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Study of a GP5-Mosaic T-cell Vaccine in Protection of Swine Against Heterologous PRRSV Strains

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Study of a GP5-Mosaic T-cell Vaccine in Protection of Swine Against Heterologous PRRSV Strains

Junru Cui, PhD

University of Connecticut, [2017]

Abstract

Porcine reproductive and respiratory syndrome (PRRS) causes significant economic losses due to reproductive failure and respiratory disease. The high genetic and antigenic diversity of PRRSV makes the control of this disease very challenging. Current vaccines cannot provide adequate protection to the circulating PRRSV strains; therefore, vaccines can confer cross-protection are in urgent demand. Two T-cell mosaic vaccines were designed based on 748 GP5 sequences using the Mosaic T-Cell Vaccine Tool Suite from the Los Alamos National Laboratory. These mosaic sequences were then used to construct DNA vaccines. Significantly higher levels of interferon-γ mRNA and virus-specific antibodies, rapid virus clearance in sera and systemic tissues such as spleen and ILN, lower lung lesion scores were recorded in GP5-Mosaic-vaccinated pigs suggested that the GP5-Mosaic vaccine was immunogenic and induced partial protection. The GP5-Mosaic vaccines complexed to cationic liposomes were administered by intramuscular injection and boosted three times at days 14, 28 and 42. Significantly higher levels of IFN- γ mRNA were detected in PBMCs of GP5-Mosaic-vaccinated pigs stimulated by 4 Genotype II PRRSV strains including VR2332, NADC9, NADC30, and SDSU73; while such responses were recorded only upon VR2332 stimulation in GP5-WT-vaccinated pigs. Pigs receiving the GP5-Mosaic vaccine were partially protected as indicated by significantly lower viral loads in sera, tissues, and lower lung lesion scores. At last, where priming with an rDNA

GP5-Mosaic and boosting with a recombinant Tet inducible Vaccinia virus GP5-Mosaic followed by heterologous virus challenge trial was performed in pigs. Similar results were recorded as GP5-Mosaic vaccination induced both humoral and cellular responses. Furthermore, the GP5-Mosaic-vaccinated pigs were cross-protected from heterologous strains as indicated by significantly lower viral loads in sera, tissues, porcine alveolar macrophages, and bronchoalveolar lavage fluids as well as lower lung lesion scores against either MN184C or VR2332 challenge. Under the same criteria, pigs receiving the GP5-WT vaccination showed higher protection only against challenge with VR2332 when compared to the vector-control group.

Overall, this dissertation reported a viable approach, GP5-Mosaic T-cell DNA vaccines, to address PRRSV diversity issue as capable of inducing both humoral and cellular responses thus providing cross-protection against heterologous PRRSV strains in pigs.

Study of a GP5-Mosaic T-cell Vaccine in Protection of Swine Against Heterologous PRRSV Strains

Junru Cui

M.S., University of Connecticut, [2012]

A Dissertation

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Junru Cui

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APPROVAL PAGE

Doctor of Philosophy Dissertation

Study of a GP5-Mosaic T-cell Vaccine in Protection of Swine Against Heterologous PRRSV Strains

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Dedication

This dissertation is dedicated to my mother Min, and to my wife Wenxi and my son Lawrence.

It is their complete understanding, utmost support, genuine caring, and selfless love

that inspired and warmed me every day

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List of Abbreviations

ADWG average daily weight gain

AR atrophic rhinitis

bp base pair

cDNA complementary deoxyribonucleic acid

CMI cell-mediated immunity

CTL cytotoxic T lymphocytes

DMEM Dulbecco's Modified Eagle's medium

DNA deoxyribonucleic acid

DPC days post challenge

E envelope protein

EP electroporation

ER endoplasmic reticulum

EU European

FBS fetal bovine serum

FIR fluorescence intensity reduction

GP glycoprotein

HIV human immunodeficiency virus

HP highly pathogenic

ID intradermal

IFN interferon

IL interleukin

ILN inguinal lymph nodes

IM intramuscular

ISG interferon-stimulated gene

JAK Janus kinase

kb kilobase

KV killed virus

M membrane protein

MARC-145 African green monkey kidney cells

MLV modified live virus

MOI multiplicity of infection

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MW molecular weight

N nucleocapsid protein

NA North American

NSP non-structural protein

OD optical density

ORF open reading frame

PAM porcine alveolar macrophage

PBS phosphate buffered saline

PBMC peripheral blood mononuclear cells

phCMV plasmid human cytomegalovirus

PRRS porcine reproductive and respiratory syndrome

PRRSV porcine reproductive and respiratory syndrome virus

RFLP restriction fragment length polymorphism

RPMI Roswell Park Memorial Institute

RTC replication and transcription complex

RT-qPCR reverse transcriptase-quantitative polymerase chain reaction

RNA ribonucleic acid

SI stimulation index

STAT signal transducer and activator of transcription

TBLN tracheobronchial lymph nodes

TCID50 tissue culture infectious dose 50

TGFβ transforming growth factor beta

Th1 type 1 T helper

Th2 type 2 T helper

Tregs regulatory T cells

VACV vaccinia virus

VN viral neutralizing

WT wild type

Chapter I Introduction

1. PRRS Overview

Porcine reproductive and respiratory syndrome (PRRS)

Porcine reproductive and respiratory syndrome (PRRS) is a major disease affecting the swine industry worldwide. PRRS causes significant economic impact with estimated annual losses of more than 600 million dollars in the United States (1) and up to 1 billion dollars elsewhere. The disease is characterized by reproductive failure in sows and frequently fatal respiratory disease in young pigs (2)(3).

History and global distribution

PRRS was first recognized as a major cause of reproductive losses and respiratory diseases in the United States in the mid-1980's and named "mystery swine disease" or “mystery reproductive syndrome”. The causative agent, PRRS virus (PRRSV) was initially isolated in the Netherlands in 1991 (4) and one year later in the United States (5). The virus isolates from the Netherlands (Lelystad virus), and the United States (VR2332) are the reference strains of the European type (type I) and the American type (type II), respectively. Currently, type I is more prevalent in Europe, whereas type II is dominantly prevalent in the United States, Canada, and Asian countries. Nonetheless, it is believed that PRRSV is present in most swine-producing regions of the world, with a few exceptions such as Switzerland, Sweden, Norway, Finland and Australia. Within infected regions, it is estimated that 60-80% of herds are typically infected.

In 2006, a highly pathogenic strain of type II PRRSV (HP-PRRSV) emerged in China (6) and spread to other Southeastern Asian countries.

Virion structure

PRRSV has been classified as a member of the family *Arteriviridae*, in the order of *Nidovirales* (7)(8). PRRSV has a single-stranded, positive-sense RNA as genome that varies in length (14,876–15,520 bp). The virions are roughly spherical with an average diameter of 54 nm and consist of a nucleocapsid surrounded by a lipid bilayer containing several proteins (9)(10) PRRSV has 10 open reading frames (ORF) which encode 8 structural proteins and at least 14 non-structural proteins (nsp) (Fig.1(11)).

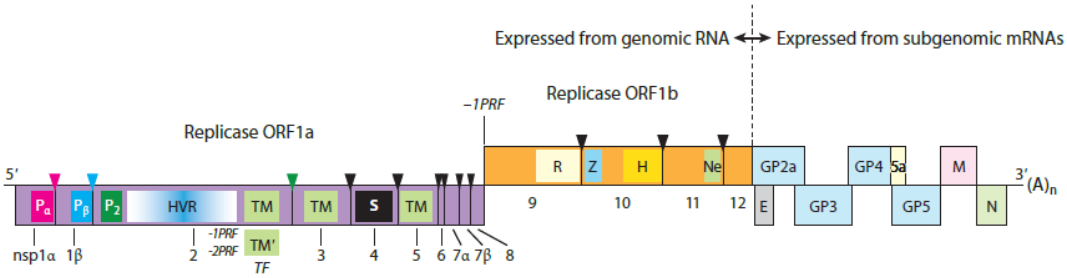

Figure 1.1 Schematic genome of PRRSV

Non-structural proteins (nsps) encoded by ORFs 1a and 1b, which constitute almost two-thirds of the genome, (such as RNA replicase and helicase), that are required for virus replication (12). At least 14 nsps are generated as a result of translation by a ribosomal frame shift between ORF1a and 1b (8)(13). Three N-glycosylated minor envelope proteins (GP2a, GP3, and GP4) are encoded by ORF2a, ORF3, and ORF4 and form heterotrimers by disulfide bond (14). This protein complex is responsible for virion attachment and entry by binding to CD163 (15). ORF2b, which is derived from a different frame within ORF2a, encodes another non-glycosylated minor protein named E (16). ORF5 encodes the major envelope glycoprotein (GP5) that forms a heterodimer with the membrane non-glycosylated protein (M) encoded by ORF6 which also participates in virion attachment by interacting with CD169 (17). The viral capsid is composed of only one nucleocapsid

protein (N), which is encoded by ORF7 and is highly immunogenic in infected animals (18). For all the major PRRSV viral proteins including structural and non-structural proteins, their characteristics and functions are summarized in Table 1 (adapted from (19)).

PRRSV replication

The PRRSV replication cycle is summarized in Figure 1.2 (11). Briefly, virus particles bind

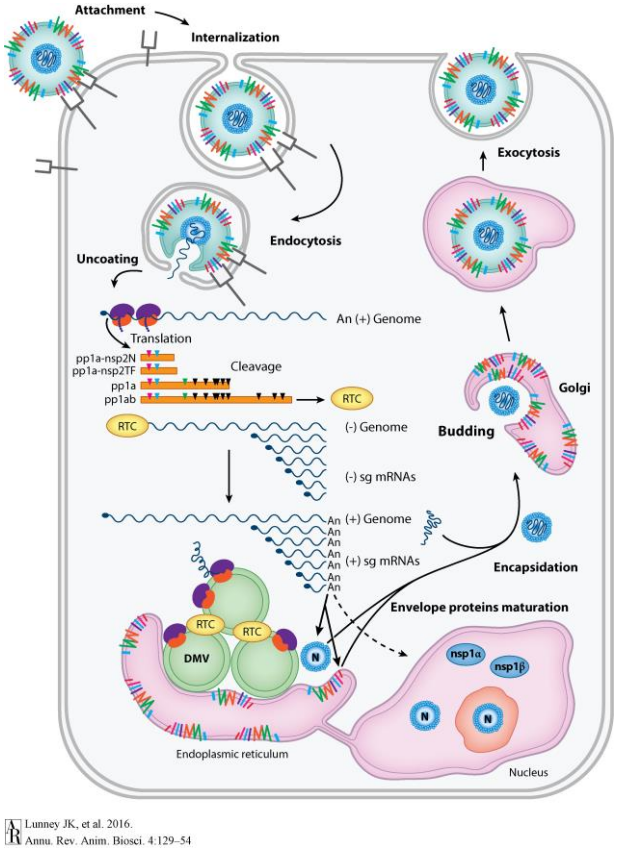

Figure 1.2. Overview of PRRSV replication (11)

to the cellular receptors and are then internalized by receptor-mediated endocytosis. After entry and uncoating, the positive-sense genomic RNA is released and subsequently acts as mRNA for synthesis of nsp polyprotein complexes polyprotein-1a and polyprotein-1b, which undergo

cleavage by viral proteases to produce 13-14 nsps. The nsps assemble into a replication and transcription complex (RTC) to catalyze replication of genomic RNA to produce full-length antigenomic RNA from which a nested transcription to produce 6 sub-genomic sense RNAs (sg mRNAs). The different sg mRNAs are translated to produce 7 structural proteins – GP2 2b, GP3, GP4, GP5, M, and N. All the structural proteins are required for viral packaging. Ultimately, newly synthesized genomes are packaged into nucleocapsids and then become enveloped. This assembly process occurs in the ER and Golgi complex. Budding of progeny virus occurs in the membranes of the endoplasmic reticulum and Golgi complex followed by exocytosis and the subsequent release of the enveloped virion particle.

