A bivalent B-cell epitope dendrimer peptide can confer long-lasting immunity in swine against foot-and-mouth disease

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Abstract

Foot-and-mouth disease virus (FMDV) causes a widely extended contagious disease of livestock. We have previously reported that a synthetic dendrimeric peptide, termed B₂T(mal), consisting of two copies of a B-cell epitope [VP1(140–158)] linked through maleimide groups to a T-cell epitope [3A(21–35)] of FMDV, elicits potent B- and T-cell-specific responses and confers solid protection in pigs to type O FMDV challenge. Longer duration of the protective response and the possibility of inducing protection after a single dose are important requirements for an efficient FMD vaccine. Herein, we show that administration of two doses of B₂T(mal) elicited high levels of specific total IgGs and neutralizing antibodies that lasted 4–5 months after the peptide boost. Additionally, concomitant levels of IFN-γ-producing specific T cells were observed. Immunization with two doses of B₂T(mal) conferred a long-lasting reduced susceptibility to FMDV infection, up to 136 days (19/20 weeks) post-boost. Remarkably, a similar duration of the protective response was achieved by a single dose of B₂T(mal). The effect on the B₂T(mal) vaccine of RNA transcripts derived from non-coding regions in the FMDV genome, known to enhance the immune response and protection induced by a conventional inactivated vaccine, was also analysed. The contribution of our results to the development of FMD dendrimeric vaccines is discussed.

KEYWORDS

dendrimer peptide, FMDV, protection, swine, vaccine

1 | INTRODUCTION

Development of safe, cost-effective vaccines is a desirable goal in practically all areas of human and animal health. In the latter field, foot-and-mouth disease (FMD) is paradigmatic of the challenge of designing strategies alternative to the conventional vaccines still used to prevent this highly transmissible, devastating livestock disease (Grubman & Baxt, 2004). Classical FMD vaccines rely on chemically inactivated FMD virus (FMDV) administered with an adjuvant and require about 7 days to induce a protective immunity that requires

re-vaccination after 6–12 months to maintain its protective effect (Doel, 2005; Rodríguez & Grubman, 2009). High levels of circulating neutralizing antibodies are considered as the main correlate for FMDV protection, albeit this relationship is not absolute and differences in resistance to virus challenge can be found in animals with similar levels of neutralizing antibodies (McCullough et al., 1992). Duration of the protective immunity is essential in establishing the fitness of any FMD vaccine (Smitsaert & Bergmann, 2017), and boosting at approximately 6 monthly intervals is often required to maintain a protective response in pigs (Cox, Aggarwal, Statham, & Barnett, 2003). Endogenous or

therapeutically induced type I interferon (IFN) may confer protection until adaptive immunity is fully activated and contribute with its immunomodulatory properties to improve the quality of the specific response elicited (Curiel, Mason, Dryden, Maurer, & Bigley, 1998; Wu, Brum, Caron, Koster, & Grubman, 2003). Transcripts corresponding to non-coding regions (NCR) of FMDV RNA stimulate innate responses and exert an immunomodulatory effect in mice and swine (Borrego et al., 2013, 2015; Rodriguez-Pulido, Borrego, Sobrino, & Saiz, 2011). This immunomodulation can result in the induction of more robust and long-lasting B- and T-cell immune responses elicited by a suboptimal dose of a commercial FMD vaccine, increasing the rate of protection against viral challenge (Borrego et al., 2017).

Subunit vaccines that completely avoid the handling of the infectious agent (Brun et al., 2011; Purcell, McCluskey, & Rossjohn, 2007) constitute the best alternative to conventional ones, particularly in terms of safety. Among the various subunit vaccine approaches, those based on peptides are advantageous, particularly for FMD, due *inter alia* to (a) total absence of infectious agent, hence of any biological risk; (b) joint display of B- and T-cell epitopes on a single scaffold; (c) clear differentiation of infected from vaccinated animals (DIVA condition); (d) efficient chemical synthesis allowing characterization as a pharmaceutical (Blanco, Andreu, & Sobrino, 2017); and (e) simple (no cold chain) and safe transport and storage.

