RESEARCHREPORT
SWINE HEALTH

Title: Evaluation of immunodominant B- and T-cell epitopes as inducers of protective immunity against porcine reproductive and respiratory syndrome virus **(NPB #14-214)**

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Industry Summary:

Since its emergence in domestic pigs in the late 1980's, porcine reproductive and respiratory syndrome virus (PRRSV) has caused significant economic losses to swine producers worldwide. A recent study in the US has estimated annual losses of approximately 650 million dollars to the pork industry due to PRRSV infection. Control of PRRSV and consequent reduction of the burden posed by the disease to swine producers and the pork industry have been major challenges to veterinarians and scientists working with swine health research both in industry- and academic-settings.

Control of PRRSV has been hindered by the lack of an effective vaccine capable of **1)** eliciting early effector humoral- and cell-mediated immune responses against PRRSV; and of **2)** providing cross-protection against heterologous PRRSV isolates. Currently available PRRSV vaccines (modified-live virus [MLV] and killed virus [KV]) elicit delayed neutralizing antibody- (NA) and cell-mediated responses and provide only partial protection to heterologous PRRSV strains. The complex interactions of PRRSV with the host immune system coupled with the high genetic and antigenic diversity of the virus are the main factors contributing to the failure of current vaccines in controlling PRRSV. **Therefore identification of regions of the PRRSV genome (antigens/proteins and/or epitopes), capable of eliciting protection would facilitate the design and development of the next generation of PRRSV vaccines.**

Recent studies have identified multiple antigenic epitopes on PRRSV genome. These studies create opportunities for rational design of improved subunit PRRSV vaccines. In the present study we sought to evaluate the role of antigenic PRRSV epitopes on protection against PRRSV infection and disease. The overall goal of this study is to evaluate the immunogenicity of antigenic epitopes of PRRSV and to evaluate their ability to induce protection against PRRSV. The objectives of the study are:

Objective 1: To develop and express polyepitope proteins of PRRSV encoding B and T cell epitopes.

Objective 2: To assess the immunogenicity of PRRSV polyepitope proteins in pigs and their ability to induce protective immunity after PRRSV challenge.

PRRSV B and T-cell multi-epitope proteins were generated and expressed in a virus vector or as recombinant proteins in bacteria. These two approaches were used to deliver the PRRSV antigens in pigs and their ability to provide protection was evaluated after PRRSV challenge.

Antibody responses were detected against PRRSV epitopes by ELISA. Responses against individual epitopes were evaluated in serum samples collected at different time points post-immunization. Antibodies were detected against 11 of the 16 PRRSV antigens included in our recombinant protein construct. Notably, following challenge infection, no differences were observed in the clinical parameters evaluated between immunized and non-immunized animals, suggesting that the levels of antibodies generated might not be enough for protection or that these epitopes do not play an important role for PRRSV protection. No detectable cell mediated responses were observed in immunized animals, suggesting a low overall immunogenicity of the T-cell antigenic determinants used in our study. Taken together the results from our indicate an overall low immunogenicity of individual PRRSV epitopes. Identification of PRRSV strains capable of eliciting broad cross-protection and use if these strains to produce broadly protective vaccines might still be the best approach to develop the next generation of improved PRRSV vaccines.

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Scientific Abstract:

The goal of this study was to evaluate the role of PRRSV B- and T-cell epitopes as inducers of protective immune responses against PRRSV. Several immunodominant

PRRSV B and T-cell epitopes have been described, however the contribution of these epitopes for protection against remains unknown. The most direct way to assess the of these epitopes for protection would be to immunize pigs with these antigenic determinants and challenge the animals with PRRSV. In this study we used two different approaches to deliver immunodominant B- and T-cell epitopes in pigs and assess their role on protection against PRRSV infection. First we designed polyepitope minigenes encoding 16 and 17 PRRSV B and T-cell epitopes, respectively and inserted those minigenes into the genome of a poxviral vector. The recombinant poxvirus was purified and used to immunize pigs. The second approach involved a combination of recombinant protein (pB) and plasmid DNA (pT) delivery systems. Animals were immunized with recombinant purified pB and a plasmid DNA encoding the pT proteins. Following immunization with the recombinant vector or with recombinant pB plus plasmid DNA, animals were challenged with virulent PRRSV strains (FL12 or NADC20, respectively). Immune responses elicited by immunization were assessed by ELISAs and lymphocyte proliferation assays. Parameters of disease and PRRSV infection including rectal temperature, lung pathology and viremia levels were monitored after challenge infection. Immunization of pigs with the viral vector expressing pB and pT did not elicit detectable immune responses against the PRRSV epitopes. This occurred inspite of efficient immunization with the vector, as evidenced by high levels of antibodies detected against an internal control protein (GFP) that was co-expressed by the viral vector. Immunization with the recombinant purified pB in combination with a water in oil adjuvant, on the other hand, elicited robust antibody responses against pB and against 11 out of the 16 epitopes that composed the B cell polyepitope protein. Notably, following challenge infection with PRRSV no evidence of protection was observed, as animals from the immunized groups presented similar levels of lung pathology and viremia when compared to control non-immunized pigs. Results of this study indicate that immunodominant B and T-cell epitopes of PRRSV have a low immunogenicity when delivered outside the context of PRRSV infection. Additionally, results from the immunization study using the recombinant pB protein suggest that the levels of antibodies elicited against the peptides were not sufficient for protection against PRRSV or perhaps, that these epitopes do not play a role on PRRSV protection.

Introduction:

Since its emergence in domestic pigs in the late 1980's, porcine reproductive and respiratory syndrome virus (PRRSV) has caused significant economic losses to swine producers worldwide[1]. A recent study in the US has estimated annual losses of approximately 650 million dollars to the pork industry due to PRRSV infection[2]. Control of PRRSV and consequent reduction of the burden posed by the disease to swine producers and the pork industry have been major challenges to veterinarians and scientists working with swine health research both in industry- and academic-settings.

Vaccines are the most cost-effective control measure for infectious diseases[3]. However, PRRSV vaccines, which have been used since mid 1990's, have been ineffective in

controlling PRRSV[4,5]. The major limitations of currently available vaccines are the induction of delayed effector immune responses against PRRSV and the lack of cross protection against heterologous PRRSV strains[4–7]. These limitations are a result of immune evasion by live attenuated vaccines, inherent low immunogenicity of killed-virus vaccines, and the genetic diversity of PRRSV isolates circulating in the field[4–6]. The next generation of PRRSV vaccines will need to overcome these limitations inducing rapid effector- (humoral and cellular) and broadly protective-immune responses[5,8]. Identification of antigenic determinants of PRRSV capable of inducing protective immunity is a critical step towards development of effective PRRS vaccines.

A number of B- and T-cell epitopes have been identified in different proteins of PRRSV[9–13, 20–22]. Notably, several B-cell epitopes located in the viral glycoproteins are capable of eliciting neutralizing antibodies[13] while T-cell epitopes have been shown to induce recall IFNγ production by PBMCs of PRRSV-immunized pigs[6,10,11]. However, the contribution of these immunodomiant epitopes (B- and T-cell) for the overall protective immunity against PRRSV remains unknown. The most direct approach to assess the role of these epitopes on protection would be to immunize pigs with these antigenic determinants and to evaluate protection against challenge infection with a virulent PRRSV strain. **The goal of this study is to evaluate the immunogenicity of B- and T-cell epitopes of PRRSV and to assess their contribution for protection.**

Objectives:

The overall goal of this study is to evaluate the immunogenicity of immunodominant PRRSV B- and T-cell epitopes and their ability to induce protective immunity against PRRSV. The objectives of the study are:

Objective 1: To develop and express polyepitope proteins of PRRSV encoding B and T cell epitopes.