Pathogenicity of the PRRS virus

It has been reported that PRRSV has a very restricted cell tropism both *in vitro* and *in vivo*. PRRSV was originally isolated in primary cell cultures of porcine alveolar macrophages (PAMs) (4) and to date, differentiated monocytes, along with PAMs, are the only porcine cells that can be effectively used for virus propagation. In addition, MARC-145 and CL2621 cells that are subclones of MA104 are also commonly used for propagating PRRSV *in vitro. In vivo*, the primary target cells of PRRSV are PAMs in the lung, interstitial macrophages, and those found in the heart, spleen, thymus and Peyer's patches. The clinical signs of PRRSV are reproductive failure in sows and often fatal respiratory disease in young pigs. PRRSV infection in sows can cause severe reproductive problems including premature farrowing, litters with weak, stillborn or mummified offspring and delayed return to service (20). In the United States, there were increased outbreaks later described as atypical PRRS or "swine abortion storm" (21). The spontaneous abortion rate was up 10-50%, and the mortality rate of the breeding herd has been found to be up to 5-10%

during an outbreak (22). In young pigs, they can display a variety of clinical signs such as dyspnea, tachypnea and death (up to 100% mortality). There were major outbreaks related to Isolates 184, RFLP 1-8-4 or Strain MN184 in 2001 and 2006 (23)(24). These outbreaks caused high morbidity (50%) and mortality (20%) rates. The analysis of ORF5 nucleotide sequence and comparison with other type 2 PRRS virus strains, demonstrated that MN184 was significantly different to previous strains. Also, three highly variable regions were identified, corresponding to nsp1β, nsp2, and ORF5. Nsp2 shared only 66-70% amino acid similarity to other North-American PRRS virus nsp2 proteins.

Table 1.1 PRRSV structural and non-structural proteins major characteristics and functions

Proteins	Genes	No. of AA(NA)	Characteristics and functions
Nsp1	ORF1a	383	Non-structural multifunctional regulatory protein; proteolytic activities; IFN inhibition
Nsp2		980	Non-structural, the largest PRRSV replicative protein; the major genetic differences between NA and EU strains; ideal marker for monitoring genetic variation and for developing differential diagnostic tests; proteolytic activities; IFN inhibition
Nsp9	ORF1b	640	Non-structural proteins; virus transcription and replication; nsp9: RNA-dependent RNA polymerase and NTPase; nsp10: helicase; nsp11: IFN inhibition
Nsp10		441	
Nsp11			
GP2a	ORF2a	256	Minor structural protein; contains 2 highly conserved N-linked glycosylation sites; essential for virus infectivity; incorporated into virions as a multimeric complex; viral attachment protein
E	ORF2b	73	Minor unglycosylated structural protein; essential for virus infectivity; incorporated into virions as a multimeric complex
GP3	ORF3	254	Minor structural protein, one of the most variable PRRSV proteins; highly glycosylated with 7 N-linked oligosaccharides; highly antigenic and may be involved in viral neutralization; essential for virus infectivity; incorporated into virions as a multimeric complex
GP4	ORF4	178	Minor structural highly glycosylated protein (4 sites); essential for virus infectivity; key glycoprotein for formation of the multiprotein complex incorporated into virions; mediates interaction between the complex and GP5; viral attachment protein and may be involved in viral neutralization

GP5	ORF5	200	Major structural, transmembrane protein with a variable number of potential N-glycosylation sites; the most variable structural protein in the PRRSV genome with GP3; involved in virus neutralization and protection; the covalent association of GP5 and M protein is crucial for virus assembly viral attachment protein; involved in the entry of virus into the host cells and in the apoptosis process
M	ORF6	174	Major unglycosylated structural protein which is the most conserved; play a key role in virus assembly and budding; GP5-M heterodimerization is crucial for virus infectivity
N	ORF7	123	Major unglycosylated, phosphorylated and structural protein; highly immunogenic and a suitable candidate for the detection of virus-specific Abs and diagnosis of the disease; the sole component of the viral capsid and interacts with itself through covalent and non-covalent interactions; able to localize in the nucleus and interact with cellular transcription factor

ORF, open reading frames; EU, European PRRSV strains; NA, North American PRRSV strains

In 2006, highly pathogenic PRRS (HP-PRRS) outbreaks provoked high losses to the pig production in China and later spread to other Asian countries. The causative agent of HP-PRRS was characterized as a variant of NA-PRRS virus (PRRSV) with a higher pathogenicity/virulence, which is called HP-PRRSV (6). The HP-PRRSV infection showed more severe reproductive failures in pregnant sows, higher morbidity (usually 50%-100%), and the mortality rates ranged from 20% to 100% in pigs of all ages. More than 40% of the infected pregnant sows exhibited abortions. All the infected pigs displayed a high fever of over 41°C. Total mortality rates were recorded in Laos, and they ranged from 6.02% in boars to 91.28% in piglets.

PRRSV infection and immune responses

Host innate immune responses play an instrumental role against early viral infection. Especially, type I IFNs are a critical component of the innate immunity against viral infections and play an essential role in the induction of adaptive immunity (25)(26). PRRSV sensitivity to type I IFNs was demonstrated in a study where IFN-α had the ability to reduce lung lesions and delay viremia in pigs (27). Some of the PRRSV proteins including nsp1, nsp2, nsp11, and N are found to inhibit

IFN induction and IFN signaling pathways (28). nsp1α and nsp1β were shown to inhibit IFN-β, specifically expression (29)(30).

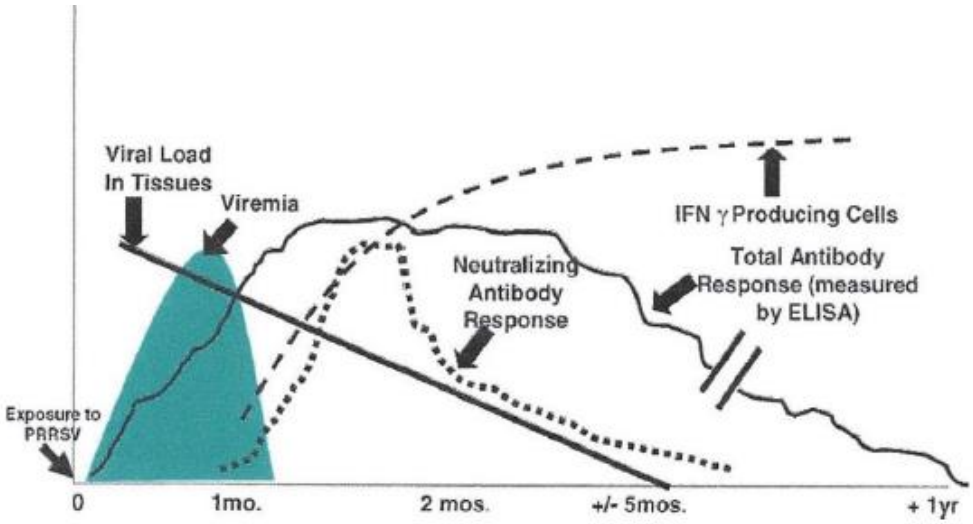

Figure 1.3 Immune responses to PRRSV infection (from Lopez et al.)

In another study, a strain dependent inhibition or activation of type I IFNs and IFN-induced proteins such as Mx-1 was reported (31). IFNs bind to their receptors on the cell surface and activate JAK/STAT pathway, leading to the expression of IFN-stimulated genes (ISGs) in both MARC-145 cells and PAM cells (32)(33)(34).

There is evidence that PRRSV infection results in delayed immune responses (Fig.1.3(35)). Viral neutralizing (VN) antibodies are usually not detected until four weeks post-infection (36)(37), and they peak at 10-18 weeks post-infection with relatively low titers (37)(38). GP5 contains the most critical neutralizing epitope, yet the presence of multiple glycosylation sites might interfere with the access of antibodies to GP5. Moreover, the existence of a decoy epitope on GP5 that induces non-neutralizing antibodies with a delayed production of VN antibodies for at least three or four weeks (39). Some studies have shown that viremia may be resolved before the presence of VN

antibodies (38). Other evidence supports that VN antibodies are important for protection, although the mechanism of action remains unclear (40)(41)(39)(42)(43). In PRRS, prolonged viremia correlates with a weak innate antiviral response (32)(28), high levels of IL-10 and TGFβ (44), low levels of IFN-γ coupled to sub-optimal adaptive immunity (45)(39). A study also showed that the levels of IFN-γ and IL-10 were satisfactory markers of polarization of the adaptive response, and the level of IL-10 correlated directly with the level of infected macrophages (46).

The adaptive cellular response can be assessed by lymphocyte recall stimulation (47) and detection of antigen-specific cytotoxic T lymphocytes (CTL). Several studies have shown that cell-mediated immune responses are weak and delayed in PRRSV-infected pigs (45). In addition, IL-10 can induce regulatory T cells that may down-regulate the production of IFN-γ producing cells, result in the shift of Th1 response to Th2 response (48)(49)(50).

2. Vaccine Design Strategy

Next Generation Vaccine Technologies

The control of PRRS relies largely on vaccination with conventional modified live-attenuated vaccines and killed vaccines. Additionally, some local farm-specific autogenous vaccines are used in the United States (51). Since the protection by current vaccines is limited to homologous PRRSV strains, the demand for vaccines that protect against diverse strains is very high. Unfortunately improving vaccine coverage is difficult due to the vast genetic and antigenic diversity of the virus. Next generation vaccine strategies that increase both breadth and depth for highly variable viruses such as consensus sequence vaccines, multi-subunit vaccines, molecular breeding by DNA shuffling and screening strategies for PRRSV, and mosaic T-cell epitope vaccines are being intensively investigated (52)(53)(54)(55)(56)(57)(58)(59)(60)(61)(62). Mosaic

T-cell epitope vaccines can be generated using a Mosaic Vaccine Tool Suite that was originally designed to address HIV sequence diversity at Los Alamos National Laboratory (61). Briefly, the resulting mosaic sequences are generated from *in silico* "recombination" of natural strains of virus and are constrained in the same fashion as natural virus sequences. Studies show that these Mosaic T-cell epitope vaccines improve the immune responses to the globally circulating strains of HIV-1 by broadening the range of recognized epitopes and increasing responses to high-frequency epitopic variants (63)(64).

Mosaic Vaccines

A mosaic protein, is a recombinant protein derived from a library of proteins of interest through

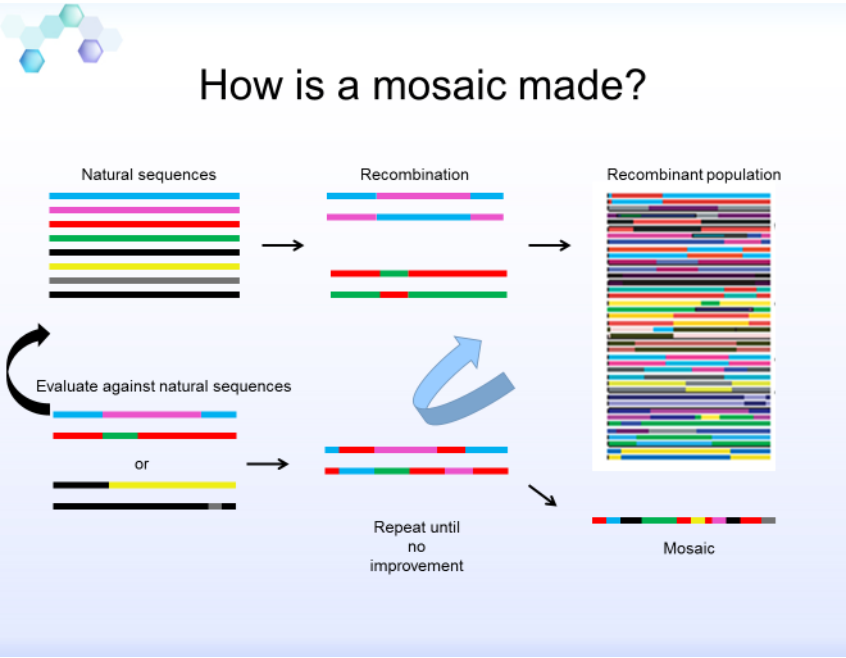

Figure 1.4. The process of generation of mosaic sequences (courtesy of Caitlin O'Connell)

many cycles of recombination. As the building blocks for the mosaic protein, (N-mer) peptides that make up the mosaic sequence by overlapping each other exit naturally within the protein

library. Values of N between 9 and 12 are recommended because that is the size of peptides preferentially recognized by cytotoxic T-cells (Figure 1.4).

