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Poly(I:C) combined with multi-epitope protein vaccine completely protects against virulent foot-and-mouth disease virus challenge in pigs

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ABSTRACT

We designed a series of epitope proteins containing the G–H loops of three topotypes of foot-and-mouth disease virus (FMDV) serotype O and promiscuous artificial Th sites and selected one epitope protein (designated as B4) with optimal immunogenicity and cross-reactivity. Three out of five pigs immunized intramuscularly with this B4 were protected against virulent FMDV challenge after a single inoculation, while all pigs co-immunized with B4 and polyinosinic–cytidylic acid [poly(I:C)] conferred complete protection following FMDV challenge. Additionally, we demonstrated that all pigs co-immunized with B4 and poly(I:C) elicited FMDV-specific neutralizing antibodies, total IgG antibodies, type I interferon (IFN-α/β) and cytokines IFN-γ. In contrast, some pigs immunized with B4 alone produced parameters mentioned above, while some not, suggesting that poly(I:C) reduced animal-to-animal variations in both cellular and humoral responses often observed in association with epitope-based vaccines and up-regulated T-cell immunity often poorly observed in protein-based vaccines. We propose that poly(I:C) is an effective adjuvant for this epitope-based vaccine of FMDV. This combination could yield an effective and safe candidate vaccine for the control and eradication of FMD in pigs.

1. Introduction

Foot-and-mouth disease virus (FMDV) causes a highly infectious disease of cloven-hoofed animals that has significant global socioeconomic impact. Foot-and-mouth disease (FMD) severely affects the welfare and productivity of high-value farm animals that are important to food security, including cattle, sheep and pigs (Alexandersen et al., 2003). Due to the devastating economical consequences that could arise from an FMD outbreak, this disease is considered a potential bioterrorism weapon (Hietela and Ardans, 2003).

Vaccination against FMDV is a major strategy to control the disease in endemic areas. Current FMDV vaccines are serotype-specific and consist of inactivated virus formulated in oil or aluminum hydroxide adjuvants (Doel, 2003). Although these vaccines can induce strong protective humoral immunity, there are major drawbacks associated with their use, namely, the requirement of propagating virulent virus in containment facilities and the associated risk of escape from manufacturing sites (Barteling and Vreeswijk, 1991), the failure of differentiating infected from vaccinated animals (DIVA) (Mackay et al., 1998; Sorensen et al., 1998; Shen et al., 1999). For all these reasons, much effort has been made to develop alternative vaccines that are both efficient and safe based on either recombinant proteins, peptides, replicating vectors or plasmid DNA (Grubman, 2005).

Epitope-based vaccines are promising candidates for the control of viral diseases by vaccination (Wang et al., 2002; Shao et al., 2011), as they pose no risk for pathogen replication and, if appropriately formulated, they may function as DIVA vaccines. An epitope vaccine should ideally include epitopes recognized both by B and T cells, and take into account the MHC restriction of the T-cell response. The development of epitope vaccine for FMDV has been limited mainly by difficulties associated with poor immunogenicity of simple peptide and the hypervariability of the immunodominant G–H loop domain. In order to overcome these restrictions, we have designed a series of FMDV multi-epitope proteins with different composition and permutation of the neutralization antigenic epitopes from three lineages of O-type FMDV. Two universal T-cell epitopes were also used to enhance the immunogenicity of these multi-epitope proteins.

Poly(I:C) is a synthetic double-stranded polyribonucleotide and an interferon (IFN) inducer (Longhi et al., 2009), which has long been known as an antiviral agent. Poly(I:C) can also serve as an

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effective adjuvant for improving humoral and cellular immunity with protein-based vaccines (Tewari et al., 2010). Previously, we demonstrated that the administration of multi-epitope protein with poly(I:C) as an adjuvant could significantly increase the neutralizing antibody response against FMDV in mice (Cao et al., 2012). In this study, we further detected the adjuvant effects of poly(I:C) on a selected epitope protein vaccine in a virulent FMDV challenge model in pigs.

2. Materials and methods

2.1. Design and synthesis of the multi-epitope genes

To enhance the immunogenicity of the multi-epitope proteins, two universal T-cell epitopes were used, namely, a pan-human leukocyte antigen DR-binding peptide (PADRE) (Agadjanyan et al., 2005) and an invasin immunostimulatory sequence taken from *Yersinia* (Invasin) (Wang et al., 2002). The entire G–H loop domain and C-terminal of VP1 structural protein of O/Mya/98 (Southeast Asia topotype), O/HN/CHA/09 (Cathay topotype), O/HN/CHA/93 (vaccine strain of Cathay topotype), O/Tibet/99 (PanAsia topotype) and O/IRN/2010 (PanAsia 2 topotype) FMDVs (Table 1) were linked together by two glycine (G) residues according to the order in Table 2. Three different DNA sequences of the designed multi-epitope proteins (Table 2) were synthesized by GenScript Incorporation (www.genscript.com) according to the most commonly occurring codons in *Escherichia coli*.

2.2. Construction and expression of the multi-epitope genes

The synthesized genes were subcloned into the prokaryotic expression vector pET-28a(+) (Merck, Darmstadt, Germany) using the *Nco* I and *Hind* III restriction sites to yield the recombinant expression plasmids pET-B2, pET-B3 and pET-B4. The fusion proteins produced by these recombinant expression plasmids were named correspondingly B2, B3 and B4.

The fusion proteins were produced in *Escherichia coli* BL21 (DE3) cells (Merck, Darmstadt, Germany). The bacteria were grown to an OD_{600} of 0.4–0.6, followed by induction with 1 mM of isopropyl-b-D-thiogalactoside (IPTG) for 5 h at 37 °C. The cells were harvested by centrifugation and the pellet was analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Cao et al., 2009).

For the Western blot analysis, the separated proteins were electro-transferred onto a nitrocellulose membrane for 2.5 h at 160 mA. The membrane was blocked and then incubated with anti-FMDV cattle serum (1:160). The serum was collected at 60 dpi from a cattle infected with FMDV, strain O/Mya/98. After several washes, the membrane was incubated with horseradish peroxidaseconjugated anti-cattle IgG (1:20,000; Sigma, St. Louis, MO). The reaction was visualized using a 3, 3′, 5, 5′-tetramethylbenzidine substrate (Sigma).

2.3. Vaccine preparation

Cells induced by IPTG were resuspended and disrupted by sonication. The inclusion bodies were pelleted and dissolved in solubilization buffer (50 mM *N*-cyclohexyl-3-aminopropanesulfonic acid and 4.5 M urea, pH 11.0). The solution was centrifuged at 10,000*g* for 10 min and the supernatant was purified by Ni–NTA His Bind Resins (Merck, Darmstadt, Germany). The purified proteins were dialyzed with 20 mM Tris–HCl (pH 8.5) containing 0.1 mM dithiothreitol for 24 h and quantified by a BCA Protein Assay Kit (Generay Biotech, Shanghai, China).