Objective 2: To assess the immunogenicity of PRRSV polyepitope proteins in pigs and their ability to induce protective immunity after PRRSV challenge.

Materials & Methods:

Analysis and design of the PRRSV B- and T-cell polyepitope proteins. The concept that we proposed to evaluate in our study was based on previously published work that identified immunodominant B- and T-cell epitopes of PRRSV[9,10]. Based on these studies we selected immunodominant B and T-cell epitopes of PRRSV. A complete list of epitopes incorporated in the design of PRRSV polyepitope proteins here are presented on Table 1. Sixteen immunodominant B-cell epitopes located in PRRSV glycoproteins (GP2, GP3, GP4, and GP5), were incorporated in the design of the B-cell polyepitope proteins (pB). Whereas 17 immunodominant T-cell epitopes were selected and incorporated in the T-cell polyepitope proteins (pT), based on their ability to induce IFN-γ production by PBMCs of PRRSV- infected animals.

Table 1 – Select immunodominant B- and T-cell epitopes of type-II PRRSV.

Epitope polyepitope protein	Protein	aa Sequence	Position	Type	Property	Reference
1 - pB	GP2	LPSLAGWWSSASDWF	41-55	B-cell		[9]
2 - pB	GP2	GSPSQDGYWSFF	37-48	B-cell	Neutralizing	[9,13]
3 - pB	GP2	KAGQAAWKQVVSEAT	121-135	B-cell	Neutralizing	[9,13,26]
4 - pB	GP3	QAAAEVYEPGRSLWC	61-75	B-cell	Neutralizing	[9,13,27]
5 - pB	GP3	RSLWCRIGHDRCSED	71-85	B-cell		[9]
6 - pB	GP3	RCSEDDHDDLGFMVP	81-95	B-cell		[9,27]
7 - pB	GP3	ELLMSIPSGYDN	89-100	B-cell		[27]
8 - pB	GP3	GFMVPPGLSSEGHLT	91-105	B-cell		[9]
9 - pB	GP3	GYDNLKLEGYYA	97-108	B-cell		[27]
10 - pB	GP3	GYYAWLAFLSFS	105-116	B-cell		[27]
11 - pB	GP4	SCLRHGDSSSQTIRK	51-65	B-cell		[9]
12 - pB	GP5	MLGRCLTAGCCSRLL	1-15	B-cell		[9]
13 - pB	GP5	ANSNSSSHLQLIYNL	31-45	B-cell	Neutralizing	[9,28]
14 - pB	GP5	YQYIYNLTICEL	41-52	B-cell	Neutralizing	[9,29]
15 - pB	GP5	KLGKAEVDGNLV	165-176	B-cell		[30]
16 - pB	GP5	TPLTRVSAEQWGRL	187-200	B-cell		[9,30]
1 - pT	nsp2	SLYKLLLEV	589-597	T-cell		[31]
2 - pT	nsp5	LLNEILPAV	1929-1937	T-cell		[31]
3 - pT	nsp9	KEEIALSAQIIQACDIR	119-135	T-cell		[10]
4 - pT	nsp9	VRGNPERVKGVLQNTRF	151-167	T-cell		[10]
5 - pT	nsp10	VRILAGGWCPGKNSFLD	209-225	T-cell		[10]
6 - pT	nsp10	CPGKNSFLDEAAYCNHL	217-233	T-cell		[10]
7 - pT	GP4	CLFAILLAT	170-178	T-cell		[32]
8 - pT	GP4	FLLAGAQHI	9-17	T-cell		[32]
9 - pT	GP5	LAALICFVIRLAKNC	117-131	T-cell		[11,32]
10 – pT[a]	GP5	KGRLYRWRSPVII/VEK	149-163	T-cell		[11,32]
11 - pT	M	CHDSTAPQKVLLAFS	9-23	T-cell		[11,32]
12 - pT	M	ALKVSRGRLLGLLHL	33-47	T-cell		[12]
13 - pT	M	FGYMTFAHFQSTNKV	57-71	T-cell	$CD8^+$ CDL	[12]
14 - pT	M	KFITSRCRLCLLGRK	93-107	T-cell	$CD8^+$ CDL	[12]
15 - pT	N	KPEKPHFPL	49-57	T-cell		[12]
16 – pT	N	VRHHLTQTE	63-71	T-cell		[34,32]
17 – pT	N	FMLPVAHTVRLIRVTST	104-120	T-cell		[32,33]

Construction of PRRSV B- and T-cell polyepitope proteins. To assess the immunogenicity and determine the ability of immunodominant B- and T-cell epitopes to elicit protective immune responses against PRRSV, we undertook two approaches: **1)** we constructed a recombinant ORFV-based vector encoding PRRSV B- and T-cell polyepitope proteins (ORFV/PRRSV-pE) by homologous recombination; and **2)** we cloned the polyepitope proteins into a bacterial (pB) and mammalian expression plasmids (pT).

To construct the poxvirus vectoring PRRSV pB and PT, DNA fragments encoding the polyepitope proteins (pB: containing 16 PRRSV B-cell epitopes; and pT: containing 17 PRRSV T- cell epitopes) were designed using universal codons. A Kozak consensus sequence (gccacc) was incorporated upstream the start codons and adjacent epitopes

were joined by spacer sequences of highly conserved Histidyl-tRNA synthase (319-GFGLPEEK-326)[37]. Sequences of HA- and FLAG-tag B-cell epitopes were incorporated at both ends of pB and pT, respectively to monitor expression of polyepitope proteins by the recombinant poxviral vector (Figure 2A). A single DNA fragment containing both pB and pT coding sequences under control of individual poxvirus early/late promoter was chemically synthesized (GeneScript, Piscataway, NJ) and cloned into the poxviral transfer vector pSPV/L-R/EGFP. The green fluorescent protein gene (EGFP) was also inserted in the recombinant virus and used a selection marker and an internal control on the immunogenicity studies.

Recombinant ORFV-PRRSV/pE virus was obtained by homologous recombination between the a wild type ORFV and the transfer vector pZippy/LR-pB/pT as previously described. Plaque purified recombinant ORFV-PRRSV/pE virus was tested for the presence of PRRSV pB and pT coding sequences by PCR and subjected to DNA sequencing to confirm integrity of inserted PRRSV polyepitope minigenes. Replication and growth characteristics of ORFV-PRRSV/pE recombinant virus were assessed in porcine and ovine cell lines. After *in vitro* characterization was complete, the recombinant ORFV-PRRSV/pE was used to immunize pigs.

In addition, the PRRSV pB protein was chemically synthesized and cloned in fusion with a polyhistidine tag into a bacterial expression vector pET28a. The recombinant PRRSV pB protein was expressed in *E. coli* and purified by nickel-NTA affinity column chromatography. Given that T cell epitopes need to be processed inside the cells and presented to T cells via major histocompatibility complex (MHC) after intracellular processing, we cloned the PRRSV pT polyepitope protein into a mammalian expression vector pcDNA3.1 (pcDNA-PRRSV-pT) and used this construct as a DNA delivery system. The purified recombinant pB protein and the plasmid encoding the pT protein were used to immunize pigs.