Potential T cell epitope targets on PRRSV

There are more than a dozen T-cell epitopes that have been identified in several viral proteins of PRRSV including non-structural proteins nsp2, nsp9 and nsp10, structural proteins GP5, M and the capsid protein N which have been reported by different research groups (Table 1.2). Among these proteins, nsp2 and nsp10 are excluded due to their ability to suppress the host immune

Table 1.2 T cell epitopes identified on PRRSV

Protein	#	T cell epitope	Induce IL-10	Reference
Nsp9	4	TMPPGFELY (198–206) VLPGVLRLV (258–266) exists in mosaic protein delete? 119KEEIALSAQIIQACDIR 151VRGNPERVKGVLQNTRF	yes	Xia, 2011 Mateu, 2013 Osorio, 2011
GP5	2	^{117}LAALICFVIRLAKNC ^{149}KGRLYRWRSPVIVEK		Zuckermann, 2008 Diaz, 2009
M	4	K_{93}FITSRCRL, F_{57}GYMTFVHF $_{9}$CNDSTAPQKVLLAFS, $_{33}$ALKVSRGRLLGLLHL $_{57}$FGYMTFVHFESTNRV, $_{93}$KFITSRCRLCLLGRK		Wu, 2011 in mice Tong, 2011 in pigs
N	3	^{49}NPEKPHFPL (Both I, II) ^{63}VRHHFTPSE (Both I, II) ^{104}FSLPTQHTVRLIRATAS $_{104}$FSLPTHHTVRLIRVTAS (VR2332)		Diaz, 2009

response. GP5 is the perfect candidate based on the fact that it has two reported T-cell epitopes (65)(66) and a major neutralizing epitope (39)(67). It forms a heterodimer with M protein, and together they play a vital role in virus infectivity. GP5 is highly variable and thus it is a major target for phylogenic analysis and vaccine development. Additionally, the most extensive sequence

information on hand is for GP5. Combining what is known at this time, we believe that GP5 is our best choice as a candidate to develop mosaic T-cell epitope vaccine to address the diversity issue of PRRSV. A method that is readily available for rapid vaccine evaluation is the use of expression plasmid DNA as the backbone to deliver and test viral antigens.

3. DNA Vaccine

DNA vaccine plasmid

DNA vaccination induces immune responses to protein(s) of interest expressed *in vivo* following the introduction of DNA plasmid encoding the protein sequence of candidate antigens (68). Normally, the target protein is expressed under a strong promoter such as human cytomegalovirus (CMV) as it activates high levels of transgene expression *in vivo* hence facilitating antigen presentation (69). The vaccine plasmids are readily produced in bacterial culture, purified, quantified, formulated when necessary and then used as a vaccine.

Advantages of DNA vaccine

DNA vaccines have several notable advantages over traditional vaccination strategies such as modified-live virus and killed virus vaccines. 1) As a safe, non-living vaccine approach, DNA vaccines have no risk of infection. 2) Target antigen can be presented by both class I and class II major histocompatibility complex (MHC) molecules. As a result, both $CD4^+$ and $CD8^+$ T cell responses are generated. Therefore, DNA vaccines can induce both humoral (70) and cell-mediated immune responses (CMI) (71), 3) The relative ease of DNA vaccine design and production allows for the quick and efficient development of immunogens via recombinant DNA technology. 4) DNA vaccines are also stable and can be used with multiple immunizations. Lastly,

5) *In vivo* expression ensures protein more closely resembles normal eukaryotic structure, with accompanying post-translational modifications and both cytosolic sand endosomal processing and presentation.

Mechanism of DNA Vaccination

The mechanism by which DNA vaccines induce adaptive immune responses has been well studied. The gene of interest can be delivered either intradermally, subcutaneously, or directly into muscle

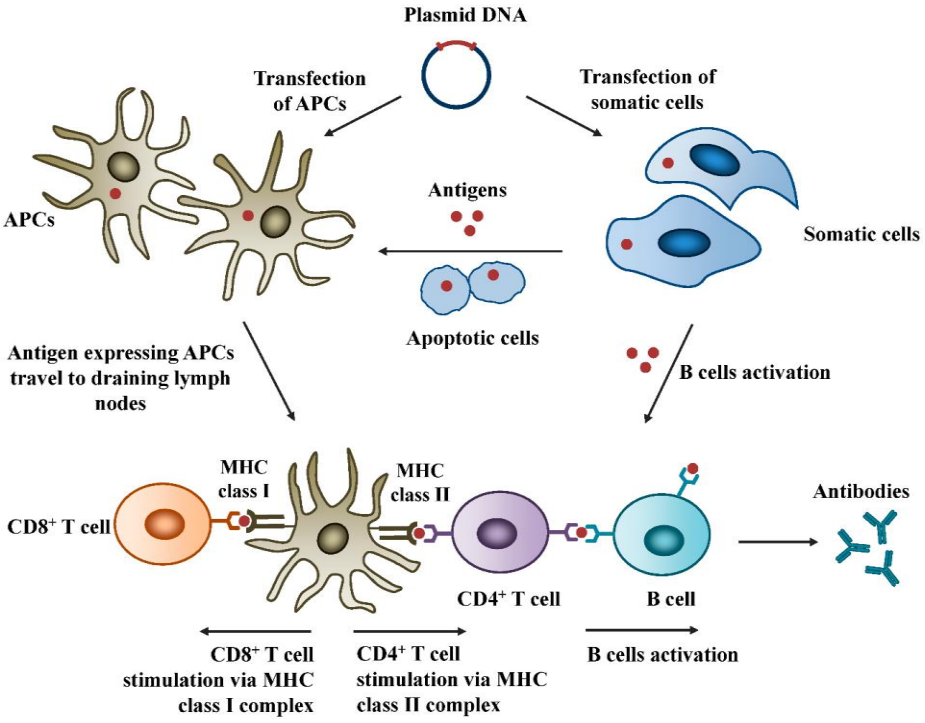

Figure 1.5. Mechanisms of action of DNA vaccines (72)

by different delivery methods including gene gun, electroporation, or needle-free injection systems. It is widely recognized that local myocytes and keratinocytes, including resident APC populations, can be directly transfected by DNA vaccines or enter via either phago- or pinocytosis (73). Facilitated by host cellular machinery in the nucleus, the gene of interest is transcribed and

translated (74). This process is then sequentially followed by the expression of polypeptides, and finally, by processing and presentation on MHC I or MHC II molecules, depending on whether the antigen is transported endogenously or exogenously. Later in the immune response, mature antigen presenting cells (APCs) travel to the draining lymph nodes where they encounter and stimulate naive T-cells (73). This process also provides the secondary signals that are essential to initiate an immune response, facilitating T-cell activation, either towards Th1 or Th2, which subsequently activates cytotoxic T cells or B cells and antibody production cascades (Figure 1.5, (72)).

In this manner, DNA vaccines are capable of inducing both humoral and cellular immune responses. The successful expression of antigen *in vivo* is very crucial in conferring protective immunity following DNA vaccination. Antigen is produced directly and naturally by host cells, and thus allows target proteins to undergo well-regulated translation processes, which facilitate correct conformational folding, along with post-translational modifications, such as glycosylation. This assures accurate mimicking of natural pathogen infection without possible safety risks (75)(76). Furthermore, plasmid DNA contains unmethylated deoxycytidylate-phosphate-deoxyguanylate (CpG) motifs, which have shown to be able to activate APC populations, thus increasing their ability to stimulate antigen-specific $CD4^+/CD8^+$ T cells, which result in enhanced immune responses.

4. Hypothesis

Our hypothesis is that mosaic T cell vaccines can present a wide array of T cell epitopes representing many PRRSV strains, which can increase the breadth and depth of immune recognition and ultimately broaden the protection against divergent PRRSV strains.

5. Project Overview

This project includes the collection of ORF5 sequence information, generation of GP5-Mosaic sequences, construction of GP5-Mosaic vaccine, characterization of the GP5-Mosaic vaccines,

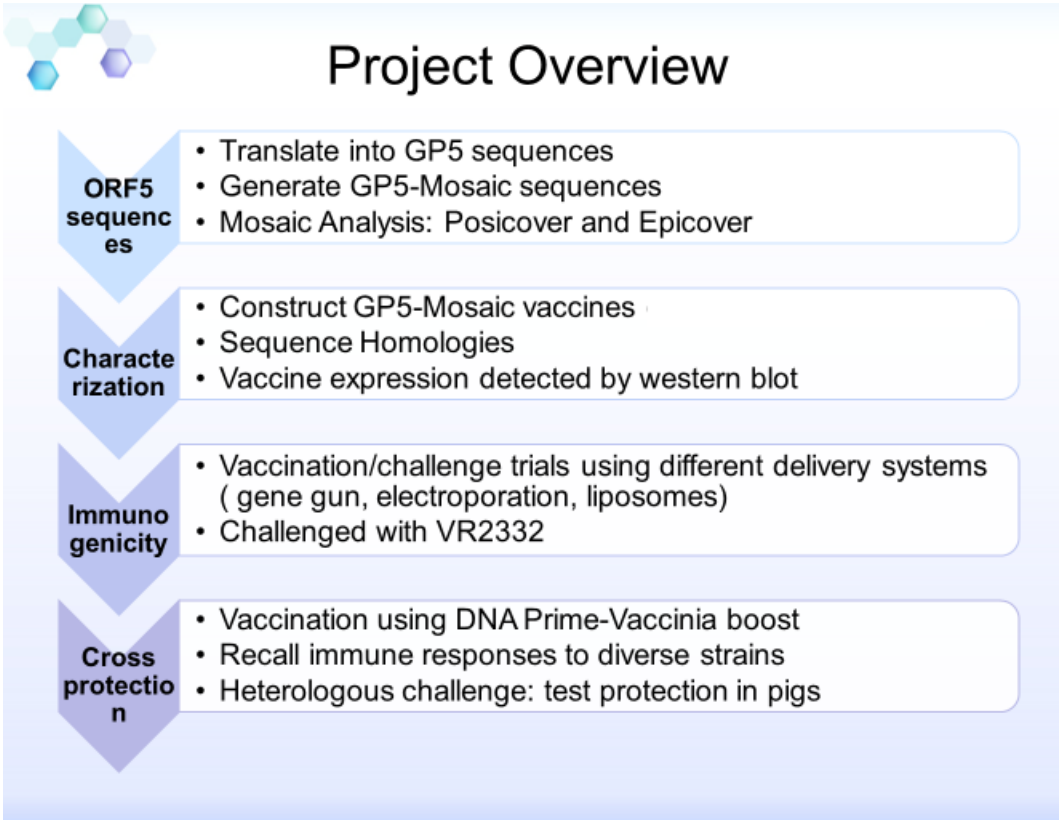

Figure 1.6 Project Overview

and antigen expression *in vitro*. The second step was to test the immunogenicity of the GP5-Mosaic vaccines in pigs. Three vaccination/challenge trials were conducted using different delivery systems such as gene gun, electroporation or liposomes. In the first two trials, the experimental pigs were challenged with VR2332 to evaluate the efficacy of the GP5-Mosaic vaccine. Their capability to induce cross reactivity ex vivo was tested by stimulation of PBMCs and expression of IFN-γ. The final trial tested the ability of the GP5-Mosaic vaccine to confer cross protection in

pigs. For this purpose, the experimental pigs were challenged with two PRRSV strains which have more than 10% difference in GP5 sequence VR2332 and MN184C (shown in Figure 1.6).