Initial studies raised legitimate concerns about the immunogenicity of FMD peptide vaccines, as linear peptides encompassing B- and/or T-cell epitopes barely achieved protection levels presaging potential commercial application (Bittle et al., 1982; Taboga et al., 1997). Such issues have been addressed by various improved strategies (Rueckert & Guzman, 2012) (Lee et al., 2017), notably

by molecular platforms where B- and/or T-cell epitopes are jointly and multiple displayed. Chief among such strategies are multiple antigenic peptides (MAPs) (Tam, 1988), in which several peptide epitopes are radially tethered to a branching, usually Lys-based core. Our groups' contribution in this regard is a dendrimeric construction, generically termed B_nT, where several copies of an FMDV B-cell epitope from capsid protein VP1 (GH loop, residues around positions 140–160) (Acharya et al., 1989; Pfaff, Mussgay, Bohm, Schulz, & Schaller, 1982) are linked to a Lys matrix elongating into an FMDV viral T-cell epitope corresponding to residues 21–40 from non-structural protein 3A (Blanco et al., 2001). The B-cell epitope shows amino acid variations among different FMDV serotypes, and it elicits neutralizing antibodies that are restricted to antigenically related isolates within a given serotype (Domingo, Baranowski, Escarmis, & Sobrino, 2002). Thus, introduction of GH loop sequences from different FMDVs in our B_nT dendrimeric vaccine platform could increase the protection spectra they confer. The T-cell epitope was selected as it was able to stimulate in vitro PBMCs from outbred pigs sequentially infected with two FMDVs of different serotypes; the responses were mainly abolished by an anti-class II antibody, and it specifically induced FMDV heterotypic neutralization (Blanco et al., 2001). The sequence of this T-cell epitope in 3A is highly conserved among FMDV serotypes, and therefore, it can evoke heterologous responses, hence contributing to extent the antigenic spectrum of the protective response conferred by this dendrimeric peptide.

The initial prototype, B₄T (thi), with *n* = 4 copies of the [VP1(136–154)] B epitope linked to the Lys core plus to the T epitope [3A(21–35)] via thioether bonds (Table 1), elicited high titres of FMDV-neutralizing antibodies in pigs and IFN-γ releasing activated

TABLE 1 B_nT dendrimeric constructs

B _n T prototypes		
Name	B ₄ T (thi) ^a	B ₂ T (mal) ^b
General structure		
MW ^c	11,236.2 Da	6,742.8 Da
Valency	4	2
HPLC ^d	7.1 min (95%)	6.9 min (98%)
B epitope [VP1(140–154)]: PVTNVRGDLQVLAQKAARTC-amide		
T epitope [T3A(21–35)]: AAIEFFEGMVHDSIK-amide		

Note: C-terminal Cys residue of B epitope connected to Lys core via.

^aA thioether (–CH₂–CO–) linkage, or

^bA 3-maleimidopropionic acid unit ()

^cExperimental mass by LC/MS.

^dRetention time on C₁₈ (Luna, 4.6 mm × 50 mm column, 3 μm; Phenomenex) eluted with a 20%–60% linear gradient of 0.036% TFA in MeCN into 0.045% TFA in H₂O over 15 min. In parenthesis, homogeneity of purified material.

T cells, hence performing as an effective vaccine with solid protection against FMDV challenge (Cubillos et al., 2008). Interestingly, and somewhat counter-intuitively, a simplified prototype, B₂T(mal), with $n = 2$ copies of a B epitope—from O/UKG/11/01 isolate (Sumption, Rweyemamu, & Wint, 2008)—linked to the Lys core-[T-cell epitope] by simpler and more effective maleimide-based conjugation (Table 1), elicited potent B- and T-cell-specific responses in swine and confers solid protection to homologous challenge (Blanco et al., 2016). In both B₄T (thi) and B₂T(mal) instances, protection was observed upon initial immunization followed by a peptide boost 3 weeks after. In these previous studies, the protection conferred by B₂T(mal) was assessed in pigs receiving two vaccine doses and FMDV challenge was conducted 20–30 days after the second immunization.