Characterization of vectors expressing PRRSV pB and pT. Expression of PRRSV polyepitope proteins (pB and pT) by ORFV-PRRSV/pE recombinant virus was assessed by immunofluorescence and western blot. Primary swine (STu) and ovine turbinate cells (OFTu) were infected with the recombinant ORFV-PRSV/pE and cells were harvested at different time points post-infection (2, 4, 6, 8, 12 and 24 h pi). Expression of pB and pT were assessed by western blot using anti-Flag and –HA tag antibodies following standard protocols. Additionally, sample collected at 24 h pi was also probed with anti-PRRSV swine hyperimune serum, to assess reactivity of anti-PRRSV swine serum with the protein expressed by the recombinant virus (ORFV-PRRSV/PE).

Expression of pB and pT were also assessed by immunofluorescence assay. Ovine turbinate cells were infected with the recombinant virus and fixed with formaldehyde 24 h pi. Cells were probed with anti-Flag and –HA epitope antibodies and expression of pB and pT evaluated under a fluorescence microscope.

Expression of the recombinant pB in *E. coli* was assessed in by SDS-PAGE followed by comassie blue staining or western blot. Expression of pT by the mammalian expression vector was assessed in transfected cells by immunofluorescence staining.

Animal immunization-challenge experiments. To assess the potential role of PRRSV B- and T-cell epitopes on protection we have performed two animal immunization-challenge experiments. The first experiment was used to evaluate protective efficacy of pB and pT delivered by the ORFV-PRRSV/pE virus, while the second experiment was conducted to evaluate the protective efficacy of the recombinant pB expressed in *E. coli* or the pT protein delivered by DNA immunization.

Immunization-challenge study with the ORFV-PRRSV-pE recombinant virus was conducted at UNL, following the protocols established by the UNL Institutional Animal Care and Use Committee. Eighteen 3-week-old weaned pigs were obtained from the UNL research farm, a PRRSV-free facility. Animals were housed in BSL-2 animal facilities and randomly allocated to three experimental groups, consisting of sham-immunized/PRRSV-challenged (PBS/PRRSV strain FL12; Group 1 – n = 6), sham-immunized/PRRSV-challenged (ORFV-GFP/PRRSV strain FL12; Group 2 – n = 6), and polyepitope protein-immunized/PRRSV-challenged (ORFV-PRRSV-pE/PRRSV strain FL12; Group 3 – n = 6). Immunization were performed by intramuscular (IM) injection of 1 ml of recombinant virus suspension (ORFV-PRRSV-pE – immunized groups) containing $10^{7.5}$ $TCID_{50}$/ml in MEM. Animals were boosted as above on day 21 post-primary immunization. Challenge infection was performed on day 49 post-immunization by IM inoculation of a PRRSV suspension containing 10^5 $TCID_{50}$ of the strain FL12. Blood and serum samples were collected before immunization (day 0), and on days 14, 21, 28 (post-primary immunization), 35, 42 and 49 (post- booster immunization). Blood samples were processed for isolation of peripheral blood mononuclear cells (PBMCs) and isolated PBMCs cryopreserved

Immunization-challenge study with the pB expressed in *E. coli* and plasmid DNA expressing PRRSV pT (pcDNA-PRRSV-pT) was conducted at SDSU Animal Resource Wing, following the protocols established by the Institutional Animal Care and Use Committee. Ten 3-week-old weaned high health pigs were obtained from Midwest Research Swine, a SPF facility. Animals were housed in BSL-2 animal facilities and randomly allocated to two experimental groups, consisting of sham-immunized/PRRSV-challenged (PBS/PRRSV strain NADC20; Group 1 – n = 5), and pB/pcDNA-PRRSV-pT protein-immunized/PRRSV-challenged (ORFV-PRRSV-pE/PRRSV strain NADC20; Group 2 – n = 5). Immunization was performed by intramuscular (IM) injection of 2 ml of recombinant protein ~800 µg of recombinant protein in PBS (PRRSV-pB – immunized groups) mixed (1:1) with Seppic montanide ISA 50 V2 adjuvant. Additionally, animals were immunized IM with plasmid DNA encoding the pT protein (pcDNA-PRRSV-pT; ~500 µg of DNA) mixed (80/20, vol/vol) with Seppic Montanide Gel 01 adjuvant. Animals were boosted as above on day 21 post-primary immunization. Challenge infection was performed on day 35 post-immunization by IM inoculation of a PRRSV suspension containing 2 x 10^5 $TCID_{50}$ of

the strain NADC20 (half IM and half IN). Blood and serum samples were collected before immunization (day 0), and on days 14, 21 (post-primary immunization), 28, 35, 42 and 49 (post- booster immunization).
Immune responses elicited by immunization with pB constructs were assessed by ELISA. The recombinant pB expressed in *E. coli* was used as antigen to coat ELISA plates and each B-cell epitope listed in Table 1 was chemically synthesized and used to coat plates used for peptide ELISAs. T cell responses were assessed by flow cytometry using CFSE proliferation assays. A pool of synthetic T-cell epitopes listed in Table 1 were used to stimulate PBMCs *in vitro*. The mitogen PHA was used as a positive control in the proliferation assays.

After challenge infection animals were monitored clinically and parameters of PRRSV infection and disease recorded. Primary parameters that were used to evaluate the animal's health status and to assess the protective potential of the PRRSV polyeptope proteins included: (i) change in body temperature; (iii) viremia; and (iv) lung pathology. Serum viremia levels were determined by quantitative RT-PCR or virus titration, with serum samples collected on days 0, 3, 7, 10 and 14 post-challenge.

Results:

<u>Objective 1: To develop and express polyepitope proteins of PRRSV encoding B and T cell epitopes.</u>

Construction and characterization of poxvirus expressing PRRSV pB and pT. A recombinant poxvirus containing PRRSV B- and T-cell polyepitope minigenes was generated by homologous recombination. The recombinant virus containing PRRSV pB and pT sequences was selected/purified by limiting dilution followed by plaque assays. The green fluorescent protein was used as a selection marker. Insertion of PRRSV polyepitope minigenes into the ORFV genome was confirmed by PCR (using polyepitope minigene specific primers; **Figure 1**) and DNA sequencing (data not shown).

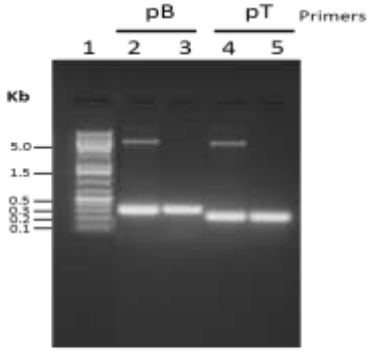

Figure 1 – Characterization of recombinant ORFV-PRRSV-pE virus. PCR reactions to confirm insertion of PRRSV polyepitope B (pB) and polyepitope T (pT) coding sequences into the genome of ORFV-PRRSV-pE. 1. 1 Kb DNA ladder; 2. Positive control plasmid DNA (pZ-PRRSV-pB/pT); 3. ORFV-PRRSV-pE DNA; 4. Positive control plasmid DNA (pZ-PRRSV-pB/pT); 5. ORFV-PRRSV-pE DNA. pB: poly-B gene specific primers; pT: poly-T gene specific primers.

Replication characteristics of the recombinant virus (ORFV-PRRSV-pE) were assessed in ovine and swine cells **(Figure 2)**. The recombinant virus presented normal replication kinetics in ovine turbinate cells; however, a marked growth defect was observed in swine turbinate cells **(Figure 2)**. These results are consistent with previous studies in our laboratory using wild type ORFV virus and ovine turbinate- and porcine kidney cells (PK15) (data not shown).