6. Summary

This thesis tested the ability of a GP5-Mosaic T-cell vaccine generated using a Mosaic Tool Suite that was originally developed for HIV-1 in Los Alamos National Laboratory, to cross-protect pigs from heterologous virus challenge. The characterization of GP5-Mosaic sequences, the evaluation of immunogenicity and protection in pigs, the ability to induce cross-reactivity *ex-vivo*, and eventually the efficacy against heterologous PRRSV challenge are discussed.

References

1. Bøtner A, Strandbygaard B, Sørensen KJ, Have P, Madsen KG, Madsen ES, Alexandersen S. Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. Vet Rec. 1997 Nov 8;141(19):497-9
2. Done SH, Paton DJ. Porcine reproductive and respiratory syndrome: clinical disease, pathology and immunosuppression. Vet Rec. 1995 Jan 14;136(2):32-5.
3. Holtkamp DJ, Kliebenstein JB, Neumann EJ, et al. Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. J Swine Health Prod 2013;21(2):72-84.
4. Wensvoort, G., C. Terpstra, J. M. Pol et al. 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet. Q. 13:121–130
5. Collins, J. E., D. A. Benfield, W. T. Christianson et al. 1992. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J. Vet. Diagn. Invest. 4:117–126
6. Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D., Tian, X., Liu, D., Zhang, S., Deng, X., Ding, Y., Yang, L., Zhang, Y., Xiao, H., Qiao, M., Wang, B., Hou, L., Wang, X., Yang, X., Kang, L., Sun, M., Jin, P., Wang, S., Kitamura, Y., Yan, J., Gao, G.F., 2007. Emergence of Fatal PRRSV Variants: Unparalleled Outbreaks of Atypical PRRS in China and Molecular Dissection of the Unique Hallmark. PLoS ONE 2.
7. K.K. Conzelmann Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group Virology, 1993, Vol.193(1), p.329
8. E.J. Snijder. The molecular biology of arteriviruses. The Journal of general virology, 1998, (Pt 5), p.961
9. Faaberg KS, Balasuriya UB, Brinton MA, Gorbalenya AE, Leung FC, Nauwynck H, Snijder EJ, Stadejek T, Yang H, Yoo D. Family Arteriviridae. In: King AMQ, editor. Virus taxonomy: classification and nomenclature of viruses: ninth report of the International Committee on Taxonomy of Viruses. Amsterdam: Elsevier/Academic Press; 2012. p. 796–805.

10. Johnson CR, Griggs TF, Gnanandarajah J, Murtaugh MP. Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative ORF5 present in all arteriviruses. J Gen Virol. 2011;92:1107–16.
11. Joan K. Lunney, Ying Fang, Andrea Ladinig, Nanhua Chen, Yanhua Li, Bob Rowland, and Gourapura J. Renukaradhya Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction with the Immune System Annual Review of Animal Biosciences Vol. 4: 129-154
12. R Allende, T L Lewis, Z Lu, D L Rock, G F Kutish, A Ali, A R Doster, F A Osorio North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. J. Gen. Virol., February 1999 80: 307-315, doi: 10.1099/0022-1317-80-2-307
13. Snijder EJ. The arterivirus replicase. The road from RNA to protein(s), and back again. Adv Exp Med Biol. 1998; 440:97-108
14. Wissink EH, Kroese MV, van Wijk HA, Rijsewijk FA, Meulenberg JJ, Rottier PJ. Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus. J Virol. 2005 Oct;79(19):12495-506.
15. Van Breedam W, Delputte PL, Van Gorp H, Misinzo G, Vanderheijden N, Duan X, Nauwynck HJ. Porcine reproductive and respiratory syndrome virus entry into the porcine macrophage. J. Gen. Virol. 2010.91: 1659 –1667
16. Wu WH, Fang Y, Farwell R, Steffen-Bien M, Rowland RR, Christopher-Hennings J, Nelson EA. A 10-kDa structural protein of porcine reproductive and respiratory syndrome virus encoded by ORF2b. Virology. 2001 Aug 15;287(1):183-91
17. Mardassi H, Massie B, Dea S. Intracellular synthesis, processing, and transport of proteins encoded by ORFs 5 to 7 of porcine reproductive and respiratory syndrome virus. Virology. 1996 Jul 1;221(1):98-112.
18. Dea,S.; Gagnon,C.A.; Mardassi,H.; Pirzadeh,B.; Rogan,D. Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates Archives of virology , 2000, Vol.145(4), p.659

19. Music N, Gagnon CA. The role of porcine reproductive and respiratory syndrome (PRRS) virus structural and non-structural proteins in virus pathogenesis Anim Health Res Rev. 2010 Dec; 11(2):135-63.

20. Rossow, K.D., 1998. Porcine Reproductive and Respiratory Syndrome. Veterinary Pathology 35, 1–20.

21. Mengeling, W.L., Lager, K.M., Vorwald, A.C., 1998. Clinical consequences of exposing pregnant gilts to strains of porcine reproductive and respiratory syndrome (PRRS) virus isolated from field cases of "atypical" PRRS. American Journal of Veterinary Research 59, 1540–1544.

22. Thanawongnuwech R, Thacker EL, Halbur PG. Effect of porcine reproductive and respiratory syndrome virus (PRRSV) (isolate ATCC VR-2385) infection on bactericidal activity of porcine pulmonary intravascular macrophages (PIMs): in vitro comparisons with pulmonary alveolar macrophages (PAMs). Vet Immunol Immunopathol. 1997 Nov;59(3-4):323-35

23. Yoon KJ, Chang CC, Zimmerman J, Harmon K. Genetic and antigenic stability of PRRS virus in pigs. Field and experimental prospectives. Adv Exp Med Biol. 2001;494:25-30

24. Han J, Wang Y, Faaberg KS. Complete genome analysis of RFLP 184 isolates of porcine reproductive and respiratory syndrome virus. Virus Res. 2006 Dec;122(1-2):175-82.

25. González-Navajas JM, Lee J, David M, Raz E. Immunomodulatory functions of type I interferons. Nat Rev Immunol. 2012 Jan 6;12(2):125-35

26. Akaoka A, Yanai H. Interferon signalling network in innate defence. Cell Microbiol. 2006 Jun;8 (6):907-22.

27. Brockmeier SL, Lager KM, Grubman MJ, Brough DE, Ettyreddy D, Sacco RE, Gauger PC, Loving CL, Vorwald AC, Kehrli ME Jr, Lehmkuhl HD. Adenovirus-mediated expression of interferon-alpha delays viral replication and reduces disease signs in swine challenged with porcine reproductive and respiratory syndrome virus. Viral Immunol. 2009 Jun;22(3):173-80.

28. Chen Z, Lawson S, Sun Z, Zhou X, Guan X, Christopher-Hennings J, Nelson EA, Fang Y. Identification of two auto-cleavage products of nonstructural protein 1 (nsp1) in porcine reproductive and respiratory syndrome virus infected cells: nsp1 function as interferon antagonist. Virology. 2010 Mar 1;398(1):87-97.

29. Shi, X., Chen, J., Xing, G., Zhang, X., Hu, X., Zhi, Y., Guo, J., Wang, L., Qiao, S., Lu, Q., Zhang, G., 2012. Amino acid at position 176 was essential for porcine reproductive and respiratory syndrome virus (PRRSV) non-structural protein 1α (nsp1α) as an inhibitor to the induction of IFN-?? Cellular Immunology 280, 125–131.

30. Wang, R., Zhang, Y.-J., 2014. Antagonizing Interferon-Mediated Immune Response by Porcine Reproductive and Respiratory Syndrome Virus. BioMed research international 2014, 315470.

31. Overend C, Mitchell R, He D, Rompato G, Grubman MJ, Garmendia AE. Recombinant swine beta interferon protects swine alveolar macrophages and MARC-145 cells from infection with Porcine reproductive and respiratory syndrome virus. J Gen Virol. 2007 Mar;88(Pt 3):925-31.

32. Patel D, Nan Y, Shen M, Ritthipichai K, Zhu X, Zhang YJ. Porcine reproductive and respiratory syndrome virus inhibits type I interferon signaling by blocking STAT1/STAT2 nuclear translocation. J Virol. 2010 Nov;84(21):11045-55.

33. Wang R, Nan Y, Yu Y, Zhang YJ. Porcine reproductive and respiratory syndrome virus Nsp1β inhibits interferon-activated JAK/STAT signal transduction by inducing karyopherin-α1 degradation. J Virol. 2013 May;87(9):5219-28.

34. Wang R, Nan Y, Yu Y, Yang Z, Zhang YJ. Variable interference with interferon signal transduction by different strains of porcine reproductive and respiratory syndrome virus. Vet Microbiol. 2013 Oct 25;166(3-4):493-503.

35. Lopez, O.J., Osorio, F.A., 2004. Role of neutralizing antibodies in PRRSV protective immunity. Veterinary Immunology and Immunopathology. doi:10.1016/j.vetimm.2004.09.005

36. E. Mateu, I. Diaz. The challenge of PRRS immunology Vet. J., 177 (2008), pp. 345–351

37. Benfield DA, Nelson E, Collins JE, Harris L, Goyal SM, Robison D, Christianson WT, Morrison RB, Gorcyca D, Chladek D. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332) J Vet Diagn Invest. 1992 Apr;4(2):127-33.

38. Nelson EA, Christopher-Hennings J, Benfield DA. Serum immune responses to the proteins of porcine reproductive and respiratory syndrome (PRRS) virus. J Vet Diagn Invest. 1994 Oct; 6(4):410-5.

39. Robinson SR, Figueiredo MC, Abrahante JE, Murtaugh MP. Immune response to ORF5a protein immunization is not protective against porcine reproductive and respiratory syndrome virus infection. Vet Microbiol. 2013 Jun 28; 164(3-4):281-5.

40. Lopez OJ, Oliveira MF, Garcia EA, Kwon BJ, Doster A, Osorio FA. Protection against porcine reproductive and respiratory syndrome virus (PRRSV) infection through passive transfer of PRRSV-neutralizing antibodies is dose dependent. Clin Vaccine Immunol. 2007 Mar; 14(3):269-75.

41. Osorio FA, Galeota JA, Nelson E, Brodersen B, Doster A, Wills R, Zuckermann F, Laegreid WW. Passive transfer of virus-specific antibodies confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. Virology. 2002 10; 302 (1): 9-20.

42. Labarque GG, Nauwynck HJ, Van Reeth K, Pensaert MB. Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. J Gen Virol. 2000 May;81(Pt 5):1327-34.