Longer duration of the response and the possibility of inducing protection after a single dose are both critical features for an efficient FMD vaccine (Robinson et al., 2016) and have not been addressed in previous studies with dendrimeric vaccines (Blanco et al., 2016; Cubillos et al., 2008). The objective of this study has been the assessment of the duration of the protective response elicited by B₂T(mal) (thereafter B₂T). We have shown that upon administration of two B₂T doses, high levels of neutralizing antibodies and IFN- γ -producing specific T cells were detected up to 22 weeks (5 months) post-immunization (pi). This effect was not substantially modified when B₂T was given with an immunomodulatory transcript corresponding to the IRES NCR of FMDV RNA. In tune with the long-lasting antibody levels, a lower susceptibility to FMDV afforded by B₂T was observed up to 19 weeks (~4.5 months) pi in 3 out of 4 pigs. Remarkably for vaccine purposes, long-lasting and protective B- and T-cell responses were also observed in pigs that received a single dose of B₂T.

2 | MATERIALS AND METHODS

2.1 | Peptides

Peptide B₂T, displaying B- and T-cell epitopes of FMDV O/UKG/11/01, was made by conjugation in solution of two precursors, previously assembled by solid-phase synthesis and purified to homogeneity by HPLC: (a) a B epitope (VP1, residues 140–158) with an additional C-terminal Cys (free thiol form) and (b) a T epitope (3A, residues 21–35), N-terminally elongated with two Lys residues followed by an additional Lys branching into two maleimide groups (Table 1). The B₂T construct (10 mg) was obtained in a clean, efficient thiol-maleimide ligation (94% of conversion) at pH 6.0, purified by HPLC (collected fractions above 95% of purity) and characterized by mass spectrometry (Blanco et al., 2016; Cubillos et al., 2008; Monso, de la Torre, Blanco, Moreno, & Andreu, 2013).

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

(IBRS-2 cells) was used. The resulting viral stock maintained the consensus sequence at the capsid region and 1.6×10^4 plaque-forming units (PFU) was shown to produce overt FMD clinical signs when intradermal inoculated in pigs (Blanco et al., 2016).

2.3 | Animals and experimental design

The immune response to B₂T was assessed in fifteen 9- to 12-week-old White cross-bred Landrace female pigs (~20 Kg). Experimental procedures were in accordance with the guidelines of directive 2010/63/EU for animal experiments, approved by the INIA Committees on Ethics of Animal Experiments and Biosafety permit numbers (CBS2014/015 and CEEA2014/018) and by the National Committee on Ethics and Animal Welfare (PROEX 218/14). Unless otherwise stated, animals were housed in a conventional farm facility at Departamento de Reproducción Animal, INIA. In a first experiment, a group of four animals (pigs 76, 77, 78 and 79) was immunized twice at days 0 and 39 pi by intramuscular injection with 2 ml of Montanide ISA 50V2 emulsion (Seppic) containing 2 mg of B₂T. Two additional pigs were PBS-inoculated and kept as controls. Blood samples were collected at days 1, 14, 21, 28, 91, 126 and 154 p.i. for serum separation and peripheral blood mononuclear cells (PBMCs) purification. In a second experiment, eight pigs were randomly assigned to two groups of four animals, which were immunized at day 0 and boosted, due to logistical considerations, at day 29 pi. In one group, the four animals (pigs 1435, 1437, 1447 and 1445) were immunized and boosted with 2 mg of B₂T + 500 μ g of RNA transcript corresponding to the IRES of FMDV (Borrego et al., 2017). In the other group, two animals (pigs 1449 and 1436) were immunized twice with 2 mg of B₂T alone, and two more (pigs 1443 and 1438) received a single dose of 2 mg of B₂T at day 29. One additional animal (pig 1439) was inoculated with RNA transcript alone and kept as control. Blood samples were collected at days 10, 15, 29, 50 and 100 p.i. Animals were transferred to high-containment facilities at CISA where each of the two groups was located in a separate unit and challenged at day 165 pi [136 days post-boost (pb)] with 1.6×10^4 pfu of FMDV O/UKG/11/01, by inoculation at two sites of the coronary matrix of both main claws of the right-forefoot pad (0.1 ml/site). As control, pig 1439, which remained negative in all the assays performed, was housed independently and similarly challenged. Animals were monitored for clinical signs of disease during 10 days and then euthanized. The criteria used to distinguish among protected, partially protected and non-protected animals were based on the lesion score, as shown in Table 2.