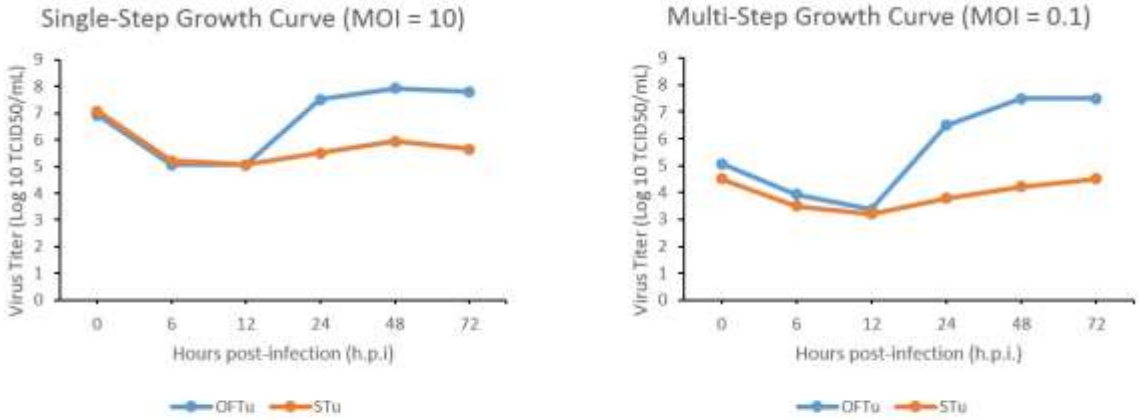

Figure 2 – Replication characteristics of recombinant ORFV-PRRSV-pE. Single (left) or multi-step (right) growth curves of ORF-PRRSV-pE in primary ovine fetal turbinate cells (blue) or primary swine turbinate cells (orange). Cells were inoculated with multiplicity of infection of 10 (single step) or 0.1 (multi-step) harvested at the indicated time points and viral titers determined by tissue culture infectious dose 50 method (log 10 TCID50/mL).

Expression of PRRSV B- and T-cell polyepitope proteins (pB and pT, respectively) was assessed by immunofluorescence using anti-HA and anti-Flag epitope antibodies. HA and Flag-epitope tags were added at the N- and C-terminus of pB and pT, respectively to allow detection of expressed proteins. **Figure 3** shows expression of both PRRSV pB and pT by the recombinant poxvirus vector (ORF-PRRSV-pE). These results demonstrate that the poxviral vector is capable of expressing high levels of PRRSV polyepitope proteins.

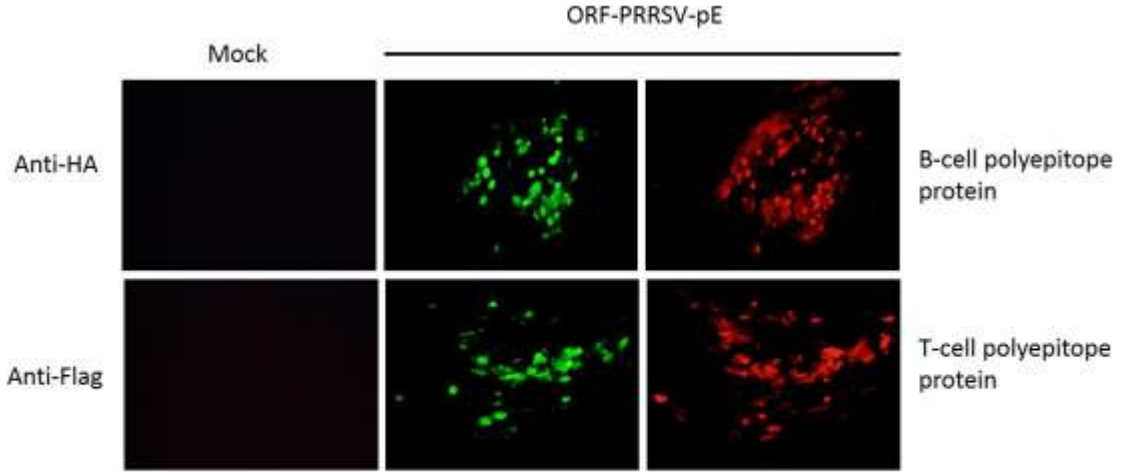

Figure 3 – Expression of PRRSV polyepitope proteins by the recombinant ORF-PRRSV-pE. Primary ovine turbinate cells were infected with ORF-PRRSV-pE. At 24 h post infection cells were fixed and processed for immunofluorescence assays. Cells were stained with anti-HA antibody (top panels) to detect expression of PRRSV B-cell polyepitope protein or with an anti-Flag antibody (bottom panels) to detect expression of PRRSV T-cell polyepitope protein. Red fluorescence staining indicate expression of PRRSV pB and pT by the recombinant ORF-PRRSV-pE. Green fluorescence staining indicates expression of the GFP reporter gene by the recombinant ORFV-PRRSV/pE virus.

The kinetics of pB and pT expression by the recombinant virus was investigated in primary ovine- and swine turbinate cell cultures (**Figure 4**). Expression of pB and pT was detected as early as 4 hours post infection in both ovine and swine turbinate cells (**Figure 4**). These results demonstrate that, despite the replication defect in swine turbinate cells (**Figure** 2), the recombinant ORF-PRRSV-pE is capable of expressing high levels of PRRSV pB and pT in primary swine cells.

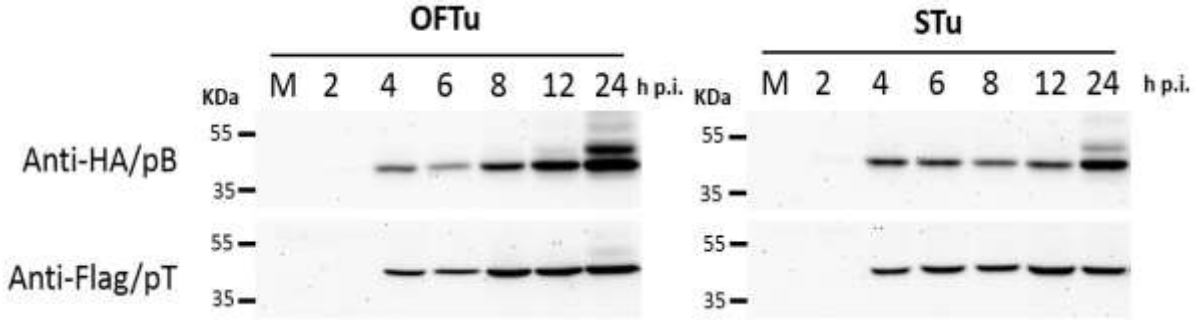

Figure 4 – Expression kinetics of PRRSV B- and T-cell polyepitope proteins by ORF-PRRSV-pE virus. Primary ovine turbinate cells (OFTu) or primary swine turbinate cells (STu) were infected with ORF-PRRSV-pE virus and harvested at indicated time points post infection (h p.i.: hours post-infection). Cells were lysed and protein extracts subjected to SDS-PAGE. Resolved protein extracts were blotted to nitrocellulose membranes and expression of PRRSV pB and pT detected with an anti-HA and anti-Flag antibodies respectively. M: mock infected negative control cells.