2.4. Animal inoculation

Female BALB/c mice (6–8 weeks old) were purchased from the Animal Center, Lanzhou Institute of Biological Products, Lanzhou, China. Commercial inactivated FMDV type O vaccines were provided by China Agricultural Vet. Bio. Science and Technology, Lanzhou, China. All epitope vaccine was emulsified with ISA 206 adjuvant (Seppic, Shanghai, China) before vaccination.

Animal studies were approved by the Review Board of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Permission No.: SYXK-GAN-2004-0005). All animals used in the present study were humanely bred during the experiment and euthanasia was carried out at the end of the experiment.

Table 1
G–H loop and C-terminal of VP1 from prevalent FMDV O isolates.

Epitopes	Sequence	Origin of epitopes
PADRE	AKFVAAWTLKAAA	Universal T cell epitopes (Agadjanyan et al., 2005)
$VP1_{132-160}$	GSSKYGDTSTNNVRGDLQVLAKKAERALP	G–H loop of O/HN/CHA/09 (Cathay topotype)
$VP1_{193-211}$	AIQPSTARHKQKIVAPAKQ	C-terminal of O/HN/CHA/09 (Cathay topotype)
$VP1_{132-160}$	GNCKYGESPVTNVRGDLQVLAQKAARTLP	G–H loop of O/Tibet/99 (PanAsia topotype)
$VP1_{193-211}$	AIHPNEARHKQKIVAPVKQ	C-terminal of O/IRN/2010 (PanAsia 2 topotype)
$VP1_{132-160}$	GDCKYGESRTTNVRGDLQVLAQKAATTLP	G–H loop of O/IRN/2010 (PanAsia 2 topotype)
$VP1_{132-160}$	GSCKYSDARVSNVRGDLQVLAQKAERALP	G–H loop of O/HN/CHA/93 (Cathay topotype)
$VP1_{194-211}$	IQPSDARHKQKIVAPAKQ	C-terminal of O/HN/CHA/93 (Cathay topotype)
$VP1_{132-160}$	GNCKYAGGSLTNVRGDLQVLDQKAARPLP	G–H loop of O/Mya/98 (Southeast Asia topotype)
$VP1_{193-211}$	AVHPSAARHKQKIVAPVKQ	C-terminal of O/Mya/98 (Southeast Asia topotype)
Invasin	TAKSKKFPSYTATYQF	Universal T cell epitopes (Wang et al., 2002)

Table 2
The composition of multi-epitope proteins.

Epitopes	Amino acid sequence
B2	PADRE–GG–(O/HN/CHA/93)$VP1_{132-160}$–GG–(O/HN/CHA/93)$VP1_{194-211}$–GG–(O/HN/CHA/09)$VP1_{132-160}$–GG–(O/Mya/98)$VP1_{132-160}$ –GG–(O/Mya/98)$VP1_{194-211}$ –GG–Invasin–6×His
B3	(O/HN/3/09)$VP1_{132-160}$–GG–(O/HN/3/09)$VP1_{193-211}$–GG–PADRE–GG–(O/Tibet/99)$VP1_{132-160}$–GG–(O/IRN/2010)$VP1_{193-211}$–GG–(O/IRN/2010)$VP1_{132-160}$–GG–Invasin–GG–(O/Mya/98)$VP1_{132-160}$–(O/Mya/98)$VP1_{193-211}$–6×His
B4	PADRE–GG–(O/HN/CHA/09)$VP1_{132-160}$–GG–(O/HN/CHA/09)$VP1_{193-211}$–GG–(O/Tibet/99)$VP1_{132-160}$–GG–(O/IRN/2010)$VP1_{193-211}$–GG–(O/IRN/2010)$VP1_{132-160}$–GG–(O/Mya98)$VP1_{132-160}$–GG–(O/Mya98)$VP1_{193-211}$–GG–Invasin–6×His

2.4.1. Comparison of the immunogenicity of B1, B2, B3 and B4 in mice

Two separate experiments were carried out in mice model for selection of functional multi-epitope protein. In Experiment 1, 32 mice were divided randomly into four groups with eight mice per group. Two groups were injected intramuscularly with 50 μg of B1 epitope protein described previously (Cao et al., 2012), and B2 epitope proteins at days 0, 21, respectively. The two groups were injected intramuscularly with 0.2 ml of phosphate-buffered saline (PBS) as negative control or 0.2 ml of commercial inactivated type O FMDV (O/HN/CHA/93) vaccine containing approximately 2.0 μg/ml whole virus antigen as positive control.

Serum samples were prepared and pooled for each group on days 21 and 35 post-vaccination and analyzed for neutralizing antibody titers by using a micro-neutralization assay with monolayers of BHK-21 cells (Cao et al., 2009). The epitope protein with the highest neutralizing antibody titers was selected for further experiments.

In Experiment 2, 30 mice were divided randomly into five groups with six mice per group. Three groups were injected intramuscularly with 50 μg of selected epitope protein in experiment 1(B2), B3 and B4 at day 0, 21, respectively. The two groups were injected intramuscularly with 0.2 ml of phosphate-buffered saline (PBS) or 0.2 ml of commercial inactivated vaccine of type O FMDV (O/HN/CHA/93). Serum samples were prepared and pooled for each group on days 21 and 42 post-vaccination and analyzed for neutralizing antibody titers against different lineages of O type FMDV. The epitope protein with the highest neutralizing antibody titers was selected for further experiments.

2.4.2. Evaluation of the immunogenicity of selected multi-epitope protein in pigs

Fifteen white pigs aged 60 days, approximately 30–40 kg, were selected from clinical healthy pig herd without vaccination with FMDV vaccine. Before experiment all pigs were tested to be negative for antibodies against serotype O and Asia 1 FMDV by liquid phase blocking ELISA (LPB-ELISA) and against FMDV non-structural protein (NSP) 3ABC by a 3ABC-ELISA test. All these pigs were separated randomly into three groups with five pigs per group: Group 1 was inoculated intramuscularly with 300 μg of selected epitope protein (B4); Group 2 was inoculated with 300 μg of B4 plus 300 μg of poly(I:C) (Sigma); Group 3 was inoculated with 2 ml PBS as negative control. Serum samples were prepared and pooled for each group on days 7, 14, 21, 28 post-vaccination and used for serological assays. Whole blood samples were also collected 28 days post-vaccination for peripheral blood mononuclear cells (PBMC) isolation. On the same day, they were transferred to the Biosafety Level 3 containment facility of our Institute and were challenged by muscularly injected at ear rear with 2000 PID50 of pig-adapted O/Mya/98 FMDV. Swine were examined daily for clinical signs for 10 days post-challenge. This FMD virus challenge model have been well developed for FMD vaccine potency test in our institute (Li et al., 2010a,b), and had also been adopted by all FMD vaccine manufacturers in China as a standard method for potency test of all commercially inactivated vaccine for pigs. So we did not involve an inactivated vaccine as positive control in this primary test.