2.4 | Virus neutralization

Neutralization assays were performed in 96-well culture plates. Serial twofold dilutions of each serum sample (in DMEM containing 2% foetal bovine serum) were incubated with 100 infection units—50%

TABLE 2 Evidences for protection in immunized pigs

Inoculum	Animal	VNT ^a	3ABC ELISA ^b	Fever ^c	Lesion Score ^d	Other signs ^e	Protected ^f
B ₂ T (2 doses)	1436	3.8	7	40.2 (5)	0	7	++
	1449	4.1	12	40.5 (7)	3 (6)	3	+
B ₂ T (1 dose)	1438	1.9	12	39.6 (7)	2 (7)	7	+
	1443	1.1	6	41.1 (4)	6 (3)	3	–
B ₂ T + RNA (2 doses)	1435	1.1	7	39.3 (5)	4 (3)	3	–
	1437	3.1	12	39.2 (3)	1 (3)	3	++
	1445	2.6	7	40.0 (3)	3 (3)	3	–
	1447	1.5	7	39.9 (3)	3 (3)	3	–
None	1439	1.1	7	39.7 (3)	5 (3)	2	–

^aAt the day of challenge.^bDay of first positive ELISA. Sera tested at 7 and 12 days pc, except animal 1443 sampled at day 6 pc, then euthanized.^cTemperature (°C) and (in parenthesis) day pc when maximum temperature registered. No fever: ≤39.0°C–39.5°C.^dAnimals monitored up to 14 days pc for lesions. Lesion score (maximum value of 7): 1 point/vesicle in foot other than inoculated (up to 3 points); 1 point/mouth, tongue or snout lesion; and 1 point/>2 lesions of diameter ≥10 mm. In parenthesis, day pc when lesions first observed.^eDay pc when lameness, ataxia and/or anorexia first observed.^fAnimals considered fully protected if lesion score ≤ 1. (++) fully protected: lesion score < 1; (+) partially protected: mild/delayed disease; and (–) non-protected.

tissue culture infective doses (TCID₅₀)—of FMDV O/UKG/11/01 for 1 hr at 37°C. Then, a cell suspension of IBRS-2 cells in DMEM was added and plates were incubated for 72 hr. Monolayers were then controlled for development of cpe, fixed at 96 hr pi and stained. Virus neutralization titres (VNTs) were calculated as the reciprocal of the final serum dilution that neutralized 100 TCID₅₀ of homologous FMDV in 50% of the wells.

2.5 | Detection of anti-FMDV antibodies by ELISA

Specific antibodies were assayed by ELISA as described (Blanco et al., 2016) using plates coated with peptide B (1 µg) that were incubated with threefold dilutions of serum and detected using HRP-conjugated protein A. Plates were read at 450 nm, and titres expressed as the reciprocal of the last serum dilution giving an absorbance range of two standard deviations above the background (serum at day 0) plus 2 SD.

Specific IgG1 and IgG2 titres to peptide B were measured and expressed as above using isotype-specific mAbs (Serotec) that were detected with HRP-labelled anti-mouse (Thermo Fisher). Antibodies against the non-structural protein precursor 3ABC were detected basically as described (Blanco et al., 2016; Borrego et al., 2017).

2.6 | PBMC isolation and IFN-γ detection by ELISPOT

Porcine PBMCs were isolated from blood samples collected in Vacutainer tubes EDTA-K2, diluted 1:1 in PBS and then

used to obtain PBMC by density-gradient centrifugation with Histopaque 1077 (Sigma) and Leucosep tubes (Greiner Bio-One) as described (Borrego et al., 2017). For the IFN-γ ELISPOT assay, 2.5 × 10⁵ PBMCs were shed in triplicate wells of Immobilon-P plates (Merck Millipore) coated as reported (Blanco et al., 2016) and in vitro stimulated with 50 µg/ml of their respective immunogenic peptides. As controls, cells were incubated with 10 µg/ml of phytohaemagglutinin (Sigma) or with medium alone. After 48 hr at 37°C and 5% CO₂, plates were washed and incubated with a biotinylated anti-mouse IFN-γ (clone P2C11, BD) followed by HRP-streptavidin (BD). The frequency of peptide-specific T cells was expressed as the mean number of spot-forming cells/10⁶ PBMCs, with background values (number of spots in negative control wells) subtracted from the respective counts of stimulated cells. These experiments were performed using outbred domestic pigs with different individual genetic backgrounds. In any case, the levels of animal-to-animal variation did not exceed those observed in other related studies (Taboga et al., 1997).