Swine convalescent sera generated against type I (EU) or type II PRRSV (NA) were used in western blot assays to evaluate the reactivity of anti-PRRSV antibodies with PRRSV polyepitope proteins expressed by ORF-PRRSV-pE. As shown in **Figure 5** only the anti-type II sera specifically reacted with ORF-PRRSV-pE infected cells. Control western blot using anti-HA and Flag tag antibodies shows efficient expression of pB and pT by the recombinant virus.

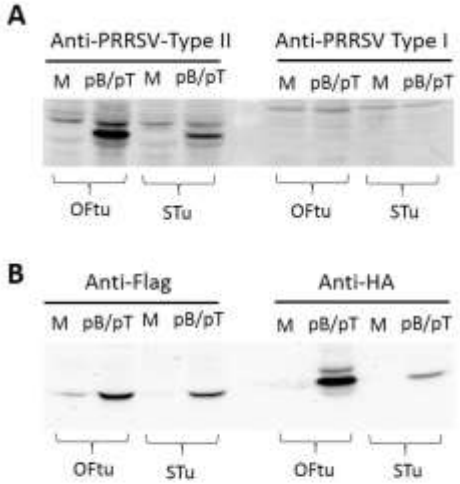

Figure 5 – Reactivity of anti-PRRSV convalescent sera with pE protein expressed by poxvirus recombinant virus. (A) western blot demonatrsting reactivity of anti-PRRSV type II sera with pE. (B) Control western blot using anti-Flag and –HA antibodies demonstrating expression of pB and pT by ORFV-PRRSV-pE

Construction and purification of PRRSV pB recombinant protein and pT recombinant plasmid DNA. In addition to the poxviral vector expressing PRRSV pB and pT described above, we also constructed two expression plasmids encoding the sequences of pB and pT. The pB expression plasmid consisted of a bacterial expression plasmid pET28, while pT was cloned into a mammalian expression plasmid (pcDNA3.1) and used as a DNA plasmid on immunization studies. The pB was expressed in *E. coli* and purified using Nickel-NTA chromatography columns (Figure 6A). Convalescent swine serum against PRRSV type II reacted against the recombinant protein in western blot assay (Figure 6B). The recombinant purified protein was used to immunize pigs to assess the role of pB epitopes on PRRSV protective immunity.

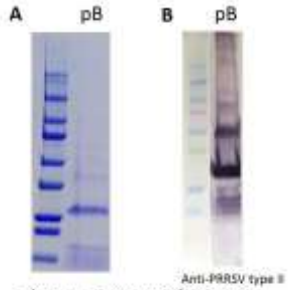

Figure 6 – Expression of PRRSV pB protein in *E. coli*. (A) SDS-Page followed by coomassie blue staining of pB protein. (B) Western blot showing reactivity of hyperimune anti-PRRSV type II (NA) serum with recombinant pB.

<u>Objective 2: To assess the immunogenicity of PRRSV polyepitope proteins and their ability to induce protective immunity after PRRSV challenge in pigs.</u>

The immunogenicity and protective potential of PRRSV B and T-cell epitopes were assessed in pigs. Two approaches were undertaken to evaluate the immunogenicity of pB and pT and to assess the potential involvement of the immunodominant B and T cell epitopes of PRRSV (Table 1) on protection. The first approach consisted on immunizing pigs with a recombinant live poxvirus vector expressing the pB and pT polyepitope (Figures 1-4) proteins of PRRSV, while the second approach consisted on immunizing pigs with a recombinant pB protein expressed in *E. coli* and with a plasmid DNA expressing the pT protein. In the second approach both pB and the plasmid DNA encoding pT were mixed with adjuvants prior to immunization. Following immunization animals were challenged with virulent PRRSV strains (FL12 or NADC20) and parameters of PRRSV infection were recorded and compared between immunized and non-immunized groups.

Assessing the immunogenicity and protective efficacy of pB and pT when the proteins were delivered intracellular by a viral vector. To evaluate the immunogenicity and protective efficacy of PRRSV B- and T-cell epitopes 3-week-old weaned pigs were immunized by the intramuscular route with the poxvirus vector expressing the pB and pT proteins (ORFV-PRRSV/pE; Figure 3 and 4). Animals were immunized on day 0 and boosted on day 21 post-primary immunization. Immune

responses elicited against pB were assessed by ELISA using the recombinant pB protein as coating antigen or by peptide ELISAs using the individual B-cell peptides to coat the ELISA plates. Additionally, serological responses against PRRSV were also assessed by immunofluorescence assay in PRRSV infected cells. Despite expressing high levels of the PRRSV polyepitope protein (Figure 3 and 4), intracellular delivery of the protein did not induce significant serological responses in immunized pigs (Figure 7A). Serological responses against an internal control green fluorescent protein (GFP) simultaneously expressed by the vector elicited robust responses in the immunized pigs (Figure 7B), as evidenced by the detection of GFP-specific antibodies in the serum of immunized pigs on days 14 and 35 post-immunization (Figure 7B). Animals from all three groups developed serum antibodies against pB following challenge infection with PRRSV strain FL12 (Figure 7A; days 59 and 63).

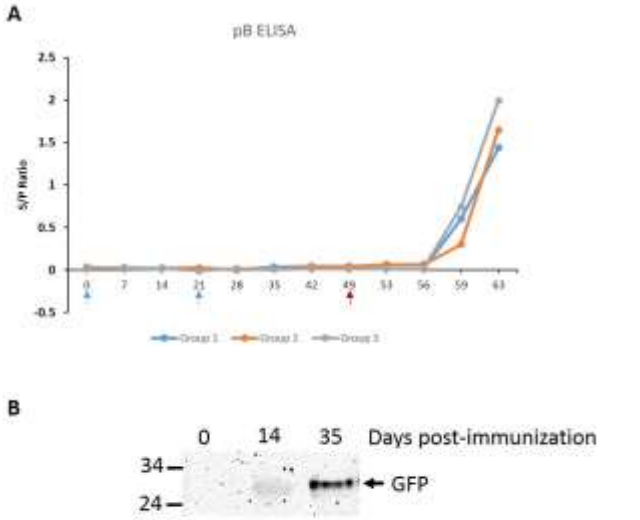

Figure 7 – Serological responses post-immunization with ORFV-PRRSV-pE. **(A)** Indirect ELISA using recombinant B-cell epitope protein to assess responses on immunized (Group 3) and control animals (Groups 1 and 2). Blue arrows indicate immunization dates. Red arrow indicates PRRSV challenge date. **(B)** Western blot demonstrating seroconversion of Group 3 animals to an internal control protein expressed by the recombinant ORFV-PRRSV-PE. Serum samples from Group 3 animals on day 0, 14 and 35 were pooled, diluted 1:500 and probed against a recombinant GFP protein on a western blot.

In addition to evaluating serum antibody responses against the polyepitope protein (pB), we also assessed the responses against each individual B-cell peptide by using peptide ELISAs. While Group 3 animals developed low levels of antibodies against some peptides (8 out of 16, on day 49 post-immunization), it was only after challenge infection with PRRSV FL12 that significant levels of antibodies were detected (Figure 8; days 53, 56 and 63). Interestingly, no antibodies were detected against peptides #3, 7 and 10 even after challenge infection. Together these results indicate an overall low immunogenicity of the PRRSV B-cell epitopes, when these they are delivered intracellularly outside the context of PRRSV infection.

