was detected in pharyngeal and nasal swabs collected after challenge (Table 2).

In conclusion, immunization with B_2T(thi) and B_2T(mal) confers higher levels of protection (80 and 100%, respectively) than those of tetravalent B_4T(thi) (66%), B_2T(mal) conferring a fully protective response that prevents virus shedding.

3.3. B_2T(mal) elicits higher anamnestic humoral responses

Specific anti-FMDV antibodies were determined by ELISA in sera from days 0, 20 and 34. All peptides elicited consistent, comparable IgG titers [400 ± 0.3, 525 ± 0.2 and 640 ± 0.2 for B_4T(thi), B_2T(thi) and B_2T(mal), respectively] after the first dose (Fig. 1A). Titers were boosted up, with slight differences among groups, after a second dose. The largest, 10-fold, increase in anti-FMDV titers after boost was recorded in pigs given B_2T(mal) (6500 ± 0.4). The other two groups showed smaller, 5–6-fold higher titers upon boosting: 3500 ± 0.5 [B_2T(thi)] and 2000 ± 0.3 [B_4T(thi)]. Despite the lack of statically significant differences ($p > 0.05$), B_2T(mal) appears to elicit 2–3 times more anti-FMDV antibodies than thioether-based constructs.

Likewise, significant VNT titers were found at day 34 after boost in sera from all pigs (Fig. 1B). Nevertheless, in contrast to IgG titers above, no clear-cut differences were found among the three constructs, with VNT titers of 370 ± 0.5 [B_4T(thi)], 240 ± 0.5 [B_2T(thi)] and 272 ± 0.3 [B_2T(mal)].

3.4. Anti-FMDV IgG1 and IgG2 serum antibody profiles

The ability of the peptides to induce specific isotype IgG1 and IgG2 antibodies was examined in sera from day 34. All three peptides induced both IgG1 and IgG2 isotypes, albeit to different extents, bivalent B_2T(thi) and B_2T(mal) being the stronger inducers (Fig. 2). This is consistent with the total IgG responses recorded above for pigs given bivalent constructs. Around 80% of pigs vaccinated with such constructs elicited IgG1 titers ≥ 3 $\log_{10}$ (3.5 ± 0.5 and 3.6 ± 0.7 in Fig. 2A and B, respectively). This rate of specific IgG1 high responders was reduced to 66% (3.2 ± 0.4) in animals immunized with B_4T(thi) (Fig. 2C).

A similar but even stronger reduction in IgG2 titers was recorded for B_4T(thi), compared to the bivalent constructs ($p > 0.05$). Only one pig (16%) given B_4T(thi) showed strong IgG2 titers (≥ 3 $\log_{10}$), while 60–66% of pigs immunized with B_2T(thi) or B_2T(mal) achieved titers of such magnitude (3.3 ± 0.9 and 3.3 ± 0.7, respectively; Fig. 2), i.e., a > 3-fold increase over B_4T(thi)-immunized pigs (2.8 ± 0.7).

In addition, only in B_2T(mal)-immunized, fully protected pigs, a significant positive correlation between specific IgG1 and IgG2 titers ($r = 0.88$, $p < 0.05$) was found. Thus, although all three peptides were able to induce both IgG isotypes, bivalent constructs, mainly B_2T(mal), appear to be capable of switching the immune response from Th2 to Th1.