2.5. Serological assays

2.5.1. Serum virus neutralizing antibodies

Sera taken from the mice and swine were analyzed for neutralizing antibody titers against three lineages of O type FMDV (O/Mya/98, O/HN/CHA/09 and/or O/Tibet/99) by a micro-neutralization assay with monolayers of BHK-21 cells (Cao et al., 2009). Double dilutions of the sera were reacted with 100 tissue culture infective dose 50% ($TCID_{50}$) of FMDV type O at 37 °C for 1 h. The cells were then added as indicators of residual infectivity. The microplates were incubated at 37 °C for 3 days prior to fixing and staining. The endpoint titers were calculated as the reciprocal of the last serum dilution to neutralize 100 $TCID_{50}$ FMDV in 50% of the wells.

2.5.2. Total IgG antibodies to FMDV

The total IgG antibodies to O type FMDV of swine sera were evaluated with a commercial LPB-ELISA kit produced by Lanzhou Veterinary Research Institute of CAAS. All procedures were performed according to the instruction of manufacturer (Cao et al., 2012).

2.5.3. Antibodies against 3ABC protein

Pre and post challenge serum samples of swine were assessed for the presence of antibodies against FMDV non-structural proteins (NSPs) 3ABC, indicative of virus replication, by use of a indirect ELISA (Lu et al., 2007).

2.6. Cellular immune detection

PBMC were isolated from the blood of swine at day 28 after immunization with Ficoll-Paque™ PLUS (Invitrogen). After being washed three times with Hank's buffer (pH 7.2), the PBMC were resuspended at 5×10^6 cells/ml with RPMI-1640 supplemented with 10% FBS, and seeded in 96 and 12-well flat-bottom plates at 100 μl and 1 ml per well, respectively. The cells were then stimulated for 48 h with 50 μl of purified B4 at a final concentration of 50 μg/ml, 50 μl RPMI-1640 as no-antigen stimulation control, 50 μl of ConA at 5 μg/ml (Sigma) as a positive control.

2.6.1. Lymphocyte proliferation assay

The proliferative activity was measured by "Cell Titer 96® Aueous One Solution Cell Proliferation Assay" protocol (Promega, USA). Briefly, 20 μl of MTS was added to the cells and the plates were further incubated for 4 h. The OD value was read at 490 nm by a plate reader. The stimulation index of the assay was defined as the ratio (R) of the absorbance signal in control and stimulated cells and calculated as follows: $R = (S - B)/B$, where S and B represent the absorbance values for the B4-stimulated and control cells, respectively (Cheng et al., 2007).

2.6.2. Cytokines assay

The level of IFN-α, IFN-β, IFN-γ, and IL-4 concentration in the culture supernatant of swine PBMC was measured by ELISA using Porcine IFN-α, IFN-β, IL-4 (R&D, Minneapolis, MN, USA) and IFN-γ (MABTECH, Cincinnati, OH, USA) ELISA kit, respectively. All procedures were performed according to the manufacturer's instruction. The net IFN-α, IFN-β, IFN-γ, and IL-4 amount was calculated by subtracting the OD value of non-antigen-stimulated (media only) samples from the OD value of stimulated samples of the same pig.

2.7. Statistical analysis

The Student's t-test was used to compare the humoral immune responses among the different groups. Cytokine data were presented as mean ± SD by GraphPad 5 software (GraphPad Software, Inc., San Diego, CA) and one-tailed t test was used for comparison of two data sets. $P < 0.05$ was used to indicate statistical significance.

3. Results

3.1. Expression, purification and Western blot analysis of the recombinant proteins

All recombinant proteins were expressed as a formation of inclusion bodies in the *E. coli* expression system, and specific bands of approximately 19.6, 25.5 and 25.5 kDa, which were consistent with the expected size of the recombinant proteins B2, B3 and B4, respectively, whereas no band was found in lysates of pET-28a (+)-transformed cells (Fig. 1A). The purified proteins were also analyzed by SDS–PAGE and the results indicated that the purity of all three recombinant proteins was above 90% of total protein amount (Fig. 1B). The recombinant proteins were recognized specifically by the anti-FMDV (type O) antibodies as shown in Western blot assay (Fig. 1C).

3.2. Selection of a functional multi-epitope protein

Based on the previously reported B1 epitope protein (Cao et al., 2012), three multi-epitope proteins, B2, B3 and B4, were designed and expressed in *E. coli*. The immunogenicity of these four epitope proteins was compared through two sequential experiments in mouse model. As Table 3 shown, B2 epitope protein induced higher level of neutralizing antibody against FMDV than B1 protein in Experiment (Exp.) 1; while B4 epitope protein elicited higher level of neutralizing antibody than B2 and B3 proteins in Exp. 2 (Table 3). These results indicated that epitope protein containing simply tandem repeats of VP1 132–160 and C terminal 194–211 amino acid sequences of three lineages of O type FMDV were more effective than the epitope protein containing additional immunostimulatory elements such as B–C and E–F loops in VP2. The location of T cell epitopes can influence the immunogenicity of the tandem repeat epitopes; the epitope protein B4 with an N and C terminal T cell epitope showed better immunogenicity than B3, but had no significant difference ($P > 0.05$) (Table 3).

B4 was selected to formulate the vaccine for pig inoculation because this epitope vaccine induced an earlier and higher neutralizing antibody response compared to the other three epitope vaccines in mouse model.

3.3. Vaccine trial

3.3.1. Poly(I:C) improves percentage of protection

Three groups of pigs, inoculated with B4 (Group 1), B4 + poly(I:C) (Group 2) and PBS (Group 3), were challenged at 28 dpv with 2000 PID50 of O/Mya/98 FMDV. All pigs in the negative control group (Group 3) developed lesions in feet at 2–3 days post-challenge (dpc). The pigs in Group 1, inoculated with B4, two pigs gave milder clinical responses than the control group and the appearance of vesicular lesions was delayed with lesions first observed at 5 dpc, the remaining pigs were completely protected. In Group 2, all five pigs were completely protected throughout the course of the experiment (Table 4). These results indicated that immunization of pigs with B4 alone conferred 60% (3/5) of solid protection against viral challenge, co-immunization B4 with poly(I:C) increased the percentage of protection to 100% (5/5).

3.3.2. FMDV neutralizing antibodies developed in pigs

Virus neutralizing antibody titers are an indicator of protection from challenge (Grubman and Baxt, 2004; McCullough et al., 1992). Sera taken from swine at 28 dpv and 10 dpc were measured for FMDV strain O/Mya/98-specific neutralizing antibody titer. All pigs in Group 2 showed measurable levels of anti-FMDV neutralizing antibody at 28 dpv, also all pigs protected against experimental challenge. In Group 1, pig 120 did not develop any obvious FMDV-specific neutralizing antibody response, other pigs showed high levels of antibody. In addition to pig 120, pig 194 which had equivalent titers to those pigs protected from disease developed clinical signs of FMD. The results demonstrated that the appearance of neutralizing antibodies does not necessarily mean protection. As expected, all pigs in Group 3 did not develop any FMDV-specific neutralizing antibody response (Table 4).