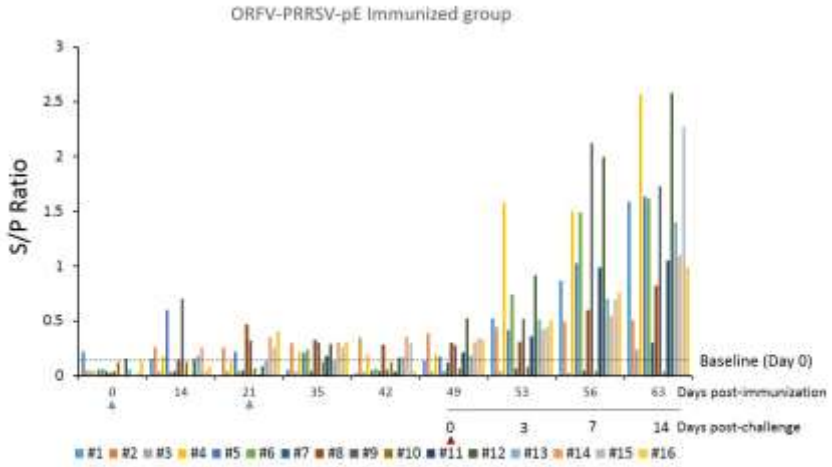

Figure 8 – Serological responses post-immunization with ORFV-PRRSV-pE against individual B-cell peptides. Serum samples from animals on group 3 (ORFV-PRRSV-PE) were pooled and tested against individual B-cell peptides by ELISA. Blue arrows indicate immunization dates. Red arrow head indicates challenge day.

The protective efficacy of the recombinant was assessed after challenge infection with PRRSV strain FL12 on day 49 post-immunization. Rectal temperatures, lung pathology and viremia were the criteria used to evaluate protection. As shown in Figure 9, so significant differences were observed in rectal temperature, lung lesions or viremia levels between treatment groups. These results indicate that the low levels of immunity elicited by immunization with pB (Figure 8) protein were not sufficient to provide protection against challenge with a virulent PRRSV strain.

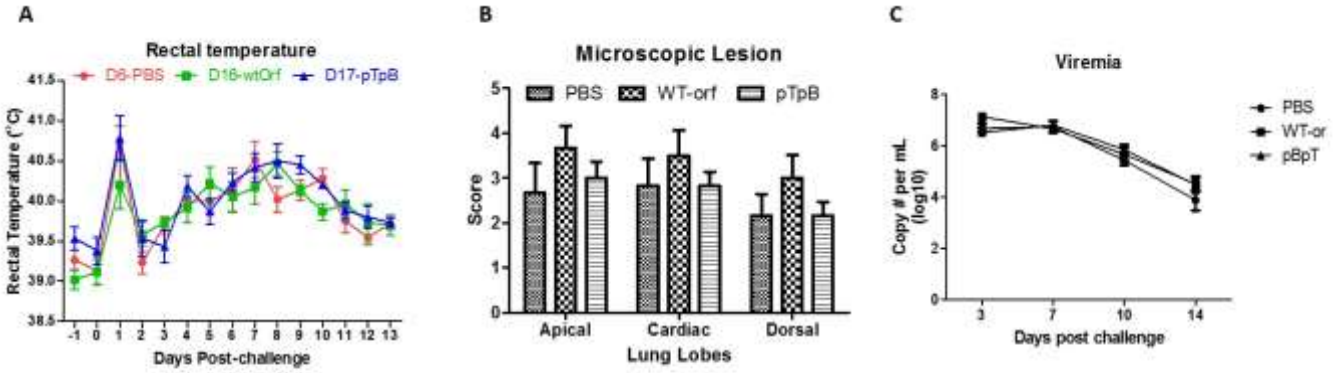

Figure 9 – Clinical and pathological findings post-PRRSV challenge. **(A)** Average daily rectal temperature per group (Group 1: PBS; Group 2, D16-wtOrf; Group 3: D17-pTpB). **(B)** Histological changes observed in the lung of animals on day 14 post-challenge. Average lesion scores observed on tha apical, cardiac and dorsal lung lobes are presented for each group. **(C)** Levels of viremia detected on days 3, 7, 10 and 14 post-challenge. Results represent genome copies per ml (log 10) and were determined by real-time PCR.

Assessing the immunogenicity and protective efficacy of pB and pT when delivered in combination with adjuvants. Since we only detected antibodies against a few PRRSV B-cell epitopes after delivering the polyepitope protein intracellular with a viral vector (Figure 8) and no protection was observed (Figure 9), we decided to take a different approach and immunize animals with a recombinant B-cell polyepitope protein expressed in *E. coli* and with a DNA plasmid encoding the T-cell polyepitope protein. The recombinant pB protein was mixed with a water-in-oil (W/O) adjuvant (Seppic Montanide ISA 50 V2; 1:1) and the plasmid DNA encoding the pT protein was mixed with a gel polymer (sodium polyacrylate) in water adjuvant (Seppic Montanide

Gel 01; 80:20%). The immune responses elicited by immunization with the recombinant pB were assessed by indirect ELISA. Serum samples were tested against the recombinant protein pB used for immunization and against a pool of all 16 peptides listed in Table 1. As shown in Figure 10, the animals immunized with the recombinant protein developed a robust serological response against the protein. Additionally, when a the serum was tested against the pool of B-cell peptides, high levels of antibodies were detected (Figure 10). Animals in the control group 1, did not present antibodies against pB nor against the peptide pool (Figure 10).

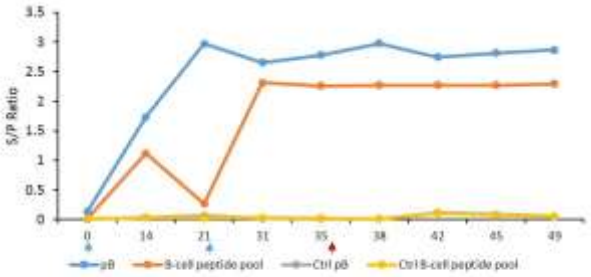

Figure 10 – Serological responses post-immunization with recombinant pB. Individual serum samples from animals on Group 1 (Control: sham immunized) and Group 2 (pB immunized) were tested by indirect ELISA using the recombinant pB protein or a pool of all 16 (Table 1) B cell peptides as the coating antigen. Blue arrows indicate immunization dates. Red arrow indicates challenge date.

The antibody response against the individual B-cell epitopes (Table 1) that compose the recombinant pB protein were evaluated by peptide ELISAs. Individual peptides were used to coat ELISA plates and serum samples from animals Group 2 (pB immunized group) were tested against each peptide. Coating conditions and antibody dilutions were optimized for each peptide using convalescent serum from PRRSV infected animals and known negative controls. As shown in Figure 11, on day 0 no antibodies were detected against any of the PRRSV B-cell epitopes. On day 14 post-immunization antibodies were detected against 8 out 16 epitopes (#1, 4, 6, 9, 11, 12, 15 and 16). On days 21 and 31 post-immunization antibodies were detected against 11 out of 16 peptides (#1, 3, 4, 6, 8, 9, 11, 12, 13, 15, and 16). After challenge infection on day 35 p.i., serum antibodies against 13 out 16 peptides (#1, 3, 4, 5, 6, 8, 9, 11, 12, 13, 14, 15 and 16). No antibodies were detected against peptides #2 (GP2, aa 37-48), 7 (GP3, aa 89-100) and 10 (GP3, 105-116), even after challenge infection with PRRSV strain NADC20. These results indicate that immunization with the recombinant pB protein resulted in specific antibody responses against 11 out of 16 PRRSV B-cell epitopes.