3.5. IgA anti-FMDV in serum and nasal swabs

The respiratory tract is considered the main target of FMDV

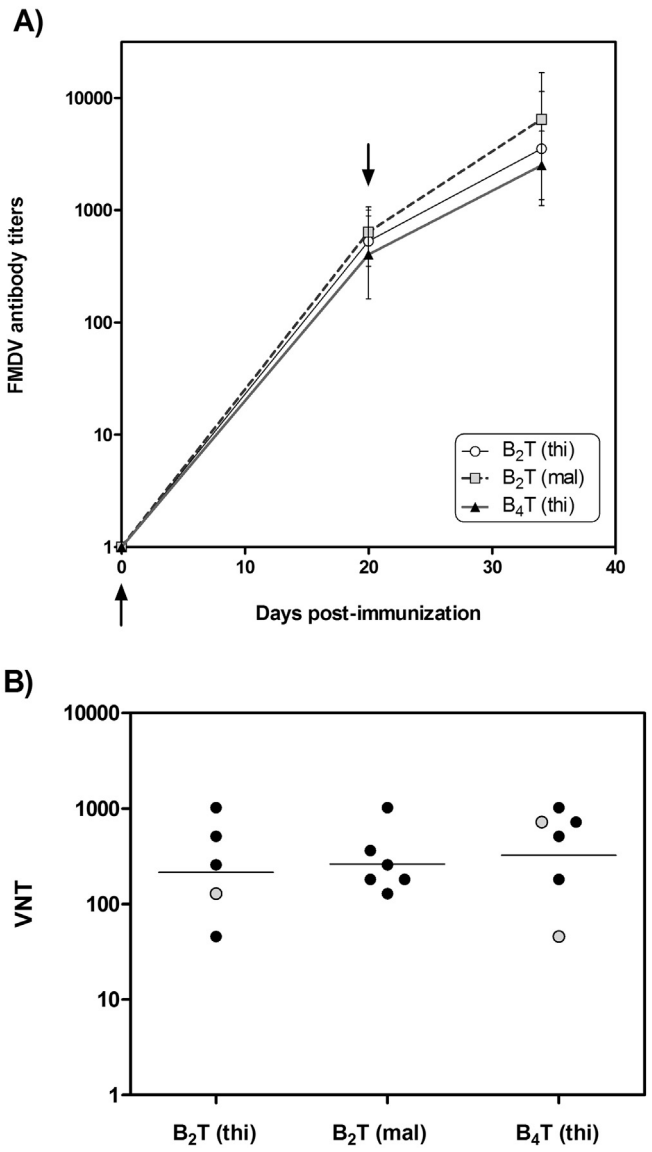


Fig. 1. Antibody responses to FMDV in peptide-immunized pigs: A) anti-FMDV response in sera collected at days 0, 20 (pre-boost) and 34 (post-boost), measured by ELISA. Each point depicts mean antibody titers ± SD for each group of pigs. Arrows show the days of immunization and boost. B) Neutralizing antibody responses at day 34 post-boost. Each symbol represents the value for an individual pig, as detailed in Table S1. Horizontal lines indicate the geometric mean for each animal group. Protected and non-protected animals, as in Table 2, are represented by black or grey circles, respectively. In no case individual spontaneous reactivity was observed in the titers determined at day 0.

infection. Therefore, we investigated the ability of bi- and tetravalent peptides to elicit systemic and local IgA responses. Serum and nasal fluids were collected at day 34 and tested in an IgA-specific ELISA (Fig. 3). Mean IgA titers in sera from B_2T(thi)- and B_2T(mal)-immunized pigs were 3.3 ± 0.9 and 3.2 ± 0.3, respectively (Fig. 3A), i.e., ca. 3-fold higher than those in B_4T(thi)-immunized pigs (2.8 ± 0.6), although differences were not statistically significant ($p > 0.05$).

Likewise, nasal IgA titers elicited by B_2T(thi) and B_2T(mal) were higher than those of B_4T(thi) (Fig. 3B) ($p > 0.05$). Indeed, no detectable local IgA responses was found in two out of six pigs immunized with B_4T(thi) (Fig. 3).

3.6. Peptides elicit FMDV-specific IFN-γ responses

We examined T cell responses at day 34 by ELISPOT analysis of IFNγ-producing PBMCs. High frequencies of IFN-γ-producing cells were found in the B_2T(thi), B_2T(mal) and B_4T(thi) groups (87 ± 32, 82 ± 27, and 40 ± 12, respectively) in response to in vitro recall with homologous peptide (Fig. 4A). Pigs immunized with bivalent B_2T(thi) and B_2T(mal) showed significantly ($p < 0.05$) higher T-cell responses than those with tetravalent B_4T(thi). All IFN-γ responses were specific, as no peptide-driven IFN-γ-producing cells were detected in non-immunized pigs (not shown).