43. Renukaradhya GJ, Alekseev K, Jung K, Fang Y, Saif LJ. Porcine reproductive and respiratory syndrome virus-induced immunosuppression exacerbates the inflammatory response to porcine respiratory coronavirus in pigs. Viral Immunology (2010). 23, 5, 457-466

44. Meier WA, Galeota J, Osorio FA, Husmann RJ, Schnitzlein WM, Zuckermann FA. Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology (2003). 309, 18-31

45. Go N, Bidot C, Belloc C, Touzeau S. Integrative model of the immune response to a pulmonary macrophage infection: What determines the infection duration? PLoS ONE (2014). 9, 9

46. Ren JQ, Sun WC, Lu HJ, Wen SB, Jing J, Yan FL, Liu H, Liu CX, Xiao PP, Chen X, Du SW, Du R, Jin NY. Construction and immunogenicity of a DNA vaccine coexpressing GP3 and GP5 of Genotype-I porcine reproductive and respiratory syndrome virus. BMC Veterinary Research (2014). 10, 1, 128

47. Wongyanin P, Buranapraditkun S, Chokeshai-Usaha K, Thanawonguwech R, Suradhat S. Induction of inducible CD4+CD25+Foxp3+ regulatory T lymphocytes by porcine reproductive and respiratory syndrome virus (PRRSV).Vet Immunol Immunopathol. 2010 Feb 15; 133(2-4):170-82.

48. Silva-Campa E, Mata-Haro V, Mateu E, Hernández J. Porcine reproductive and respiratory syndrome virus induces CD4+CD8+CD25+Foxp3+ regulatory T cells (Tregs). Virology. 2012 Aug 15; 430(1):73-80.

49. Díaz I, Darwich L, Pappaterra G, Pujols J, Mateu E. Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs. Virology. 2006 Aug 1;351(2):249-59.

50. Kimman TG, Cornelissen LA, Moormann RJ, Rebel JM, Stockhofe-Zurwieden N. Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. Vaccine. 2009 Jun 8;27(28):3704-18.

51. Jiang,P.; Jiang,W.; Li,Y.; Wu,S.; Xu,J. Humoral immune response induced by oral administration of S. typhimurium containing a DNA vaccine against porcine reproductive and respiratory syndrome virus. Vet.Immunol.Immunopathol., 2004, 102, 3, 321-328, Netherlands

52. Jiang,Y.; Xiao,S.; Fang,L.; Yu,X.; Song,Y.; Niu,C.; Chen,H. DNA vaccines co-expressing GP5 and M proteins of porcine reproductive and respiratory syndrome virus

(PRRSV) display enhanced immunogenicity. Vaccine, 2006, 24, 15, 2869-2879, Netherlands

53. Jiang,Y.; Fang,L.; Xiao,S.; Zhang,H.; Pan,Y.; Luo,R.; Li,B.; Chen,H. Immunogenicity and protective efficacy of recombinant pseudorabies virus expressing the two major membrane-associated proteins of porcine reproductive and respiratory syndrome virus. Vaccine, 2007, 25, 3, 547-560, Netherlands

54. Jiang,W.; Jiang,P.; Wang,X.; Li,Y.; Du,Y.; Wang,X. Enhanced immune responses of mice inoculated recombinant adenoviruses expressing GP5 by fusion with GP3 and/or GP4 of PRRS virus. Virus Res., 2008, 136, 1-2, 50-57, Netherlands

55. Shen,G.; Jin,N.; Ma,M.; Jin,K.; Zheng,M.; Zhuang,T.; Lu,H.; Zhu,G.; Jin,H.; Jin,M.; Huo ,X.; Qin,X.; Yin,R.;Li,C.; Li,H.; Li,Y.; Han,Z.; Chen,Y.; Jin,M. Immune responses of pigs inoculated with a recombinant fowlpox virus coexpressing GP5/GP3 of porcine reproductive and respiratory syndrome virus and swine IL-18. Vaccine, 2007, 25, 21, 4193-4202, Netherlands

56. Eko,F.O.; He,Q.; Brown,T.; McMillan,L.; Ifere,G.O.; Ananaba,G.A.; Lyn,D.; Lubitz,W.; Kellar,K.L.; Black,C.M.;Igietseme,J.U. A novel recombinant multi subunit vaccine against Chlamydia. J.Immunol., 2004, 173, 5, 3375-3382, United States

57. Yan,J.; Yoon,H.; Kumar,S.; Ramanathan,M.P.; Corbitt,N.; Kutzler,M.; Dai,A.; Boyer,J.D .; Weiner,D.B. Enhanced cellular immune responses elicited by an engineered HIV-1 subtype B consensus-based envelope DNA vaccine. Mol.Ther., 2007, 15, 2, 411-421, United States

58. Santra,S.; Liao,H.X.; Zhang,R.; Muldoon,M.; Watson,S.; Fischer,W.; Theiler,J.; Szinger, J.; Balachandran,H.;Buzby,A.; Quinn,D.; Parks,R.J.; Tsao,C.Y.; Carville,A.; Mansfield,K .G.; Pavlakis,G.N.; Felber,B.K.;Haynes,B.F.; Korber,B.T.; Letvin,N.L. Mosaic vaccines elicit CD8+ T lymphocyte responses that confer enhanced immune coverage of diverse HIV strains in monkeys Nat. Med., 2010, 16, 3, 324-328, United States

59. Zhou,L.; Ni,Y.Y.; Pineyro,P.; Sanford,B.J.; Cossaboom,C.M.; Dryman,B.A.; Huang,Y.W .; Cao,D.J.; Meng,X.J. DNA shuffling of the GP3 genes of porcine reproductive and

respiratory syndrome virus (PRRSV) produces a chimeric virus with an improved cross-neutralizing ability against a heterologous PRRSV strain. Virology, 2012, 434, 1, 96-109

60. Fischer,W.; Perkins,S.; Theiler,J.; Bhattacharya,T.; Yusim,K.; Funkhouser,R.; Kuiken,C.; Haynes,B.;Letvin,N.L.; Walker,B.D.; Hahn,B.H.; Korber,B.T. Polyvalent vaccines for optimal coverage of potential T-cell epitopes in global HIV-1 variants. Nat.Med., 2007, 13, 1, 100-106, United States
61. Thurmond,J.; Yoon,H.; Kuiken,C.; Yusim,K.; Perkins,S.; Theiler,J.; Bhattacharya,T.; Ko rber,B.; Fischer,W. Web-based design and evaluation of T-cell vaccine candidates. Bioinformatics, 2008, 24, 14, 1639-1640, England
62. J. Cui, C.M. O'Connell, J.D. Smith, J. Smyth, P. H. Verardi, Y. Pan and A. E. Garmendia "A GP5 Mosaic T-cell Vaccine for Porcine Reproductive and Respiratory Syndrome Virus Is Immunogenic and Confers Partial Protection to Pigs" Vaccine Reports 2016, 6: 77-85
63. Barouch DH, O'Brien KL, Simmons NL, King SL, Abbink P, Maxfield LF, Sun YH, La Porte A, Riggs AM, Lynch DM, Clark SL, Backus K, Perry JR, Seaman MS, Carville A, Mansfield KG, Szinger JJ, Fischer W, Muldoon M, Korber B. Mosaic HIV-1 vaccines expand the breadth and depth of cellular immune responses in rhesus monkeys. Nature Medicine (2010). 16, 3, 319-323
64. Santra S, Liao HX, Zhang R, Muldoon M, Watson S, Fischer W, Theiler J, Szinger J, Balachandran H, Buzby A, Quinn D, Parks RJ, Tsao CY, Carville A,Mansfield KG, Pavlakis GN, Felber BK, Haynes BF, Korber BT, Letvin NL. Mosaic vaccines elicit CD8+ T lymphocyte responses that confer enhanced immune coverage of diverse HIV strains in monkeys. Nature Medicine (2010). 16, 3, 324-328
65. Vashisht K, Goldberg TL, Husmann RJ, Schnitzlein W, Zuckermann FA. Identification of immunodominant T-cell epitopes present in glycoprotein 5 of the North American Genotype of porcine reproductive and respiratory syndrome virus. Vaccine (2008). 26, 36, 4747-4753
66. Plagemann PG, Rowland RR, Faaberg KS. The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Archives of Virology (2002). 147, 12, 2327-2347

67. Ulmer, J.B., et al., Heterologous protection against influenza by injection of DNA encoding a viral protein., in Science1993. p. 1745--- 1749.
68. Galvin, T.A., J. Muller, and A.S. Khan. Effect of different promoters on immune responses elicited by HIV--- 1 gag/env multigenic DNA vaccine in Macaca mulatta and Macaca nemestrina. Vaccine, 2000. 18(23): p. 2566--- 83.
69. Yang, Z.Y., et al., A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. Nature, 2004. 428(6982): p. 561--- 4.
70. Wang, R., et al., Induction of CD4(+) T cell--- dependent CD8(+) type 1 responses in humans by a malaria DNA vaccine. Proc Natl Acad Sci U S A, 2001. 98(19): p. 10817--- 22.
71. Kutzler, M.A. and D.B. Weiner, DNA vaccines: ready for prime time? Nat Rev Genet, 2008. 9(10): p. 776--- 88.
72. Lewis, P.J. and L.A. Babiuk, DNA vaccines: a review. Adv Virus Res, 1999. 54: p.129--- 88.
73. Xu, Y.; Yuen, P.-W.; Lam, J.K.-W. Intranasal DNA Vaccine for Protection against Respiratory Infectious Diseases: The Delivery Perspectives. *Pharmaceutics* 2014, *6*, 378-415.
74. Nichols,W.W., et al., Potential DNA vaccine integration into host cell genome. Ann N Y Acad Sci, 1995. 772: p. 30--- 9.
75. Manam, S., et al., Plasmid DNA vaccines: tissue distribution and effects of DNA sequence, adjuvants and delivery method on integration into host DNA. Intervirology, 2000. 43(4--- 6): p. 273--- 81.

Chapter II A GP5 Mosaic T-cell Vaccine for Porcine Reproductive and Respiratory Syndrome Virus Is Immunogenic and Confers Partial Protection to Pigs

Summary

Porcine reproductive and respiratory syndrome (PRRS) causes significant economic losses due to reproductive failure and respiratory disease. The high genetic and antigenic diversity of the causative agent PRRSV, an RNA virus, makes the control of this disease very challenging. In order to achieve broad protection towards such a divergent virus, two T-cell mosaic vaccines were designed based on 748 glycoprotein 5 sequences using the Mosaic T-Cell Vaccine Tool Suite from the Los Alamos National Laboratory. Mosaic sequences were then used to construct two DNA vaccines. The mosaics were closer in amino acid sequence to strains that differ from VR2332 by at least 10%. The T-cell epitope coverage was broadened when two mosaics were made. Expression of the mosaic sequences was verified in *E. coli* BL21 (DE3) by western blot. Two vaccination/challenge trials were performed in pigs to evaluate immunogenicity. The vaccines were delivered by gene gun (Trial 1) or electroporation (Trial 2). Significantly higher proliferative responses were detected in virus-stimulated peripheral blood mononuclear cells of GP5-Mosaic-vaccinated pigs compared to control pigs in both trials. In Trial 2, significantly higher levels of interferon-γ mRNA and lower levels of IL-10 mRNA were detected in GP5-Mosaic-vaccinated pigs, as compared to control pigs. Virus-specific antibodies were higher in GP5-Mosaic-vaccinated animals than in control animals in Trial 2. The antibodies were neutralizing. In Trial 2, the viral copy numbers in serum 5 days after challenge with VR2332 decreased over 260-fold within 2 days in GP5-Mosaic-vaccinated pigs, while the viral copy numbers increased by 1.2 to 5-fold in control animals in the same period. In Trial 2, viral loads in inguinal lymph nodes and spleen, and lung lesion scores of GP5-Mosaic-vaccinated animals

were lower compared to those of control animals. This data led us to conclude that the GP5-Mosaic vaccine was immunogenic and induced partial protection in vaccinated pigs.