As has been previously reported, there was increase in neutralizing antibody titer following challenge in all groups, including completely protected pigs (Eble et al., 2009; Golde et al., 2005). The challenge acted as a boost, and the pigs had increased neutralizing antibody titers by 10 dpc. However, there was a greater increase in neutralizing antibody titer in those pigs that showed clinical signs of FMD than in those protected from FMDV challenge, almost all unprotected pigs (except for 120 and 194 in Group 1) were above the maximum values of the assay (2.1 log10) at 10 dpc. At a group level, a clear boost in neutralizing antibody titer post-challenge could be detected in the PBS-vaccinated group followed by B4-vaccinated group.

3.3.3. Total IgG antibodies to FMDV

Total IgG antibodies to FMDV were detected by LPB-ELISA for all sera collected weekly from each group following inoculation. As Fig. 2 shown, no detectable levels of FMDV IgG antibodies were found in sera of PBS immunized pigs (Group 3) before challenge. Except for pig 120 in Group 1, no significant differences were observed between antibody titers of other vaccinated pigs, only a

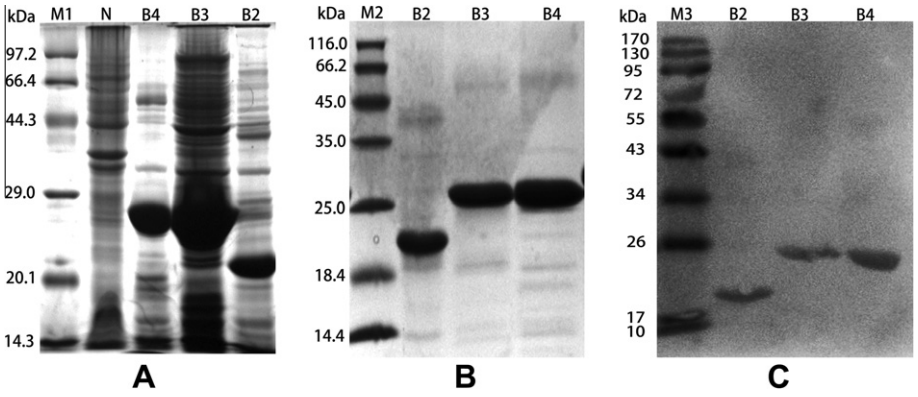

Fig. 1. Analysis of the recombinant proteins expressed in *E. coli* by SDS–PAGE (A), purification (B) and Western blot (C). M1 and M2 show different protein molecular weight standards; M3 is a prestained protein molecular weight standard; N show the lysates of pET-28a (+)-transformed cells; B2, B3 and B4 are three target proteins shown in crude lysates (A), purification (B) and Western blot (C).

Table 3
Comparison of neutralizing antibody responses against FMDV O/Mya/98, O/HN/CHA/09 and O/Tibet/99 in the mice immunized with B1, B2, B3 and B4.

Exp.	Group and dpv	Neutralizing antibody titer[a] (average value)		
		FMDV O/Mya/98	FMDV O/HN/CHA/09	FMDV O/Tibet/99
Exp. 1	Multi-epitope peptide B1			
	21	0.6, 0.6, <0.6, 0.9 (0.6)	<0.6, 0.6, 0.6, 0.6 (0.6)	ND
	35	1.2, 1.2, 1.4, 1.6 (1.35)	1.1, 1.4, 1.5, 1.5 (1.40)	
	Multi-epitope peptide B2			
	21	0.6, 0.9, 0.6, 1.2 (0.83)	0.6, 0.6, 0.6, 0.9 (0.68)	ND
	35	1.9, 1.7, 1.4, 1.6 (1.65)	1.3, 1.4, 1.7, 1.7 (1.53)	
	Inactivated vaccine (O/HN/CHA/93)			
	21	0.6, 0.9, 1.4, 1.2 (1.0)	0.6, 0.9, 1.2, 0.9 (0.9)	ND
	35	1.9, 2.4, 1.5, 1.9 (1.93)	1.7, 2.0, 1.5, 1.7 (1.73)	
	PBS			
	21	<0.6, <0.6, <0.6, <0.6	<0.6, <0.6, <0.6, <0.6	ND
	35	<0.6, <0.6, <0.6, <0.6	<0.6, <0.6, <0.6, <0.6	
Exp. 2	Multi-epitope peptide B2			
	21	0.6, 0.6, 0.9 (0.70)	0.6, 0.6, 0.6 (0.60)	0.6, 0.6, 0.6 (0.60)
	42	1.9, 2.3, 1.9 (2.07)	1.6, 1.9, 1.6 (1.70)	1.3, 1.5, 1.8 (1.53)
	Multi-epitope peptide B3			
	21	0.6, 0.6, <0.6 (0.60)	0.6, <0.6, 0.6 (0.60)	0.6, <0.6, 0.6 (0.60)
	42	1.5, 1.9, 1.8 (1.73)	1.3, 1.7, 1.5 (1.50)	1.0, 1.7, 1.4 (1.37)
	Multi-epitope peptide B4			
	21	0.6, 0.9, 1.2 (0.90)	0.9, 0.6, 0.6 (0.70)	0.9, 0.6, 0.6 (0.70)
	42	2.1, 1.9, 2.5 (2.17)	1.9, 1.6, 2.1 (1.87)	1.8, 1.3, 2.0 (1.70)
	Inactivated vaccine (O/HN/CHA/93)			
	21	0.6, 0.9, 1.2 (0.90)	0.9, 0.9, 1.2 (1.0)	0.6, 1.2, 0.9 (0.9)
	42	1.9, 2.4, 1.9 (2.07)	1.9, 2.0, 1.9 (1.93)	1.7, 2.0, 1.7 (1.8)
	PBS			
	21	<0.6, <0.6, <0.6	<0.6, <0.6, <0.6	<0.6, <0.6, <0.6
	42	<0.6, <0.6, <0.6	<0.6, <0.6, <0.6	<0.6, <0.6, <0.6

[a] Neutralizing antibody determinations were performed with sera sampled at day 21 and 35 in Experiment (Exp.) 1, and at day 21 and 42 in Exp. 2 after the primary vaccination. Serum samples were obtained from four mice in Exp. 1 and three mice in Exp. 2 from each group, respectively. Log 10 reciprocal antibody titers to Neutralize 100 TCID50 of FMDV strain O/Mya/98 (Southeast Asia topotype), FMDV strain O/HN/CHA/09 (Cathay topotype) and FMDV O/Tibet/99 (PanAsia topotype) in 50% of the wells. "ND" means "not done".

Table 4
Neutralizing antibody titers (log 10) and 3ABC antibodies of pigs and protection against FMDV challenge.