4. Discussion

Developing effective, safe marker vaccines remains a pressing need in FMD control, with peptide-based vaccines increasingly recognized as a sensible alternative towards this goal (Zhang et al., 2011). Herein, we extend previous results with serotype C dendrimeric peptides (Cubillos et al., 2008) to constructs displaying 4 or 2 copies of an established B epitope (GH loop of VP1) from pandemic O/UKG/11/01 (Knowles et al., 2001).

Given the structural minimalism of our immunogen platforms, plus the low sequence homology (~31%) between GH loops of serotypes C and O (YTASARGDLAHLTTTHAR vs. PVTNVRGDLQVLAQKAART, respectively), the extensibility of our approach was non-obvious and required experimental verification. Results show that original B_4T(thi), successfully tested against serotype C in swine (Cubillos et al., 2008), can indeed be extended to serotype O, but with limited protection. Also, in tune with previous data in mice (Monso et al., 2013) but now in an important FMDV host, bivalent B_2T(thi) and B_2T(mal) elicit potent responses with high (80%) and full (100%) protection rates, respectively, that outperform tetravalent B_4T(thi). Interestingly, despite minor variation between bivalent constructs, differences in immunogenicity were noticeable (Monso et al., 2013).

The improved immunogenicity of constructs with multiple vs. single B epitope display has been argued by positing that repetitive antigens induce direct cross-linking of surface Ig receptors in immature B cells (Bachmann et al., 1993) or that epitope multimerization promotes antigen internalization by DCs or other APCs (Zinkernagel, 2003). Although a detailed explanation of the superior immunogenicity of bivalent vs. tetravalent platforms requires further research, bivalent constructs elicit higher IFNγ-producing T cells and one can hypothesize that the more sterically crowded B_4T(thi) structure is less efficiently cross-linked to B-cell receptor, hence less immunogenic (Blanco et al., 2013).

The trend towards higher total IgG titers upon boosting observed in pigs given B_2T(thi) and—to a larger extent— B_2T(mal) was not matched by the neutralizing ability of the sera. Similar mean VNTs were observed upon boosting regardless of peptide structure. Overall, however, ELISA and VNT titers are better clustered in animals immunized with B_2T(mal).

High IgG1/IgG2 ratios have been consistently associated with poor correlation between neutralizing antibody levels and protection in naturals hosts immunized with FMDV peptides (Mulcahy et al., 1990; Taboga et al., 1997). Here, higher IgG2 titers for B_2T(mal) vs. thioether-type platforms, particularly B_4T(thio), is observed. Assuming that porcine IgG2 levels increase in response to cytokines such as IFN-γ and IL-12 (Crawley and Wilkie, 2003), our results suggest association between this Th1-biased isotype balance and improved protection. Opsonization seems to be an important mechanism for FMDV protection (Lannes et al., 2012; McCullough et al., 1988) and IgG2 isotype opsonizes and activates complement more efficiently than IgG1 (Bastida-Corcuera et al., 1999; Crawley and Wilkie, 2003). Whether this applies to porcine