Key words: PRRS, PRRSV, mosaic T-cell vaccine, swine

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a major disease affecting swine worldwide, characterized by reproductive failure and respiratory disease (1)(2). PRRS causes a significant economic impact, with annual losses of more than 660 million dollars in the United States alone (3). PRRSV, the causative pathogen, , is a member of genus *Arterivirus*, family *Arteriviridae,* order *Nidovirales* (4)(5). The genome is a single-stranded, positive sense RNA and the virion has an envelope. The viral genome is approximately 15 kb, and encodes 7 structural proteins and 14 non-structural proteins (6)(7)(8)(9). Among the structural proteins, ORF5 is attractive in diversity-related studies due to its great variability (10)(11)(12)(13)(14). ORF5 encodes for GP5, an envelope protein containing a major neutralizing epitope (15)(16) and at least two T-cell epitopes (17)(18). GP5 forms a heterodimer with the M protein, which plays a role in viral infectivity and assembly (19). GP5 is of major interest in vaccine studies (20)(21)(22)(23)(24)(25)(26)(27). There are two main genotypes, Genotype I (European type) and Genotype II (North American type) (28). Nucleotide dissimilarity between genotypes of up to 40%, and within the same genotype up to 20% have been recorded (29). This high genetic and antigenic diversity is a major challenge for control of PRRS.

The control of PRRS relies largely on vaccination with modified-live-attenuated vaccines and killed-virus vaccines. Additionally, autogenous vaccines are used (30). Next generation

vaccine strategies to increase both breadth and depth for highly variable virus include consensus sequence vaccines (31)(32)(33), multisubunit vaccines (34)(35)(36)(37), molecular breeding by DNA shuffling (38)(39)(40)(25) and mosaic T-cell epitope vaccines (41)(42)(43)(44)(45)(46). The latter, a mosaic protein, is a recombinant protein derived from a collection of protein sequences of interest. As building blocks for the mosaic protein, each and every peptide (N-mer) can be found naturally within the input proteins. Values of N between 9 and 12 are recommended because that is the size of peptides preferentially recognized by cytotoxic T-cells. A Mosaic Vaccine Tool Suite originally designed to develop HIV mosaic vaccines at Los Alamos National Laboratory (Los Alamos, NM) (41) is available. Studies show that these mosaic T-cell epitope vaccines improved the immune responses to globally circulating strains of HIV-1 by broadening the range of recognized epitopes and increasing responses to high-frequency epitopic variants (44)(45). Considering the variability similarities with HIV, a mosaic T-cell vaccine for PRRSV could represent many divergent strains and induce broad T-cell responses. The T-cell vaccine concept does not exclude the simultaneous use of vaccine components that activate humoral protective immunity. Two mosaic T-cell epitope DNA vaccines were developed based on GP5 sequences of 748 Genotype II strains. Two vaccination/challenge trials in pigs were conducted and the results are discussed.

2. Materials and Methods

2.1. Viruses and Cells

VR2332 was propagated in MARC-145 cells. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U penicillin/mL, and 100 µg streptomycin/mL. The virus was purified over

continuous cesium chloride gradients, quantified using a spectrophotometer NanoDrop 1000 (Thermo Scientific) and stored at -80°C for future use. Purified virus was used as antigen for indirect ELISA and lymphocyte proliferation assays.

2.2. Design and Construction of Mosaic T-cell DNA Vaccines

GP5-Mosaic sequences were designed using the Mosaic T-Cell Vaccine Tool Suite (Los Alamos National Laboratory) (32)(41). Genotype II ORF 5 sequences were obtained from the following sources: 67 sequences from GenBank, 678 sequences, courtesy of Dr. Fred Leung, University of Hong Kong, and 3 full-length genome sequences, courtesy of Dr. Kay Faaberg, USDA ARS. Based upon the coverage assessment tools provided in the Mosaic T Cell Vaccine Tool Suite, two mosaic outputs were selected that maximized the 9-mer epitope coverage. Porcine codon-optimized mosaic sequences with additional 3' polyhistidine tags were synthesized by DNA 2.0 (Menlo Park, CA) and subcloned into the *Xho* and *Eag* sites of plasmid phCMV1 (Genlantis, San Diego, CA), under the control of the CMV promoter (shown in Scheme 2.1). Plasmid DNA was purified using the Plasmid Giga Kit as instructed (Qiagen, Valencia, CA).

2.3. Characterization of GP5-Mosaic Sequences

2.3.1. Matched 9-mer Coverage by Position and Epitope Coverage Assessment

Alignment of 748 GP5 sequences is color coded according to potential 9-mer epitope coverage by the two designed mosaic sequences using the Posicover tool (41)(42). Mean per sequence coverage of 748 PRRSV GP5 sequences by 2 designed mosaic sequences was analyzed using the Epicover tool (41) (42).

2.3.2. Comparison of GP5-Mosaic Sequences with Divergent PRRSV Strains

The homologies between GP5 sequences of VR2332, NADC9, NADC30, SDSU73, MN184(C), Mosaic1 and Mosaic2 were evaluated using BLAST (NCBI, Bethesda, MD).

2.3.3. Western Blot

Expression of protein by the mosaic constructs was verified in *Escherichia coli* BL21 (DE3). Briefly, transformed BL21 cells were cultured in LB broth containing kanamycin. Cell lysates were resuspended in 2x Laemmli sample buffer with 5% β-mercaptoethanol (Sigma, St. Louis, MO), boiled for 5 min and subjected to SDS-PAGE on 10% gels. Proteins were transferred onto PVDF membranes, and blocked for 2 h with 5% (w/v) dry milk in 0.05% (v/v) Tween 20/Tris-buffered saline (T-TBS). The blots were probed with a PRRSV-positive swine serum pool for 2 h at room temperature. After washing, HRP-conjugated anti-pig IgG antibody (Santa Cruz Biotechnology, Dallas, TX) was added and incubated for 2 h, then washed four times (10 min each) with T-TBS. Proteins were demonstrated by chemiluminescence (GE Health, Marlborough, MA).

2.4. Vaccination and Collection of Samples

Three to four week-old, PRRSV-free, porcine circovirus-2-free, cross-bred piglets were utilized in the study. The vaccines were administered by gene gun or electroporation as described in Table 2.2. Briefly, in Trial 1, DNA vaccine coupled to 1 µm gold beads was administered with a Helios Gene Gun (Bio-Rad Hercules, CA) at day 0 and boosted at days 14 and 28. Challenge was given at day 45 and necropsies were performed at day 52. In Trial 2, the animals were

vaccinated by intramuscular injection with the DNA vaccine, followed by electroporation at day 0 and boosted the same way at days 14 and 28. Challenge was given at day 43 and necropsies performed at day 50. In both trials, challenge was both via intranasal and intramuscular routes with 10^6 $TCID_{50}$/animal of VR2332. Blood was collected at days 0, 7, 14, 17, 21, 28, 40, at the challenge day, and 5 and 7 days post-challenge. At the end of the experiment, the pigs were euthanized and the lungs evaluated macroscopically, weighed, and bronchoalveolar lavages (BAL) performed. The vaccination/challenge trial design is shown as Scheme 2.2. Tissue samples were collected from each lung lobe, tracheobronchial lymph nodes (TBLN), spleen, and inguinal lymph nodes (ILN) and then fixed in 10% neutral buffered formalin or kept frozen. Fixed tissues were sectioned, and stained with hematoxylin and eosin for histologic evaluation. Lung lesion scores were determined as previously reported (47). All the animal work was done under a protocol approved by the University of Connecticut Institutional Animal Care and Use Committee.

2.5. Lymphocyte Proliferation Test: MTT Assay

Peripheral blood mononuclear cells (PBMCs) were isolated from blood using Histopaque-1077 (Sigma, St. Louis, MO). PBMCs were suspended to $1x10^7$ /mL in RPMI medium containing 10% FBS, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 200 U/mL penicillin, and 200 µg/mL streptomycin, then seeded in 96-well flat-bottom plates (100 µL/well) in triplicate. PBMCs were stimulated with VR2332 (10 µg/mL) or media alone. Concanavalin A (ConA) (10 µg/mL) was used as a positive control. After incubation for 48 h at 37°C in a 5% CO_2 atmosphere, the proliferation responses were detected by a standard MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) method. Lymphocyte proliferation was

expressed as stimulation index (SI), from the ratio of OD_{570} nm of stimulated compared to that of unstimulated cells.

2.6. Cytokine Recall Immune Response to PRRSV

PBMCs seeded in 24-well flat-bottom plates (400 µL/well) in duplicate were stimulated with 200 $TCID_{50}$ VR2332/well or media alone for 48 h at 37°C in a 5% CO_2 atmosphere. The cells were collected and total RNA was extracted for quantitative real-time PCR analysis.

2.7. Indirect Enzyme-Linked Immunosorbent Assay

For iELISA, purified VR2332 was coated onto 96-well plates at a concentration of 2.5 µg/mL overnight at 4°C, and blocked with 10% dry milk in PBS-T (PBS containing 0.05% Tween-20) for 2 h at room temperature. The plates were washed with PBS-T and sera diluted 40-fold in PBS-T buffer containing 5% dry milk was added. After 60 min at room temperature, the plates were washed five times with PBS-T buffer and incubated with mouse HRP anti-pig IgG, (The Jackson Laboratory, Bar Harbor, ME) for 60 min at room temperature. The plates were washed five times in PBS-T, substrate was added and incubated in the dark at room temperature for 15 min. The reactions were stopped with 1 M HCl. The plates were read at OD_{450} in a microplate reader (Biotek HTK model, Winooski, VT). Archival PRRSV positive and negative sera of pigs were used as controls in each ELISA run.

2.8. Serum Neutralization

Test sera were mixed with equal volumes of DMEM containing 100 $TCID_{50}$ of VR2332 (final serum dilution 1:4). After incubation at 37°C for 1 h, the serum-virus mixtures were added to MARC-145 monolayers in 96-well plates, and incubated at 37°C in a 5% CO_2 atmosphere for 48 h. VR2332 virus plus negative serum and uninfected cells were used as virus and cell controls, respectively. Neutralization was quantified both by a fluorescence intensity reduction (FIR) test and qRT-PCR 48 h after infection. For FIR, cells 48 h after infection were washed three times with PBS and then fixed with 80% cold acetone for 15 min at 4°C. The cells were reacted with a protein-A purified PRRSV-positive swine serum for 1 h. After three washes with PBS, FITC-conjugated rabbit anti-pig IgG (Sigma, St. Louis, MO) was incubated for 1 h. After washing, fluorescence intensity was measured in a microplate reader (Biotek HTK model, Winooski, VT).

2.9. Quantitative Real-time PCR

Total RNA was extracted from 250 µL of serum or supernatants using TRIzol LS Reagent or from tissues using TRIzol Reagent (Invitrogen, Grand Island, NY). RNA was quantified using a spectrophotometer NanoDrop 1000 (Thermo Scientific). cDNA was synthesized using random primers (Invitrogen, Grand Island, NY) in a 20 µL reaction mixture. The reaction was run in a thermocycler (Applied Biosystems GeneAmp PCR System 2400) as follows: 26°C for 10 min, 42°C for 45 min and 75°C for 10 min. SYBR Green real-time PCR was then performed, using the cDNA as a template and 5'-ATG ATG RCC TGG CAT TCT-3'and 5'-ACA CGG TCG CCC TAA TTG- 3' as the forward and reverse primers for ORF7, respectively. The PCR reaction was performed at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 61°C for 1 min using the ABI 7500 detection system. For each assay, a standard

curve was generated using serially diluted RNA which contained 10^2–10^7 copies/μL. In each run, positive and negative reference samples were run along with the test samples. The viral loads were determined by plotting the Ct values against the standard curve. Melting curves were analyzed to verify the specificity of the PCR.

For cytokine evaluation, total RNA was extracted from virus-stimulated or media-treated PBMCs using TRIzol Reagent. cDNA synthesis and real-time PCR followed the same protocol described above. GAPDH (forward primer: 5'-CGT CCC TGA GAC ACG ATG GT- 3'and reverse primer: 5'-CCC GAT GCG GCC AAA T -3') was used as internal control to calculate the changes of interferon-γ (IFN-γ) (forward primer: 5'-TGG TAG CTC TGG GAA ACT GAA TG - 3'and reverse primer: 5'- GGC TTT GCG CTG GAT CTG -3'), or Interleukin-10 (IL-10) (forward primer: 5'- TGA GAA CAG CTG CAT CCA CTT C- 3'and reverse primer: 5'-TCT GGT CCT TCG TTT GAA AGA AA -3') by the delta-delta method (48).