2.7 | Statistical analyses

Differences among peptide-immunized groups in FMDV-antibody titres and number of IFN-γ-producing cells were analysed using the Student's *t* test. Values are cited in the text as mean ± SD. All *p* values are two sided, and *p* values < .05 were considered significant. Statistical analyses were conducted using GraphPad Prism Software 5.0.

3 | RESULTS

3.1 | Peptide B₂T elicits long-lasting specific antibody response and activation of T cells

To explore the duration and quality of the immunity elicited by B₂T, four domestic pigs (numbered 76, 77, 78 and 79) were immunized and boosted (day 39) with B₂T peptide and subjected to a longitudinal analysis of specific antibody elicited. A consistent and long-lasting response of total IgG antibodies specific to peptide B was observed in the serum from all pigs from day 14 pi (Figure 1a). Antibody titres in the four animals were high, reaching ca. 4 log units, and were maintained up to day 154 pi. In addition, similar titres of peptide-specific IgG1 and IgG2 antibodies were observed at days 28 and 154 pi (Figure 1b,c), as reported for anti-virus IgG1 and IgG2 in B₂T-immunized pigs (Blanco et al., 2016). Consistently, significant VNTs were also observed from day 14 pi, which increased in all four animals after booster vaccination (day 91 pi) (Figure 1d). Interestingly, by day 154 pi, average VNTs similar to those observed before boost were detected and maintained up to day 184 pi (~20 weeks pb; data not shown). As expected, control animals did not show any specific humoral response.

When the induction of circulating IFN- γ -producing T cells was analysed, a significant frequency of B₂T-specific spots was observed in all immunized animals from day 14 up to day 154 pi (Figure 1e). Significant responses were also observed when peptide 3A(21–35) was used to stimulate the cultures (Figure 1f), confirming the recognition of 3A(21–35) as a T-cell epitope by porcine lymphocytes. Upon boost, this epitope-specific response was also observed in three out of four animals.

These results demonstrate that administration of two doses of B₂T evokes long-lasting FMDV-neutralizing antibodies and T-cell responses.

3.2 | Co-administration of a transcript corresponding to the FMDV IRES does not modify the response elicited by B₂T

RNA transcripts mimicking structural domains in the NCR of the FMDV RNA act as potent type I IFN inducers and can enhance the immunogenicity and protection evoked by a suboptimal dose of a conventional FMD vaccine in swine (Borrego et al., 2017). To assess the effect of the combined administration of a transcript from an FMDV NCR on the quality and duration of the protective immunity elicited by B₂T peptide, eight domestic pigs were immunized with either peptide alone or in combination with 500 μ g of RNA transcripts corresponding to the FMDV IRES (see Table 2). The four animals immunized with B₂T + RNA (pigs 1435, 1437, 1447 and 1445) were similarly boosted at day 29 pi. In addition, two animals (pigs 1449 and 1436) were immunized twice with B₂T alone, and two more (pigs 1443 and 1438) received a single dose of B₂T at day 29. A consistent response of total IgG antibodies specific to peptide B was observed in serum for all animals (Figure 2a,b). In pigs receiving B₂T + RNA, titres in the 2.5 to 3.5 range were observed before boost (Figure 2a). Upon peptide boost, an

increase in antibody titres was only observed in two animals (1437 and 1445). Among the animals immunized twice with B₂T alone, a similar response was observed in pig 1449, while pig 1436 showed higher titres both before peptide boost and at day 50 pi (Figure 2b). The two pigs that received a single B₂T dose (1443 and 1438) elicited an IgG response at day 21 pi (corresponding to day 50 pi in Figure 2b) similar to those of animals 1445, 1447 and 1435 before boost, and these titres were maintained 80 days after immunization (day 100 pi in Figure 2b).

The ratio of IgG1- and IgG2-specific antibody titres was similar in the animals studied with the exception of pig 1445 that showed 10-fold higher IgG2 titres at day 98 pi (Figure 2c,d). This observation is similar to that reported for other B₂T-immunized pigs (Blanco et al., 2016).