Group/inoculum (protection rate)	Animal #	Neutralising antibody titer[a]		Day of onset [b]	Lesion score[c]		3ABC results	
		28 dpv	10 dpc[d]		Onset	10 dpc	28 dpv	10 dpc
Group 1	184	1.1	1.9	–	0	0	Neg	Neg
B4	194	1.2	1.9	5	1	4	Neg	Pos
3/5(60%)	193	1.7	>2.1	–	0	0	Neg	Neg
	149	1.8	>2.1	–	0	0	Neg	Neg
	120	<0.6	1.9	5	1	4	Neg	Pos
Group 2	140	0.6	0.9	–	0	0	Neg	Neg
B4 + poly(I:C)	187	0.6	1.1	–	0	0	Neg	Neg
5/5(100%)	186	1.8	1.9	–	0	0	Neg	Neg
	188	1.0	1.3	–	0	0	Neg	Neg
	170	0.9	1.9	–	0	0	Neg	Neg
Group 3	161	<0.6	>2.1	3	4	4	Neg	Pos
PBS	119	<0.6	>2.1	2	2	4	Neg	Pos
0/5	129	<0.6	>2.1	3	4	4	Neg	Pos
	139	<0.6	>2.1	2	2	4	Neg	Pos
	182	<0.6	>2.1	2	4	4	Neg	Pos

[a] Neutralizing antibody titers (log 10) and 3ABC antibodies of vaccinated pigs on the day of challenge and 10 days later.
[b] Days of onset of lesion in feet.
[c] The clinical score was determined by the number of physical parts that vesicular lesions appeared in the dpc when lesions were first observed and in the 10 dpc. The physical parts included four feet. The maximum score is 4.
[d] dpc, days post-challenge.

few pigs (149 in Group 1, 140 and 188 in Group 2) produced detectable levels of FMDV-specific IgG antibodies at 7 dpv, most pigs elicited FMDV-specific IgG antibodies at 14 dpv and almost all pigs produced high levels of specific IgG antibodies at 21 dpv. As expected, FMDV challenge acted as a boost, there was increase in IgG titers in all tested pigs at 10 dpc. In contrast, highly increase in IgG titers was observed in those pigs that showed clinical signs of FMD, with titers reaching at least 3 log 10 units. There is a good correlation between the virus neutralizing antibody titers and IgG antibody titers.

3.3.4. Immunization with multi-epitope protein allows differentiation of infected and vaccinated pigs

A key feature of infected animals is the induction of antibodies against NSPs of FMDV (Sun et al., 2004). Using a 3ABC ELISA, we showed that all pigs had no obvious 3ABC nonstructural protein antibodies at 28 dpv. By the end of the experiment at 10 dpc, all sera from those pigs presented clinical signs of FMDV (all pigs in group 3, pigs 194 and 120 in group 1) were 3ABC positive and all sera from protected pigs were 3ABC negative (Table 4). Those results indicated that administration of the multi-epitope protein

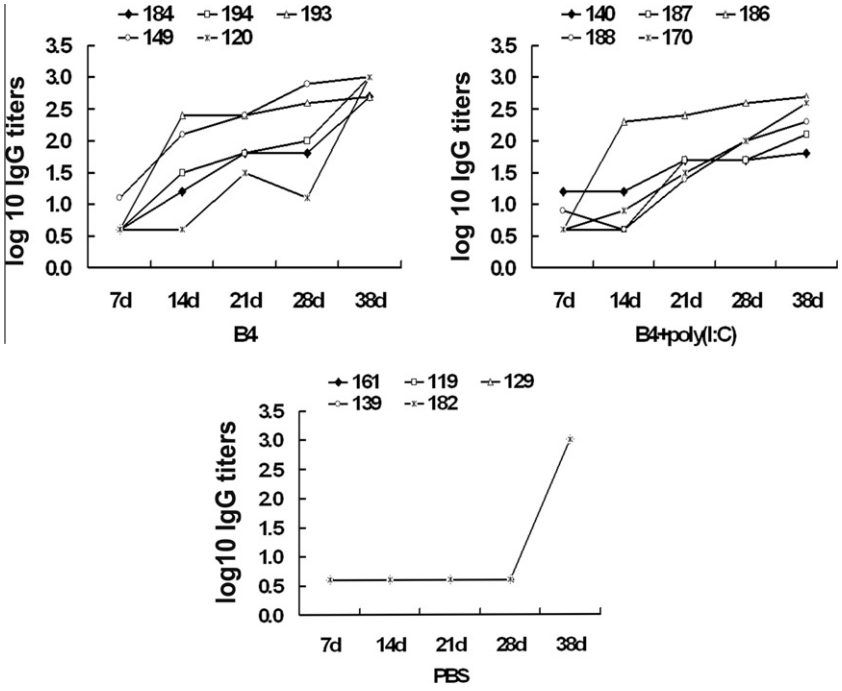

Fig. 2. Total IgG antibodies to FMDV in sera analyzed by LPB-ELISA. Pigs were immunized on day 0 and challenged on day 28. Sera were pooled on days 7, 14, 21, 28 and 38 post-vaccination and detected by LPB-ELISA. IgG titers were expressed as the reciprocal log10 of the serum dilutions giving 50% of the absorbance recorded in the control wells (wells with virus but without serum) using inactivated O/Mya/98 FMDV as antigen. Antibody titers that below the sensitivity of the assay (0.9) were adjusted to 0.6 before making figure.

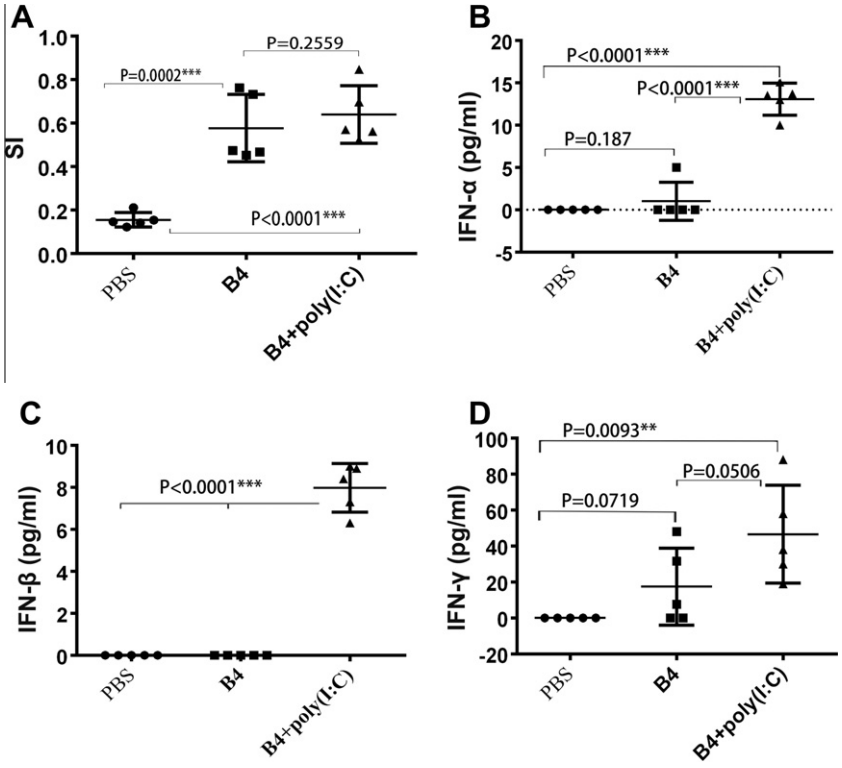

Fig. 3. Stimulation of lymphocytes proliferation in vitro by B4 and cytokines production. PBMC were isolated at 28 dpv from all groups and stimulated by B4. The proliferative responses were shown as SI (A). The levels of IFN-α, IFN-β and IFN-γ concentration in the culture supernatant were measured by ELISA. The net amount of IFN-α (B), IFN-β (C) and IFN-γ (D) was calculated as methods shown in Section 2.