2.10. Lung Lesion Scoring

Lung lesion scoring was done by a board certified veterinary pathologist, blinded to the treatment groups as previously described (47).

2.11. Statistical Analysis

Student t-test or Two-way ANOVA was used to evaluate the differences between the samples within or between groups. The Pearson product-moment correlation coefficient was calculated. The data were analyzed using GraphPad Prism (version 5.0).

3. Results

3.1. Characterization of Mosaic T-cell Epitope Vaccines

3.1.1. GP5 Mosaic Vaccine Increased Epitope Coverage Over VR2332

The amino acid sequences of Mosaic 1 and 2 were 88 % identical; while those of Mosaic 1 and VR2332 GP5 and Mosaic 2 and VR2332 GP5 were 93 % and 90.5 % identical, respectively (Fig. 2.1A). Homologies between GP5-Mosaic sequences with VR2332, NADC9, NADC30, SDSU73 and MN184(C) are shown in Table 2.1. Mosaic 2, has an average amino acid sequence identity of 90.25%, which is better coverage than both Mosaic 1 (average identity of 85.75%) or VR2332 (average identity of 86.25%) towards the other 4 strains examined. Mosaics were optimized for greatest coverage of 9-mer epitope over the peptide set. Two mosaic sequences increased both positional coverage compared to one mosaic sequence, as shown by more yellow-colored squares and fewer black-colored ones (Fig. 2.1B), and a better epitope coverage (66.4% vs 53.4% in exact match, 88.8% vs 82.4% in off-by-1 and 96.5% vs 93.6% in off-by-2) (Fig. 2.1C).

3.1.2. GP5-Mosaic Vaccine Expression Was Confirmed *in vitro*

The expression of GP5-Mosaic vaccines 1 and 2 *in vitro* was confirmed in *Escherichia coli* by western blot (Fig. 2.1D). The western blot showed specific protein bands with approximate molecular weight of 20 kDa consistent with GP5, whereas no bands were detected with negative control serum (data not shown).

3.2. GP5-Mosaic Vaccine Activated Cellular Responses

Mosaic vaccination activated lymphocyte proliferative responses as detected 12 days after the second boost in Trial 1 and 10 days after the second boost in pigs in Trial 2. In Trial 1, significantly higher ($p<0.05$) levels of proliferation were detected in PBMCs from both GP5-VR2332 (GP5-WT), and GP5-Mosaic vaccinated animals, as compared with vector-control animals (Fig. 2.2A). In Trial 2, similar results were observed. Virus stimulation induced significantly higher lymphocyte proliferative responses in GP5-Mosaic-vaccinated animals compared to vector-control and electroporation-control animals (Fig. 2.2B).

IFN-γ and IL-10 relative fold-changes are shown in Fig. 2.2C and 2.2D, respectively. Higher IFN-γ mRNA expression was detected in GP5-Mosaic-vaccinated pigs 5 days after challenge compared to those in electroporation-control ($p<0.05$) or vector-control pigs ($p=0.054$) (Fig. 2.2C). In contrast, significantly lower IL-10 mRNA expression was observed in GP5-Mosaic-vaccinated pigs than in electroporation-control pigs at the same time point ($p<0.05$) (Fig. 2.2D).

3.3. GP5-Mosaic Vaccine Induced Virus-specific Antibodies

GP5-Mosaic vaccines delivered by electroporation in Trial 2, induced significantly higher levels of antibodies, as detected in sera collected at the challenge day and 5 days after challenge, than those of control animals ($p<0.05$) while gene gun in Trial 1 did not (Figs. 2.3A and 2.3B). In Trial 2, sera collected at the same time points from GP5-Mosaic-vaccinated animals neutralized virus (Figs. 2.3C and 2.3D), demonstrating that the neutralizing epitope remained functional. The levels of neutralizing antibodies in sera from GP5-Mosaic-vaccinated pigs were higher ($p<0.01$) compared to those in sera from control animals (Fig. 2.3C). The viral copy numbers were significantly lower ($p<0.05$) when virus was mixed with sera from GP5-Mosaic-

vaccinated animals than with sera from control pigs (Fig. 2.3D). The Pearson product-moment correlation coefficient of these two assays for the full range was 0.69 (Fig. 2.3E).

3.4. Rapid Clearance of Virus in GP5-Mosaic-vaccinated Animals

In Trial 2, viral loads in the GP5-Mosaic-vaccinated group were higher than those in vector-control or electroporation-control groups 5 days after challenge. However, viral loads in all GP5-Mosaic-vaccinated animals were reduced by more than 260-fold two days later. In contrast, the mean viral loads increased 1.2 to 5-fold in vector-control or electroporation-control groups, respectively (Fig. 2.4A). There were no significant differences in viral loads in lungs or TBLNs between groups in Trial 2 (Fig. 2.4B). However, viral loads in ILNs and spleens of GP5-Mosaic-vaccinated animals were lower than those in control animals ($p<0.05$ * and $p<0.01$ **). In Trial 2, viral loads in sera at necropsy were significantly lower ($p< 0.05$) in GP5-Mosaic-vaccinated pigs than those in Trial 1 (Fig. 2.6).

3.5. Lower Lung Lesion Scores Detected in GP5-Mosaic-vaccinated Animals

Lung lesion scores were significantly lower ($p<0.05$) in GP5-Mosaic-vaccinated animals in Trial 2 than those in vector-control animals when evaluated with either 7 or 9 sections of lung (Fig. 2.4C). No differences were observed in Trial 1 (data not shown).

3.6. Higher Average Daily Weight Gain (ADWG) Recorded in GP5-Mosaic-vaccinated Animals

ADWG were 1.44, 0.78 and 0.61 in GP5-Mosaic, vector-control and EP-control groups respectively in Trial 2 as shown in Fig 2.5. ADWG in GP5-Mosaic group was higher (p=0.056) than those in EP-control group.

4. Discussion

The high genetic and antigenic variability of PRRSV prevents current vaccines from conferring broad protection. To address this challenge, several strategies including consensus sequence vaccines, multisubunit vaccines, molecular breeding by DNA shuffling, mosaic T-cell epitope vaccines are being investigated (26)(31)(32)(33)(34)(35) (36)(37)(38)(39)(40)(41)(42)(43)(44)(45)(46). The similarities between HIV and PRRSV in terms of genetic variability made the mosaic T-cell vaccine an attractive and logical alternative to address such diversity for the latter. Mosaic vaccines for HIV-1 have been shown to be capable of broadening epitope recognition and increasing responses to high-frequency epitopic variants (44)(45). Mosaic T-cell vaccines were pursued in the present study for PRRS.

Two PRRSV GP5-Mosaics developed in this study were aligned with divergent strains. GP5-Mosaic 2 showed higher sequence homology with those strains than VR2332 which is the parental strain of a number of commercial MLV vaccines (Table 2.1). When GP5-Mosaic sequences were compared to previously reported GP5 T-cell epitopes ^{117}LAALICFVIRLAKNC and ^{149}KGRLYRWRSPVIVEK in both genotypes [17](18), we found the corresponding T-cell epitopes ^{117}LAALTCFVIRFAKNC and ^{149}KGRLYRWRSPVIIEK in GP5-Mosaic 1 and ^{117}LAALICFVIRLAKNC and ^{149}KGKLYRWRSPVIIEK in GP5-Mosaic 2. In GP5-Mosaic 1, there were T121I and F127L substitutions in the first epitope and I161V in the second epitope. In GP5-Mosaic 2, the first epitope had a perfect match with the reference epitope and there were

K151R and I161V substitutions in the second epitope. The effect of substitutions such as T121I, F127L and K151R is likely minimal since mosaic vaccines were used as a pool. Moreover, I161V substitution may not cause a significant difference because of the highly similar structures between them.

The lymphocyte proliferation, a general assessment tool for cellular responses (22)(24)(49)(50)(24)(51), indicated that GP5-Mosaic vaccines elicited such responses, regardless of the delivery method. Moreover, cytokine profiling, is helpful to evaluate the overall cellular response. In PRRS, prolonged viremia correlates with a weak innate antiviral response (52)(53), high levels of IL-10 and TGFβ (54), low levels of IFN-γ (55) coupled to sub-optimal adaptive immunity (16). A study also showed that the levels of IFN-γ and IL-10 were good markers of polarization of the adaptive response, and the level of IL-10 correlated directly with the level of infected macrophages (56). Therefore, determining IFN-γ and IL-10 levels is useful to evaluate vaccine performance. Finding that IFN-γ mRNA levels of PRRSV stimulated PBMCs were significantly increased on 5 DPC, indicated that the GP5-Mosaic vaccine-induced cellular responses which may be relevant to protection (57)(58)(59). The higher levels of IL-10 mRNA detected in control pigs suggested that inhibition of adaptive responses could be responsible for the slower PRRSV clearance in these animals. The observation that levels of IFN-γ and IL-10 tended to be opposite in animals of the same group agreed with a previous study (60).

Detecting neutralizing antibodies in GP5-Mosaic-vaccinated animals indicated the preservation of a relevant B-cell epitope (15)(16) in a T-cell mosaic vaccine background. This is also reflected, by sequence data, that Mosaic1 has a single mutation (L39F) within the neutralizing epitope while Mosaic 2 has none. The effect of the L39F mutation on Mosaic 1 on

neutralization was not specifically tested as the two mosaics were mixed to vaccinate, and Mosaic 2 may have compensated potential deficits of Mosaic 1.

The rapid virus clearance in sera at necropsy in GP5-Mosaic-vaccinated pigs in Trial 2 but not in Trial 1, together with the fact that further evidence showed that viral copy numbers in serum of GP5-Mosaic vaccinated pigs at 7DPC in Trial 2 were significantly lower than those of GP5-Mosaic vaccinated pigs in Trial 1 (Fig 2.6), suggested that electroporation was more effective than gene gun delivery. Electroporation utilizes a short high-voltage pulse to increase DNA uptake and transfection efficiency (61)(62)(63). In Trial 2, viral loads were lower in ILNs and spleens of GP5-Mosaic-vaccinated animals than those in control animals ($p<0.05$) but not in lungs or TBLNs. There were no significant differences in viral loads in PAMs or BAL fluid among groups. A possible explanation is that there were much higher viral loads in lungs upon challenge (64) and since necropsy was performed 7 days after challenge, there was likely not enough time to allow the host to clear the virus in local tissues (65)(66). It is possible that systemic responses seen in GP5-Mosaic-vaccinated animals cleared circulating virus more effectively and reduced the loads in ILNs and spleen.

The lower lung lesion scores and the higher ADWG in GP5-Mosaic-vaccinated animals in Trial 2 are further evidence that a level of protection was achieved. Testing protection against divergent PRRSV strains will further determine the value of the GP5-Mosaic vaccine to control PRRS.