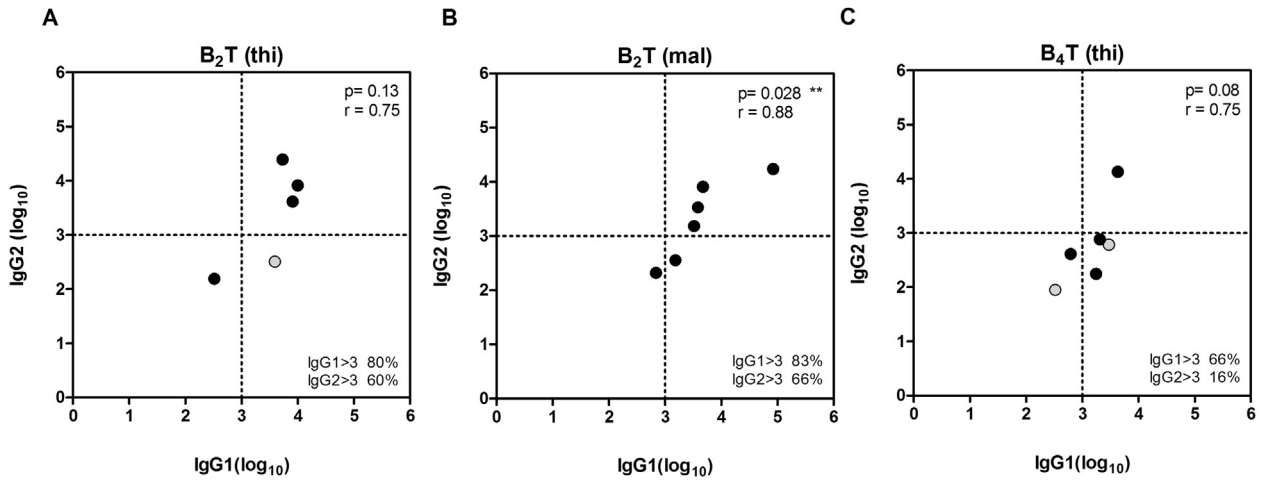


Fig. 2. IgG1 and IgG2 anti-FMDV profiles in pigs immunized with B_2T(thi) (A), B_2T(mal) (B) or B_4T(thi) (C). Endpoint titers are expressed as the reciprocal of serum dilution (log_{10}) giving the absorbance recorded in control wells (sera collected at day 0) + 2 × SD. Each symbol represents the IgG1 and IgG2 titers (X and Y values respectively) for an individual pig, as detailed in Table S1. The lower right quadrant shows the percentage of pigs with titers above than 3 log_{10}. Protected and non-protected animals are represented by black or grey circles, respectively, as in Table 2. In no case individual spontaneous reactivity was observed in the titers determined at day 0.