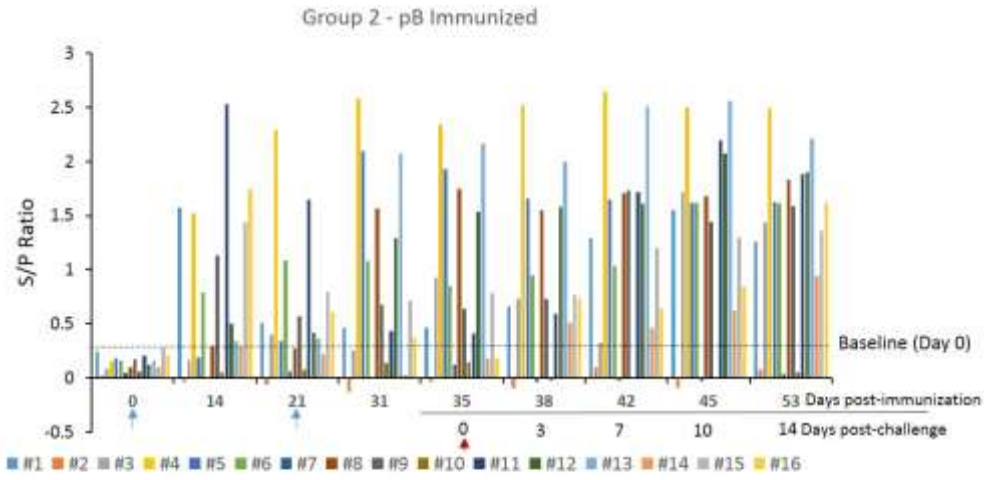

Figure 11 – Serological responses post-immunization with recombinant pB. Individual serum samples from animals on Group 1 (Control: sham immunized) and Group 2 (pB immunized) were tested by indirect ELISA using the recombinant pB protein or a pool of all 16 (Table 1) B cell peptides as the coating antigen. Data is presented as average group sample to positive (S/P) ratio. Blue arrows indicate immunization dates. Red arrow indicates challenge date.

Given that four epitopes included in the polyepitope protein evaluated here have been described as neutralizing epitopes (#2, 3, 4, 14 and 14; Table 1), PRRSV-specific neutralizing antibody responses were evaluated by virus neutralization assays. Interestingly despite the detection of antibodies against epitopes #3, 4, and 13 on day 35 post-immunization by peptide ELISA (Figure 11), no neutralizing activity was detected in the serum of polyepitope protein immunized animals (data not shown).

Cell mediated responses were assessed by using carboxyfluorescein diacetate succinimidyl ester (CFSE) staining assays to monitor lymphocyte proliferation. While stimulation of peripheral mononuclear cells (PBMCs) with phytohemagglutinin (PHA), a known mitogen used as a positive control, led to marked proliferation of PBMCs (Figure 12, middle panels), no significant proliferative responses were observed when PBMCs of control and immunized pigs were stimulated with a pool of PRRSV T-cell peptides present in the T-cell polyepitope protein (Figure 12; top right and bottom right panels). These results suggest that delivery of the plasmid DNA expressing the PRRSV pT in combination with the gel adjuvant was inefficient in inducing T-cell responses against the PRRSV T-cell epitopes. This can be due to: 1. the overall low immunogenicity of the T-cell epitopes when expressed outside the context of PRRSV infection; and/or 2. incorrect processing and presentation of the T-cell epitopes to the immune system.

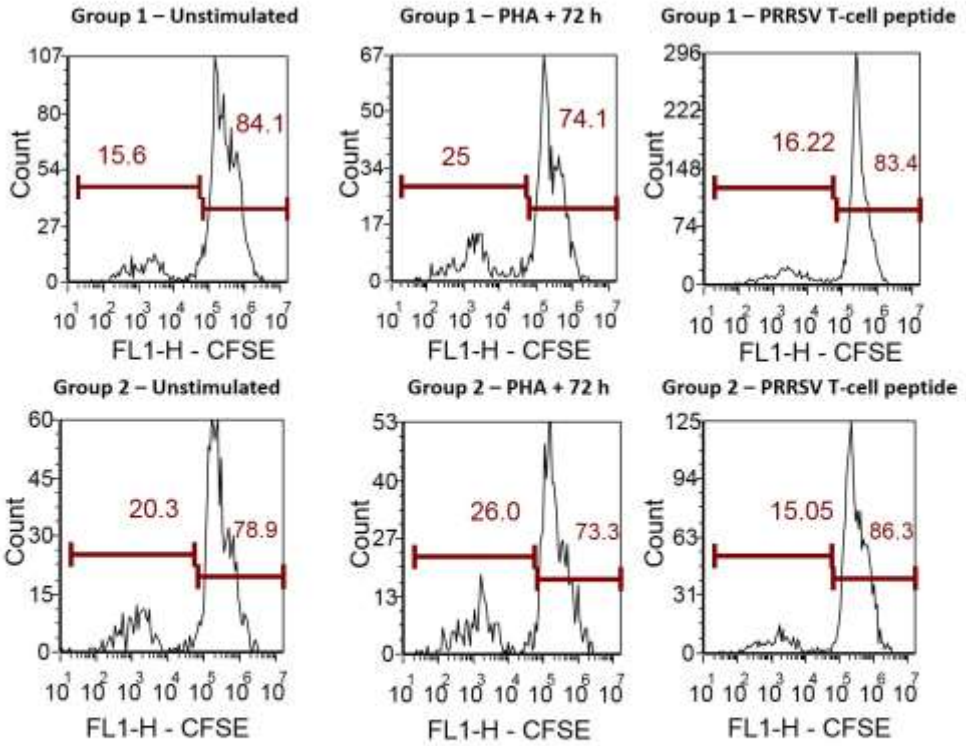

Figure 12 – Lymphocyte proliferation assay. Peripheral blood mononuclear cells were collected from control sham immunized animals (Group 1) and pB/pT immunized animals on day 35 post-immunization and lymphocyte proliferation was assessed by CFSE staining followed by flow cytometry. PBMCs (1 x 10^5 cells) were cultures in 96-well plates and stimulated with PHA (5 µg) or with a pool of PRRSV T-cell epitopes (20 µg; Table 1) for 72 h. Unstimulated cells were used as negative controls. After 72 h incubation, proliferation was assessed by flow cytometry analysis. Values in red represent percent number of cells. Peak on right represent non-proliferating cells while lower peaks on the left represent proliferating cells.

The ability of PRRSV B and T cell epitopes to induce protective immunity was evaluated after challenge-infection. Control (Group 1) sham-immunized animals and pB/pT immunized animals were challenged on day 35 post-immunization with PRRSV strain NADC20 (IN and IM challenge), and viremia and lung pathology were evaluated. Despite high levels of antibodies against the recombinant B-cell polyepitope protein (Figure 10) and against most individual B-cell epitopes included in the pB (Figure 11) no differences in viremia levels were observed between sham-immunized and recombinant PRRSV pB/pT immunized groups (Figure 13). These results suggest that either the levels of antibodies induced against epitopes #1, 3, 4, 6, 8, 9, 11, 12, 13, 15, and 16 (Figure 11) are not sufficient for protection or that these epitopes might not elicit protective immune responses against PRRSV.