B4 did not induce NSP 3ABC antibodies in pigs, that the 3ABC antibodies were well correlated with the development of clinical signs in pigs. Therefore, it can be easy to distinguish vaccinated from infected pigs using currently approved diagnostic tests.

3.4. Lymphocyte proliferation analysis

PBMC from all pigs of the tested groups were isolated at 28 dpv. The cells were stimulated with B4. The proliferative responses are shown as stimulation index (SI). As Fig. 3A shown, the highest levels of lymphocyte proliferation were observed in B4 + poly(I:C)-vaccinated group followed by B4-vaccinated group, however, no significant difference was observed between these two groups ($P > 0.05$). But the SI of PBMC from pigs immunized with B4 + poly(I:C) or B4 alone were significantly higher than that of PBMC from the PBS-vaccinated pigs ($P < 0.01$).

The induction of type I interferon IFN-α (Fig. 3B) and IFN-β (Fig. 3C) in PBMC samples from vaccinated pigs at 28 dpv was also determined. All pigs co-immunized with B4 and poly(I:C) showed detectable levels of IFN-α and IFN-β. However, only one pig in Group 1 vaccinated with B4 alone showed low levels of IFN-α and others did not show detectable levels of IFN-α and IFN-β. As expected, all pigs in PBS-immunized group were below the sensitivity of the assay (5 pg/ml each).

Production of IFN-γ, a Th 1-cytokine, was detected in supernatants of PBMC from immunized pigs collected at 28 dpv in response to in vitro stimulation with B4 (Fig. 3D). The individual IFN-γ amounts varied, the IFN-γ amounts of pig 149 and 120 in Group 1, all pigs in Group 3 were below the sensitivity of the assay. All pigs in Group 2 elicited high levels of IFN-γ; the mean net amount is 47 pg/ml, which is higher than that of Group 1 (with mean net amount of IFN-γ is 17.4 pg/ml).

Production of IL-4, a Th 2-associated cytokine, was also detected, although the OD values of B4-stimulated PBMC from all pigs in Group 2, 149 in Group 1 were higher than those of un-stimulated PBMC controls, the IL-4 amounts were below the sensitivity of the assay (5 pg/ml, data not shown).

4. Discussion

Previously, we have shown that co-administration of the multi-epitope protein with poly(I:C) can greatly increase the neutralizing antibody response against FMDV in mice (Cao et al., 2012). In the present work, we further optimized multi-epitope protein to improve immunogenicity and broaden cross-reactivity against antigenic variants. Three out of five pigs given a single inoculation with this multi-epitope protein alone conferred complete protection following virulent FMDV challenge, two pigs delayed disease onset and the clinical signs were less severe at the first observed day compared to control group, but all pigs co-administrated multi-epitope protein with poly(I:C) conferred complete protection. This result indicated that poly(I:C) improved protection against FMDV challenge in pigs.

As has been previously seen with synthetic peptide immunogens (Mulcahy et al., 1990; Steward et al., 1991; McCullough et al., 1992; Taboga et al., 1997; Wang et al., 2002), there was not a strong correspondence between neutralizing antibodies and protection (Table 4). Protection was found in the presence neutralizing antibodies of low titers in some vaccinated pigs, such as pigs 140 and 187 in Group 2. In contrast, pig 194 in Group 1 had a moderate titer was not protected. Other functional properties, such as antibody affinity and cell-mediated immune response, were probably involved in the protection. We have observed that neutralizing antibody responses are usually varied in individual pigs inoculated with inactivated FMDV vaccine; some vaccinated pigs with low or even no detectable levels of neutralizing antibodies could be protected from virulent FMDV challenge (Li et al., 2010a, 2010b), which was quite different from what had been reported in cattle (Moonen et al., 2004). Recently published papers also proved that cellular immunity mediated by interferon alone could completely protect pigs from virulent FMDV challenge (Moraes et al., 2007; Dias et al., 2012). Thus, for evaluation of the efficacy of multi-epitope protein-based vaccines for FMD in pigs, consideration should be needed for cellular immune response, antibody affinity and other functional properties of the antibody response, in addition to in vitro neutralizing activity (wang et al., 2002).

Poly(I:C) is an safe and effective adjuvant for cellular immunity and a potent inducer of type I IFNs (Trumpfheller et al., 2012; Alexopoulou et al., 2001; Longhi et al., 2009). Which is the first line of host defense against a viral infection, and it plays an important role in antiviral immunity (Stark et al., 1998; Goodbourn et al., 2000). Pigs inoculated with 8 mg poly (I:C) stabilized with poly-L-lysine and carboxymethyl cellulose (poly ICLC) were completely protected when challenged 24 h later with FMDV and the antiviral activity of poly ICLC is related to its ability to modulate the immune response by inducing the production of IFN-α, IFN-β and IFN-γ in vivo (Dias et al., 2012). In this study, all pigs co-administrated B4 with poly(I:C) produced a certain levels of IFN-α, IFN-β and IFN-γ. It has been reported that IFN-γ stimulates MHC expression in antigen-presenting cells and efficiently inhibits FMDV replication (Zhang et al., 2002). Meanwhile, the highest levels of lymphocyte proliferation were also observed in B4 + poly(I:C)-vaccinated group. These results suggest that co-administration multi-epitope protein with poly(I:C) up-regulated T cell-mediated immune response that efficiently contributes to FMDV protection. Therefore, better clinical protection conferred by the combination of B4 and poly(I:C) in this study, especially in the case that no sufficient neutralizing antibody titers were induced, would be due to an efficient lymphoproliferative response and cytokines release.

Animal-to-animal variations have been reported for the protective responses to epitope vaccines, including those against FMDV (Collen et al., 1991; Taboga et al., 1997; Cubillos et al., 2008). This difference is prominent in the case of vaccination with B4 alone; some pigs elicited neutralizing antibodies and total IgG antibodies as well as IFN-γ, while some pigs did not produce parameters mentioned above. It is probably due to polymorphisms in genes of class I and class II major histocompatibility complexes may affect recognition of individual epitopes, thus resulting in variations in both cellular and humoral responses among individual pigs. Interestingly, these types of variations are buffered by offering poly(I:C) as adjuvant. All B4 + poly(I:C)-vaccinated pigs elicited cellular and humoral immune response, as revealed by the parameters analyzed in this study, again all pigs turned out to be protected against FMDV challenge, suggesting that B4 with poly(I:C) can elicit B- and T-cell responses sufficient to cope with FMDV replication, thus minimizing the chances of selecting escape virus (Taboga et al., 1997).