The kinetics of VNT (Figure 2e,f) paralleled that of total IgG (Figure 2a,b). In all animals, consistent titres were found from day 15–21 pi up to day 154 pi and a clear anamnestic response, associated with the highest IgG titres (see Figure 2a), was noticed 20 days after the second dose (day 49 pi) in three of the four animals boosted with B₂T + RNA (pigs 1437, 1445 and 1447). Such secondary response was more marked in pigs boosted with B₂T alone (1449 and 1436) (Figure 2f), whose average VNTs were higher than those found in animals immunized with B₂T + RNA (Figure 2e). This difference could be, at least in part, due to the immunomodulatory effect of the RNA.

Stimulation of specific T cells was also observed, with high frequencies of IFN- γ -producing cells being detected (days 21 and 49 pi) in the four pigs immunized with B₂T + RNA and in one of the animals (pig 1449) immunized with B₂T alone (Figure 2g,h), which is consistent with the induction of a memory T-cell response. In average, pigs immunized with B₂T + RNA showed higher levels of IFN- γ -producing cells than those receiving B₂T alone. In both groups, no correlation was observed between the frequency of IFN- γ -producing cells and total IgG titre or VNT, as animals showing higher frequencies (1435 and 1449), particularly at day 49 pi, did not elicit higher antibody titres (Figure 2a,b).

Altogether, these results confirm that administration of two doses of B₂T confers long-lasting FMDV-specific antibody and T-cell responses, which are not substantially modified by co-administration of the IRES RNA transcript. Most remarkably, a single immunization with B₂T alone elicits high neutralizing antibody levels that remain detectable 5 months pi.

3.3 | B₂T peptide conferred a reduced susceptibility to FMDV at late times after immunization

The three groups of pigs immunized with one or two doses of B₂T alone or two doses of B₂T + RNA, respectively, were FMDV challenged at day 165 pi (136 days pb). As expected, control pig 1439, inoculated with IRES transcript alone, showed full FMD signs upon challenge, including vesicles in the snout and all four feet from day 3 post-challenge (pc) (Table 2). Among animals immunized with B₂T, pig 1443 (given a single dose) developed full FMD signs (like control pig 1439) and was considered as non-protected. Pig 1436 was protected while 1438 and 1449 were considered partially protected, having developed vesicles and mild clinical

FIGURE 1 Immune responses in B₂T-immunized animals (pigs 76, 77, 78 and 79): (a) anti-B peptide antibody titres in sera collected at days 0, 7, 14, 21, 28, 91, 126 and 154 pi measured by ELISA. Each point depicts mean antibody titres for each group of pigs. Arrows show the day of boost. IgG1 (b) and IgG2 (c) anti-B peptide titres in sera collected at days 28 and 154 pi. End point titres are expressed as the reciprocal of serum dilution (log₁₀) giving the absorbance recorded in control wells (sera collected at day 0) + 2 × SD. Horizontal lines indicate the geometric mean for each animal group. (d) Neutralizing antibody responses (VNTs) in sera samples as in (a). Each symbol represents the value for an individual pig. Horizontal lines indicate the geometric mean for each animal group. Two PBS-inoculated control animals did not show any specific humoral response. (e,f) Specific T-cell responses measured by an ex vivo IFN-γ ELISPOT. IFN-γ released by PBMC and stimulated in vitro with B₂T (e) or T-cell peptide 3A(21–35) (f). The frequencies of FMDV-specific IFN-γ-secreting cells (spots)/10⁶ PBMC at the days indicated in (a) are shown. Values represent the mean of triplicates of PBMCs from immunized pigs. *Denotes the statistical difference found among the groups compared (panels a–f) using Student's *t* test (*p* < .05)

signs from day 6 pc. This limited protection could be partially due to continued exposure to FMDV released by non-protected pig 1443. In the B₂T + RNA group, two of the four animals (1445 and 1447) showed fewer vesicles and milder clinical signs than those of control 1439, while pig 1437 was protected. In general, a correlation was observed between high VNT at the day of challenge and the level of protection (Table 2). Same as with VNTs levels, immunization with B₂T + RNA did not improve the protective response relative to B₂T alone (two doses and adjuvant in either case).

4 | DISCUSSION

Assuming the limited number of animals studied, which came imposed by the reduced availability at the BSL3 animal facility for

FMDV challenge, and that additional studies with a higher number of animals will be necessary to further confirm the statistical significance of our observations, our results support that a long-lasting specific immune response associated with reduced susceptibility to FMDV infection was observed at least 4.5 months after the last immunization, not only in two animals immunized with two doses of B₂T alone but also in one of two pigs immunized once with B₂T.