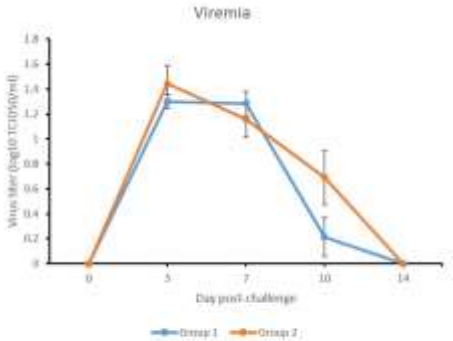

Figure 13 – Viremia post-challenge infection with PRRSV. Virus titers in serum were determined by limiting dilution according to the Spearman and Karber's method and expressed as tissue culture infectious dose 50/mL.

Discussion:

The overall goal of our study was to evaluate the immunogenicity and the ability of B- and T-cell epitopes of PRRSV to induce protective immune responses in pigs. A number of B- and T-cell epitopes have been identified in different proteins of PRRSV[9–13, 20–22]. However, the contribution of these immunodomiant epitopes (B- and T-cell) for the overall protective immunity against PRRSV remain unknown. The most direct approach to assess the role of these epitopes on protection would be to immunize pigs with these antigenic determinants and to evaluate protection against challenge infection with a virulent PRRSV strain. In our study we undertook two different approaches to assess the immunogenicity of B- and T-cell epitopes of PRRSV and to assess their contribution for protection. Initially, we constructed a poxvirus expressing PRRSV B and T-cell polyepitope proteins, in which the B and T-cell epitopes listed in Table 1 were fused into two open reading frames (pB and pT) encoding PRRSV B and T-cell epitopes, respectively. This poxvirus vector was used to deliver the PRRSV pB and pT proteins in pigs. Since this approach used a live viral vector, both PRRSV pB and pT proteins were delivered intracellularly in immunized pigs. Our second approach consisted of using a recombinant B-cell polyepitope protein expressed in *E. coli* and a plasmid DNA encoding the T-cell polyepitope protein combined with potent adjuvants to immunize pigs. The first approach using a viral vector, allowed intracellular delivery of both pB and pT (endogenous antigen), while the second approach using the recombinant pB plus the plasmid pT DNA allowed extracellular delivery of pB (exogenous antigen) and intracellular delivery of pT (endogenous antigen).

Endogenous antigens that are generated within the cells are processed into smaller fragments (peptides) by the proteasome and displayed at the cell surface associated with major histocompatibility class I (MHC-I) molecules for recognition by CD8+ T cells. Exogenous antigens are taken up by antigen presenting cells (like dendritic cells, macrophages and/or B lymphocytes), processed into smaller peptides in the lysosome and displayed at the cell surface in association with MHC-II molecules for recognition by CD4+ T helper cells. Exogenous antigens can also be presented at the cell surface of macrophages and dendritic cells as intact antigens. By using a viral vector, plasmid DNA and recombinant protein we have targeted both endogenous and exogenous antigen processing and presentation pathways.

Despite high levels of expression of both pB and pT by the poxviral vector, no immune responses were detected in immunized pigs. As shown in Figure 7 no significant antibody responses were detected against pB in immunized animals. When we evaluated the responses against individual epitopes that compose the pB protein we observed very modest antibody responses (above day 0 baseline) against some peptides on days 14, 21, 28, 35, 42 and 49 post-immunization. Notably, after PRRSV challenge infection antibody levels against pB and individual peptides was markedly higher (Figure 7 and 8). These results indicate that intracellular delivery of the PRRSV B cell polyepitope protein was not able to elicit robust immune responses in immunized animals. To assess whether the immunization with the poxvirus vector was effective, we assessed antibody responses against an internal control protein, green fluorescent protein (GFP), co-expressed by the viral vector. As shown in Figure 7B animals immunized with the viral vector encoding the pB and the internal control GFP developed high levels of antibodies against GFP (serum dilution of up to 1:500), despite the low levels of antibodies against the PRRSV B-cell epitopes. These contrasting results (high levels of antibodies against GFP and low or lack of antibodies against specific PRRSV B-cell epitopes), suggest that the PRRSV polyepitope pB protein may not have been properly processed via the endogenous antigen processing pathway, thus resulting in poor presentation and display of the individual peptides to the immune system. Alternatively, individual PRRSV B-cell epitopes may have low immunogenicity when these peptides are delivered intracellularly outside the context of PRRSV infection. Following challenge with PRRSV strain FL12, all animals got infected and exhibiting typical PRRSV lung pathology and characteristic viremia. No differences were observed between treatment groups regarding the clinical parameters evaluated.

Given the low immunogenicity of the immunodominant PRRSV epitopes when those were delivered intracelularly by a viral vector, we decided to test an alternative delivery strategy using a recombinant B-cell polyepitope protein expressed in *E. coli* and a plasmid DNA expressing the PRRSV T-cell polyepitope protein. Both recombinant pB and pT plasmid DNA were delivered in combination with potent adjuvants. Delivery of the recombinant pB in pigs resulted in robust antibody responses after intramuscular immunization (Figure 10). Additionally, antibodies against 11 out of 16 individual B-cell epitopes were detected on immunized animals (Figure 11). Interestingly, despite the fact that 5 of the B-cell epitopes present in pB have been described as neutralizing epitopes (Table 1) and IgG responses against 3 (#3, 4, and 13) out of these 5 epitopes were detected by ELISA, no neutralizing antibody responses were detected on days 21 and 35 post-immunization.

Cell mediated immune responses were evaluated by lymphocyte proliferation assays using CFSE staining and flow cytometry analysis (Figure 12). However, no proliferation was observed when PBMCs from pT vaccinated animals were incubated with a pool of PRRSV T cells epitopes, when compared to control unstimulated cells (Figure 12). These results indicate that immunization with plasmid DNA encoding PRRSV T-cell epitope did not elicit T-cell responses against the peptides. This can be attributed to

the low immunogenicity of individual peptides when expressed outside the context of PRRSV infection or to improper processing of the T cell polyepitope protein and inadequate presentation of the individual T-cell peptides to T-cells. Additionally, the genetic background of the pigs used in our experiment and the genetic diversity of the swine major histocompatibility complex, the swine leukocyte antigens (SLA) may also have contributed for the lack of detectable immune responses against the individual T-cell peptides.

Notably, despite the robust antibody responses detected against pB and 11 PRRSV B-cell epitopes (Figure 11), no protection was observed following challenge-infection with PRRSV strain NADC20. Animals from control and pB immunized groups presented similar levels of lung pathology and viremia. These results suggest that the levels of antibodies elicited against individual B-cell epitopes may not sufficient for protection or that these epitopes do not play a role on protection against PRRSV.

Here we sought to assess the role of PRRSV B- and T-cell epitopes on protection against PRRSV infection and disease. Several studies have described the presence of immunodominant epitopes in different PRRSV proteins; however the actual contribution of these epitopes for protection remain unknown. Results here show that PRRSV B and T-cell epitopes present a low immunogenicity when delivered to pigs outside the context of PRRSV infection. This was more evident for T-cell epitopes, as two delivery strategies failed to elicit detectable T-cell responses against individual PRRSV peptides. Responses against PRRSV B-cell epitopes, on the other hand, were readily detected when these epitopes were delivered to pigs as a recombinant polyepitope protein in combination with a potent water in oil adjuvant. Immunized animals developed robust antibody responses against most (11 out of 16) B-cell epitopes in pB. Notably, despite the high levels of antibodies detected against individual B-cell epitopes no protection was observed post-challenge infection with a virulent strain of PRRSV, suggesting that the levels of antibodies generated were not sufficient for protection or perhaps that these epitopes do not play a role on protection against PRRSV.