The recent development of dendrimeric peptides containing one copy of an FMDV T-cell epitope branching out into four copies of a B-cell epitope provides promise for peptide-subunit vaccine development (Cubillos et al., 2008). Pigs vaccinated twice with a dendrimeric peptide conferred a solid protection against FMDV challenge. However, ultimately to be successful any new vaccine must provide protection after a single inoculation, since in an outbreak there will presumably be no time or sufficient logistical support for multiple vaccinations. In addition to the problems derived from limited immunogenicity, the weakness of antigenic cross-reactivity is a major drawback for peptide vaccine of FMDV; Amino acid substitutions in the G–H loop domain alter the interaction of FMDV with neutralizing antibodies. The alteration occurs when

the loop epitope of a vaccine does not match that of a virus causing an FMD outbreak. The outbreak of O/Mya/98 FMDV observed in China in 2010 (Zheng et al., 2012) coincided with the widespread use of the synthesized peptide vaccine in pigs (Wang et al., 2002). To broaden the spectrum of antigenicity, we designed multi-epitope protein including four G–H loop domains from three topotypes of O-type FMDV. As Table 3 shown, this multi-epitope protein induced neutralizing antibodies in mice against FMDV O/Mya/98, O/HN/CHA/09 and/or O/Tibet/99. Further study is needed to evaluate the cross-protection of multi-epitope protein vaccine with poly(I:C) as adjuvant on pigs against different topotypes of FMDV serotype O.

The epitope vaccine is a site-specific antigenic marker vaccine. The chemically-defined immunogen elicits site-specific antibody responses that are readily distinguishable from the complex responses to infection. In our study, multi-epitope protein induced a serological response compatible with its use as a vaccine facilitating the differentiation of infected from vaccinated animals, since it did not elicit antibodies to the NSP 3ABC, which is indicative of active virus infection (i.e., active viral replication) (Mackay et al., 1998). On the other hand, all pigs immunized with B4 plus poly(I:C) was lack of seroconversion to antibody reactivity against NSP 3ABC after FMDV challenge suggests a lack of generalized infection.

5. Conclusion

Based on our results, we can conclude that poly(I:C) reduced animal-to-animal variations in both humoral and cell-mediated immune responses often observed in association with peptide-based vaccines (Collen et al., 1991; Taboga et al., 1997; Cubillos et al., 2008) and up-regulated T-cell immunity often poorly observed in protein-based vaccines (Trumpfheller et al., 2012), accordingly improved percentage of protection against virulent FMDV challenge in pigs. This multi-epitope protein combined with poly(I:C) could generate an effective and safe vaccine for the prevention and control of FMD in pigs, which can be used in combination with a diagnostic assay employing NSPs, i.e., 2C, 3A, 3AB, 3B and 3ABC, allowing unequivocal differentiation between vaccinated and infected animals. Utilizing these reagents vaccination should not be a barrier to international trade. This novel vaccine does not require high-containment facilities for production and could be made safely since no infectious FMDV is present. These laboratory results are encouraging for the development of a multi-epitope protein subunit vaccine for FMD.

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References

Agadjanyan, M.G., Ghochikyan, A., Petrushina, I., Vasilevko, V., Movsesyan, N., Mkrtichyan, M., Saing, T., Cribbs, D.H., 2005. Prototype Alzheimer's disease vaccine using the immunodominant B cell epitope from β-amyloid and promiscuous T cell epitope pan HLA DR-binding peptide. J. Immunol. 174, 1580–1586.

Alexandersen, S., Zhang, Z., Donaldson, A.I., Garland, A.J.M., 2003. The pathogenesis and diagnosis of foot-and-mouth disease. J. Comp. Pathol. 129, 1–36.

Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A., 2001. Recognition of double-stranded RNA and activation of NF-kappa B by Toll-like receptor 3. Nature 413, 732–738.

Barteling, S.J., Vreeswijk, J., 1991. Developments in foot-and-mouth disease vaccines. Vaccine 9, 75–88.

Cao, Y.M., Lu, Z.J., Li, P.H., Sun, P., Fu, Y.F., Bai, X.W., Bao, H.F., Chen, Y.L., Li, D., Liu, Z.X., 2012. Improved neutralising antibody response against foot-and-mouth-disease virus in mice inoculated with a multi-epitope peptide vaccine using polyinosinic and poly-cytidylic acid as an adjuvant. J. Virol. Methods 185, 124–128.

Cao, Y.M., Lu, Z.J., Sun, J.C., Bai, X.W., Sun, P., Bao, H.F., Chen, Y.L., Guo, J.H., Liu, X.T., Liu, Z.X., 2009. Synthesis of empty capsid-like particles of Asia I foot-and-mouth disease virus in insect cells and their immunogenicity in guinea pigs. Vet. Microbiol. 137, 10–17.

Cheng, G., Zhao, X., Yan, W.Y., Wang, W.F., Zuo, X.P., Huang, K., Liu, Y., Chen, J., Wang, J.L., Cong, W., Liu, M.Q., Gao, H.H., Chen, J.L., Lu, Y.G., Zheng, Z.X., 2007. Alpha interferon is a powerful adjuvant for a recombinant protein vaccine against foot-and-mouth disease virus in swine, and an effective stimulus of in vivo immune response. Vaccine 25, 5199–5208.

Collen, T., Dimarchi, R., Doel, T.R., 1991. A T cell epitope in VP1 of foot-and-mouth disease virus is immunodominant for vaccinated cattle. J. Immunol. 146, 749–755.

Cubillos, C., de la Torre, B.G., Jakab, A., Clementi, G., Borras, E., Barcena, J., Andreu, D., Sobrino, F., Blanco, E., 2008. Enhanced mucosal immunoglobulin A response and solid protection against foot-and-mouth disease virus challenge induced by a novel dendrimeric peptide. J. Virol. 82, 7223–7230.

Dias, C.C., Moraes, M.P., Weiss, M., Diaz-San Segundo, F., Perez-Martin, E., Salazar, A.M., de los Santos, T., Grubman, M.J., 2012. Novel antiviral therapeutics to control foot-and-mouth disease. J. Interferon Cytokine Res. 32, 462–473.

Doel, T.R., 2003. FMD vaccines. Virus Res. 91, 81–99.

Eble, P.L., Weerdmeester, K., van Hemert-Kluitenberg, F., Dekker, A., 2009. Intradermal vaccination of pigs against FMD with 1/10 dose results in comparable vaccine efficacy as intramuscular vaccination with a full dose. Vaccine 27, 1272–1278.

Golde, W.T., Pacheco, J.M., Duque, H., Doel, T., Penfold, B., Ferman, G.S., Gregg, D.R., Rodriguez, L.L., 2005. Vaccination against foot-and-mouth disease virus confers complete clinical protection in 7 days and partial protection in 4 days: use in emergency outbreak response. Vaccine 23, 5775–5782.

Goodbourn, S., Didcock, L., Randall, R.E., 2000. Interferons: cell signalling, immune modulation, antiviral responses and virus countermeasures. J. Gen. Virol. 81, 2341–2364.