The duration of the protective immunity conferred by dendrimer B₂T was close to those elicited by conventional vaccines (Robinson et al., 2016) and that recently reported for cattle immunized with a deficient human adenovirus vector expressing FMDV empty capsids (Sitt et al., 2019). Indeed, in some FMD-endemic regions, for example South America, manufacturers are required to show that oil-adjuvanted conventional vaccines are able to induce protective antibody levels for 180 days after one dose and

FIGURE 2 Immune responses in pigs immunized with B₂T alone or in combination with RNA transcripts from the NCR of the FMDV RNA. Total IgG-specific anti-FMDV antibodies measured by ELISA in sera collected at days 10, 15, 29, 50 and 100 pi from animals immunized with B₂T + RNA (a) or B₂T alone (b). Each point depicts mean antibody titres ± SD for each group of pigs. Arrows show the day of boost. IgG1 (c) and IgG2 (d) anti-FMDV antibodies in sera collected at day 98 pi. End point titres are expressed as the reciprocal of serum dilution (log₁₀) giving the absorbance recorded in control wells (sera collected at day 0) + 2 × SD. (e,f) Neutralizing antibody responses (VNTs) in sera samples as in (a) or (b), respectively. Each symbol represents the value for an individual pig. Specific T-cell responses measured by an ex vivo IFN-γ ELISPOT. IFN-γ released by PBMC and stimulated in vitro with B₂T at days 21 pi (g) and 49 pi (h). The frequencies of FMDV-specific IFN-γ-secreting cells (spots)/10⁶ PBMC are indicated. Values represent the mean of triplicates of PBMCs from immunized pigs. The statistical analysis of the differences found among the groups compared (panels A–H) is shown in Figure S1

for 1 year after revaccination, performed 6 months after the first dose (Smitsaart & Bergmann, 2017).

In addition, when the RNA transcripts were delivered together with B₂T, no significant improvement was observed in terms of the VNT elicited or the level of protection conferred by B₂T alone. Interestingly, RNA delivery increased the average amount of IFN- γ -producing specific cells, suggesting that the subset of T cells being activated in the presence of RNA transcripts did not provide a better T-cell help. RNA transcripts corresponding to the FMDV IRES induce a TLR- and RLR-engaged response in pigs being mainly sensed by RIG-I (Borrego et al., 2015). Our results suggest that IRES transcripts might decrease the specific humoral response to our dendrimeric vaccine, which could be consistent with the lack of positive impact on VNT (Figure 2). Thus, the use of immunostimulatory molecules in order to achieve better and longer responses to peptide-based vaccination needs further study, such as exploring the stimulatory activity of different cytokines other than IFN-I as an alternative to improve the promptness and duration of protective immunity induced by dendrimers. Likewise, the use of alternative adjuvants and/or the incorporation of new T-cell epitopes already identified in FMDV proteins (Blanco et al., 2001; Garcia-Briones et al., 2004) in the B₂T dendrimers could allow improvement of the protective response they confer. Such an improvement could be also achieved by the combined administration, either in a single shoot or in a prime-boost regime, of B₂T dendrimers with inactivated FMDV or recombinant immunogens such as purified empty capsids or expressed in adenovirus vectors (Rodríguez & Grubman, 2009).

Besides the advantages commented in the Introduction, peptide vaccines, such as our B₂T dendrimers, can provide strategic advantages for FMD control. As peptide synthesis is much faster than production of FMDV inactivated vaccines, peptide vaccines can play a pivotal role in a front-row, swift response to emerging FMDV strains in the context of a global control strategy for this disease. Prompt access to peptide vaccines can also be a remedial FMD control strategy in the event of conventional vaccine supply shortage, particularly in FMD-free areas such as the EU, USA, Canada or Australia, where a restriction in vaccine production plants means that vaccine supply mainly depends on the limited number of current FMD vaccine banks.

Overall, the results reported here provide additional evidence supporting the potential use of B₂T dendrimers as candidates in new FMD vaccine formulations.

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DATA AVAILABILITY STATEMENT

This is a revisions of a paper originally submitted prior to the policy's adoption.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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