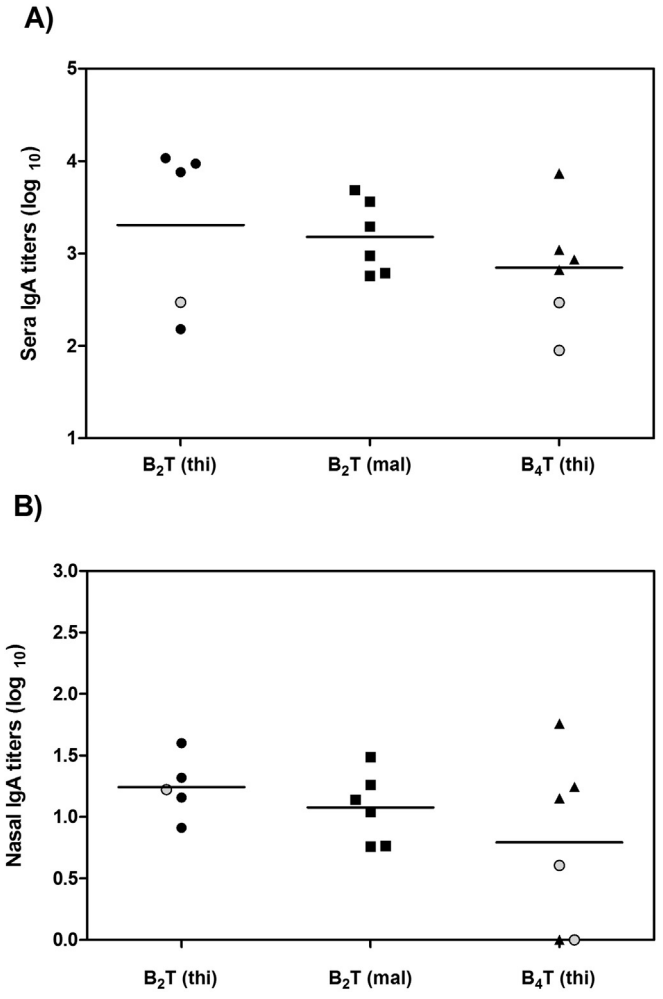


Fig. 3. Systemic and mucosal IgA-specific responses to FMDV in samples collected at day 34 post-immunization. IgA titers in sera (A) and nasal swabs (B) are as in Fig. 2. Horizontal lines indicate the geometric mean for each group of pigs. Protected and non-protected animals, as in Table 2, are represented by black or grey circles, respectively (individual values are detailed in Table S1). No individual spontaneous reactivity was observed in the titers determined at day 0.

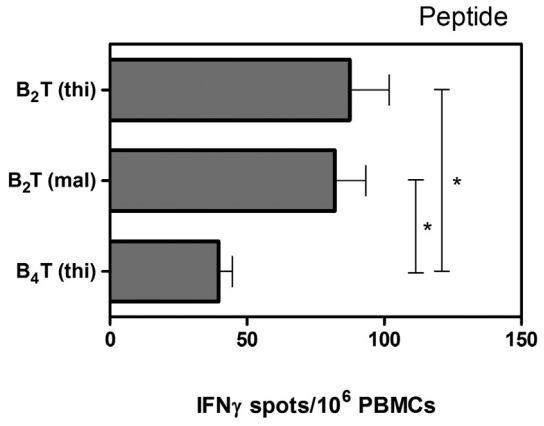


Fig. 4. Specific T-cell responses measured by an ex vivo IFN-γ ELISPOT. IFN-γ release by PBMC stimulated in vitro with the homologous peptide. The frequency of FMDV-specific IFN-γ secreting cells (spots)/10^6 PBMC in each sample was determined as detailed in Table S1. Values represent the mean of triplicates of PBMCs from pigs immunized with the indicated peptide (see individual values in Table S1).

IgG2 remains to be ascertained.

IgG2 and IgA can enhance T cell responses via an FcR-mediated mechanism that facilitates internalization and processing of antibody-complexed antigens by DCs, resulting in a more efficient epitope presentation to CD4+ and CD8+ T cells (Igietseme et al., 2004). The trend towards lower IgG2 and IgA levels elicited by B_4T(thi) could explain its lower protection scores relative to B_2T(thi) and B_2T(mal).

FMDV initiates infection through mucosal surfaces (Alexandersen et al., 2003), hence making effective mucosal IgA stimulation crucial for successful vaccination (Francis and Black, 1983; McCullough and Sobrino, 2004). As observed in swine for serotype C-based B_4T(thi) (Cubillos et al., 2008), and in mice for the same serotype O peptides studied here (Blanco et al., 2013), our constructs elicited significant IgA levels in respiratory tract secretions. IgA titers were likewise found in serum, confirming these peptides, particularly B_2T dendrimers, as good mucosal vaccine candidates, possibly capable of overcoming limitations such as local degradation, physical ejection, etc (Eble et al., 2007).

Production issues also regard consideration. Earlier (Monso

et al., 2013) we showed that the efficient thiol-ene chemistry used to make B_2T(mal) was very advantageous when compared with the sluggish conjugation-plus-purification required for B_4T(thi) (Cubillos et al., 2008). So again B_2T(mal) appears as the most favorable construct in terms of production simplicity, with positive impact in costs and adaptability, e.g., new sequences in emergency situations. Hence, these results taken together, we assuredly recommend B_2T(mal) as an ideal FMDV vaccine candidate in swine, eliciting a potent immune response in both serum and mucosae, and solid protection. A similar appraisal of the three prototypes in cattle, the other main natural FMDV host, is currently under way.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2016.03.005.

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