Grubman, M.J., 2005. Development of novel strategies to control foot-and-mouth disease: marker vaccines and antivirals. Biologicals 33, 227–234.

Grubman, M.J., Baxt, B., 2004. Foot-and-mouth disease. Clin. Microbiol. Rev. 17, 465–493.

Hietela, S.K., Ardans, A.A., 2003. Molecular weapons against agricultural vulnerability and the war on terror. J. Vet. Med. Educ. 30, 155–156.

Li, D., Lu, Z.J., Xie, B.X., Sun, P., Chen, Y.L., Fu, Y.F., Liu, Z.X., 2010a. Alternative way to test the efficacy of swine FMD vaccines: measurement of pigs median infected dose (PID50) and regulation of live virus challenge dose. Virol. J. 7, 215.

Li, D., Liu, Z.X., Sun, P., Li, Y.L., Lu, Z.J., Tian, M.N., Chen, Y.L., Xie, B.X., Bao, H.F., Fu, Y.F., Cao, Y.M., Li, P.H., Bai, X.W., Sun, J.C., Guo, J.H., Liu, X.T., Xie, Q.G., 2010b. The efficacy of FMD vaccine reduced non-structural proteins with a mAb against 3B protein. Vet. Res. Commun. 34, 445–457.

Longhi, M.P., Trumpfheller, C., Idoyaga, J., Caskey, M., Matos, I., Kluger, C., Salazar, A.M., Colonna, M., Steinman, R.M., 2009. Dendritic cells require a systemic type I interferon response to mature and induce CD^{4+} Th1 immunity with poly IC as adjuvant. J. Exp. Med. 206, 1589–1602.

Lu, Z.J., Cao, Y.M., Guo, J.H., Qi, S.Y., Li, D., Zhang, Q., Ma, J.W., Chang, H.Y., Liu, Z.X., Liu, X.T., Xie, Q.G., 2007. Development and validation of a 3ABC indirect ELISA for differentiation of foot-and-mouth disease virus infected from vaccinated animals. Vet. Microbiol. 125, 157–169.

Mackay, D.K.J., Forsyth, M.A., Davies, P.R., Berlinzani, A., Belsham, G.J., Flint, M., Ryan, M.D., 1998. Differentiating infection from vaccination in foot-and-mouth disease using a panel of recombinant, non-structural proteins in ELISA. Vaccine 16, 446–459.

McCullough, K.C., De Simone, F., Brocchi, E., Capucci, L., Crowther, J.R., Kihm, U., 1992. Protective immune response against foot-and-mouth disease. J. Virol. 66, 1835–1840.

Moonen, P., Jacobs, L., Crienen, A., Dekker, A., 2004. Detection of carriers of foot-and-mouth disease virus among vaccinated cattle. Vet. Microbiol. 103, 151–160.

Moraes, M.P., de los Santos, T., Koster, M., Turecek, T., Wang, H., Andreyev, V.G., Grubman, M.J., 2007. Enhanced antiviral activity against foot-and-mouth disease virus by a combination of type I and II porcine interferons. J. Virol. 81, 7124–7135.

Mulcahy, G., Gale, C., Robertson, P., Lysian, S., DiMarchi, R.D., Doel, T.R., 1990. Isotype responses of infected, virus-vaccinated and peptidevaccinated cattle to foot-and-mouth disease virus. Vaccine 8, 249–256.

Shao, J.J., Wong, C.K., Lin, T., Lee, S.K., Cong, G.Z., Sin, F.W.Y., Du, J.Z., Gao, S.D., Liu, X.T., Cai, X.P., Xie, Y., Chang, H.Y., Liu, J.X., 2011. Promising multiple-epitope recombinant vaccine against foot-and-mouth disease virus type O in swine. Clin. Vaccine Immunol. 18, 143–149.

Shen, F., Chen, P.D., Walfield, A.M., 1999. Differentiation of convalescent animals from those vaccinated against foot-and-mouth disease by a peptide ELISA. Vaccine 17, 3039–3049.

Sorensen, K.J., Madsen, K.G., Madsen, E.S., Salt, J.S., Nqindi, J., Machay, D.K.J., 1998. Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB, 3ABC in ELISA using antigens expressed in baculovirus. Arch. Virol. 143, 1461–1467.

Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., Schreiber, R.D., 1998. How cells respond to interferons. Annu. Rev. Biochem. 67, 227–264.

Steward, M.W., Stanley, C.M., DiMarchi, R., Mulcahy, G., Doel, T.R., 1991. High-affinity antibody induced by immunisation with a synthetic peptide is associated with protection of cattle against foot-and-mouth disease. Immunology 72, 99–103.

Sun, T., Lu, P., Wang, X., 2004. Localization of infection-related epitopes on the non-structural protein 3ABC of foot-and-mouth disease virus and the application of tandem epitopes. J. Virol. Methods 119, 79–86.

Taboga, O., Tami, C., Carrillo, E., Nunez, J.I., Rodriguez, A., Saiz, J.C., Blanco, E., Valero, M.L., Roig, X., Camarero, J.A., Andreu, D., Mateu, M.G., Giralt, E., Domingo, E., Sobrino, F., Palma, E.L., 1997. A large-scale evaluation of peptide vaccines against foot-and-mouth disease: lack of solid protection in cattle and isolation of escape mutants. J. Virol. 71, 2606–2614.

Tewari, K., Flynn, B.J., Boscardin, S.B., Kastenmueller, K., Salazar, A.M., Anderson, C.A., Soundarapandian, V., Ahumada, A., Keler, T., Hoffman, S.L., Nussenzweig, M.C., Steinman, R.M., Seder, R.A., 2010. Poly(I:C) is an effective adjuvant for antibody and multi-functional CD^{4+} T cell responses to *Plasmodium falciparum* circum sporozoite protein (CSP) and αDEC-CSP in non human primates. Vaccine 2008, 7256–7266.

Trumpfheller, C., Longhi, M.P., Caskey, M., Idoyaga, J., Bozzacco, L., Keler, T., Schlesinger, S.J., Steinman, R.M., 2012. Dendritic cell-targeted protein vaccines: a novel approach to induce T-cell immunity. J. Intern. Med. 271, 183–192.

Wang, C.Y., Chang, T.Y., Walfield, A.M., Ye, J., Shen, M., Chen, S.P., Li, M.C., Lin, Y.L., Jong, M.H., Yang, P.C., Chyr, N., Kramer, E., Brown, F., 2002. Effective synthetic peptide vaccine for foot-and-mouth disease in swine. Vaccine 20, 2603–2610.

Zhang, Z.D., Hutching, G., Kitching, P., Alexandersen, S., 2002. The effects of gamma interferon on replication of foot-and-mouth disease virus in persistently infected bovine cells. Arch. Virol. 147, 2157–2167.

Zheng, H.X., He, J.J., Guo, J.H., Jin, Y., Yang, F., Lv, L., Liu, X.T., 2012. Genetic characterization of a new pandemic Southeast Asia topotype strain of serotype O foot-and-mouth disease virus isolated in China during 2010. Virus Genes 44, 80–88.