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References

1. S H Done, D J Paton. Porcine reproductive and respiratory syndrome: clinical disease, pathology and immunosuppression. Vet Rec (1995) 136, 2, 32-35
2. Bøtner, A. Diagnosis of PRRS Veterinary. Microbiology (1997) 55, 1-4, 295-301
3. Holtkamp D.J., Kliebenstein J.B., Neumann E.J., J. J. Zimmerman, H.F. Rotto, T.K. Yoder, C. Wang, P.E. Yeske, C.L. Mowrer and C.A. Haley. Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. J Swine Health Prod. 2013; 21(2):72-84.
4. Karl-Klaus Conzelmann, Nico Visser, Peter Van Woensel, Heinz-Jürgen Thiel. Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group. Virology (1993) 193, 1, 329-339
5. E J Snijder, J.Meulenberg. The molecular biology of arteriviruses. The Journal of General Virology (1998). 79 (Pt 5), 5, 961-79
6. G. Wensvoort, C. Terpstra, J.M.A. Pol, E.A. ter Laak, M. Bloemraad, E.P. de Kluyver, C. Kragten, L. van Buiten, A. den Besten, F. Wagenaar, J.M. Broekhuijsen, P.L.J.M. Moonen, T. Zetstra, E.A de Boer, H.J. Tibben, M.F. de Jong, P. van 't Veld, G.J.R. Greenland, J.A. van Gennep, M.Th. Voets, J.H.M. Verheijden & J. Braamskamp (1991) Mystery swine disease in the Netherlands: The isolation of Lelystad virus, Veterinary Quarterly, 13:3, 121-130
7. James E. Collins, David A. Benfield, William T. Christianson, Louis Harris, Jane C. Hennings, Daniel P. Shaw, Sagar M. Goyal, Sam McCullough, Robert B. Morrison, Han Soo Joo, David Gorcyca, Dan Chladek. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. Journal of Veterinary Diagnostic Investigation (1992). 4, 2, 117-126
8. Dea S, Gagnon CA, Mardassi H, Pirzadeh B, Rogan D. Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. Archives of Virology (2000).145, 4, 659-688
9. J J Meulenberg. PRRSV, the virus. Vet Res (2000). 31, 1, 11-21

10. Mateu E, Martín M, Vidal D. Genetic diversity and phylogenetic analysis of glycoprotein 5 of European-type porcine reproductive and respiratory virus strains in Spain. Journal of General Virology (2003). 84, 3, 529-534
11. Kim HK, Yang JS, Moon HJ, Park SJ, Luo Y, Lee CS, Song DS, Kang BK, Ann SK, Jun CH, Park BK. Genetic analysis of ORF5 of recent Korean porcine reproductive and respiratory syndrome viruses (PRRSVs) in viremic sera collected from MLV-vaccinating or non-vaccinating farms. Journal of Veterinary Science (2009).10, 2, 121-130
12. Shi M, Lam TT, Hon CC, Murtaugh MP, Davies PR, Hui RK, Li J, Wong LT, Yip CW, Jiang JW, Leung FC.. Phylogeny-based evolutionary, demographical, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses. Journal of Virology (2010). 84, 17, 8700-8711
13. Brar MS, Shi M, Ge L, Carman S, Murtaugh MP, Leung FC. Porcine reproductive and respiratory syndrome virus in Ontario, Canada 1999 to 2010: genetic diversity and restriction fragment length polymorphisms. Journal of General Virology (2011). 92, 6, 1391-1397
14. Martín-Valls GE, Kvisgaard LK, Tello M, Darwich L, Cortey M, Burgara-Estrella AJ, Hernández J, Larsen LE, Mateu E. Analysis of ORF5 and full-length genome sequences of porcine reproductive and respiratory syndrome virus isolates of Genotypes 1 and 2 retrieved worldwide provides evidence that recombination is a common phenomenon and may produce mosaic isolates. Journal of Virology (2014). 88, 6, 3170-81
15. Plagemann PG1, Rowland RR, Faaberg KS. The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Archives of Virology (2002). 147, 12, 2327-2347
16. Ostrowski M, Galeota JA, Jar AM, Platt KB, Osorio FA, Lopez OJ. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. Journal of Virology (2002). 76, 9, 4241-4250
17. Vashisht K, Goldberg TL, Husmann RJ, Schnitzlein W, Zuckermann FA. Identification of immunodominant T-cell epitopes present in glycoprotein 5 of the North American Genotype of porcine reproductive and respiratory syndrome virus. Vaccine (2008). 26, 36, 4747-4753

18. Díaz I, Pujols J, Ganges L, Gimeno M, Darwich L, Domingo M, Mateu E. In silico prediction and ex vivo evaluation of potential T-cell epitopes in glycoproteins 4 and 5 and nucleocapsid protein of genotype-I (European) of porcine reproductive and respiratory syndrome virus. Vaccine (2009). 27, 41, 5603-5611
19. Xia P, Dang Z, Qiu H, Fan X, Zhou B, Cui B, Chen P. Function of PRRSV GP5 envelope protein by using pseudotyped virus. Veterinary Microbiology (2009). 138, 3-4, 297-303
20. Xue C, Wang W, Liu Q, Miao Z, Liu K, Shen H, Lv L, Li X, Chen X, Cao Y. Chimeric influenza-virus-like particles containing the porcine reproductive and respiratory syndrome virus GP5 protein and the influenza virus HA and M1 proteins. Archives of Virology (2014).159, 11, 3043-3051
21. Wu F, Peng K, Tian J, Xu X, Zhou E, Chen H. Immune response to Fc tagged GP5 glycoproteins of porcine reproductive and respiratory syndrome virus. Viral Immunology (2014). 27, 7, 343-9
22. Ren JQ, Sun WC, Lu HJ, Wen SB, Jing J, Yan FL, Liu H, Liu CX, Xiao PP, Chen X, Du SW, Du R, Jin NY. Construction and immunogenicity of a DNA vaccine coexpressing GP3 and GP5 of Genotype-I porcine reproductive and respiratory syndrome virus. BMC Veterinary Research (2014). 10, 1, 128
23. Lee JA, Kwon B, Osorio FA, Pattnaik AK, Lee NH, Lee SW, Park SY, Song CS, Choi IS, Lee JB. Protective humoral immune response induced by an inactivated porcine reproductive and respiratory syndrome virus expressing the hypo-glycosylated glycoprotein 5. Vaccine (2014). 32, 29, 3617-22
24. Zhao H, Wang Y, Ma Z, Wang Y, Feng WH. Recombinant Kluyveromyces lactis expressing highly pathogenic porcine reproductive and respiratory syndrome virus GP5 elicits mucosal and cell-mediated immune responses in mice. J Vet Sci. (2014) 15(2): 199–208.
25. Tian D, Ni YY, Zhou L, Opriessnig T, Cao D, Piñeyro P, Yugo DM, Overend C, Cao Q, Lynn Heffron C, Halbur PG, Pearce DS, Calvert JG,Meng XJ. Chimeric porcine reproductive and respiratory syndrome virus containing shuffled multiple envelope genes confers cross-protection in pigs. Virology (2015). 485, 402–413

26. Zhu S, Guo X, Keyes LR, Yang H, Ge X. Recombinant Encephalomyocarditis Viruses Elicit Neutralizing Antibodies against PRRSV and CSFV in Mice. PLoS ONE (2015). 10, 6
27. Xiong D, Song L, Zhai X, Geng S, Pan Z, Jiao X. A porcine reproductive and respiratory syndrome virus (PRRSV) vaccine candidate based on the fusion protein of PRRSV glycoprotein 5 and the Toll-like Receptor-5 agonist Salmonella Typhimurium FljB. BMC Veterinary Research (2015). 11, 121
28. Meng XJ, Paul PS, Halbur PG, Lum MA. Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two Genotypes of PRRSV in the U.S.A. and Europe. Archives of Virology (1995). 140, 4, 745-755
29. Kim WI, Lee DS, Johnson W, Roof M, Cha SH, Yoon KJ. Effect of genotypic and biotypic differences among PRRS viruses on the serologic assessment of pigs for virus infection. Veterinary Microbiology (2007). 123, 1-3, 1-14
30. Kimman TG, Cornelissen LA, Moormann RJ, Rebel JM, Stockhofe-Zurwieden N. Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. Vaccine (2009). 27, 28, 3704-3718
31. Jian Y; Yoon, H; Kumar, S; Ramanathan, M.; Corbitt, N; Kutzler, M; Dai, A; Boyer, J; Weiner, D. Enhanced cellular immune responses elicited by an engineered HIV-1 subtype B consensus-based envelope DNA vaccine. Molecular Therapy (2007) 15, 411–421.
32. Santra S, Korber BT, Muldoon M, Barouch DH, Nabel GJ, Gao F, Hahn BH, Haynes BF, Letvin NL. A centralized gene-based HIV-1 vaccine elicits broad cross-clade cellular immune responses in rhesus monkeys. Proc Natl Acad Sci (2008). 105(30): 10489–10494.
33. Vu HL, Ma F, Laegreid WW, Pattnaik AK, Steffen D, Doster AR, Osorio FA. A Synthetic Porcine Reproductive and Respiratory Syndrome Virus Strain Confers Unprecedented Levels of Heterologous Protection. Journal of Virology (2015). 89, 23, 12070-12083

34. Jiang Y, Xiao S, Fang L, Yu X, Song Y, Niu C, Chen H. DNA vaccines co-expressing GP5 and M proteins of porcine reproductive and respiratory syndrome virus (PRRSV) display enhanced immunogenicity. Vaccine (2006). 24, 15, 2869-2879
35. Jiang Y, Fang L, Xiao S, Zhang H, Pan Y, Luo R, Li B, Chen H. Immunogenicity and protective efficacy of recombinant pseudorabies virus expressing the two major membrane-associated proteins of porcine reproductive and respiratory syndrome virus. Vaccine (2007). 25, 3, 547-560
36. Shen G, Jin N, Ma M, Jin K, Zheng M, Zhuang T, Lu H, Zhu G, Jin H, Jin M, Huo X, Qin X, Yin R, Li C, Li H, Li Y, Han Z, Chen Y, Jin M. Immune responses of pigs inoculated with a recombinant fowlpox virus coexpressing GP5/GP3 of porcine reproductive and respiratory syndrome virus and swine IL-18. Vaccine (2007). 25, 21, 4193-4202
37. Jiang W, Jiang P, Wang X, Li Y, Du Y, Wang X. Enhanced immune responses of mice inoculated recombinant adenoviruses expressing GP5 by fusion with GP3 and/or GP4 of PRRS virus. Virus Research (2008). 136, 1-2, 50-57
38. Zhou L, Ni YY, Piñeyro P, Sanford BJ, Cossaboom CM, Dryman BA, Huang YW, Cao DJ, Meng XJ. DNA shuffling of the GP3 genes of porcine reproductive and respiratory syndrome virus (PRRSV) produces a chimeric virus with an improved cross-neutralizing ability against a heterologous PRRSV strain. Virology (2012). 434, 1, 96-109
39. Zhou L, Ni YY, Piñeyro P, Cossaboom CM, Subramaniam S, Sanford BJ, Dryman BA, Huang YW, Meng XJ. Broadening the heterologous cross-neutralizing antibody inducing ability of porcine reproductive and respiratory syndrome virus by breeding the GP4 or M genes. PLoS ONE (2013). 8, 6
40. Ni YY, Opriessnig T, Zhou L, Cao D, Huang YW, Halbur PG, Meng XJ. Attenuation of porcine reproductive and respiratory syndrome virus by molecular breeding of virus envelope genes from genetically divergent strains. J Virology (2013). 87, 1, 304-313
41. Fischer W, Perkins S, Theiler J, Bhattacharya T, Yusim K, Funkhouser R, Kuiken C, Haynes B, Letvin NL, Walker BD, Hahn BH, Korber BT. Polyvalent vaccines for optimal coverage of potential T-cell epitopes in global HIV-1 variants. Nature Medicine (2007). 13, 1, 100-106

42. Thurmond J, Yoon H, Kuiken C, Yusim K, Perkins S, Theiler J, Bhattacharya T, Korber B, Fischer W. Web-based design and evaluation of T-cell vaccine candidates. Bioinformatics (2008). 24, 14, 1639-1640
43. Kong WP, Wu L, Wallstrom TC, Fischer W, Yang ZY, Ko SY, Letvin NL, Haynes BF, Hahn BH, Korber B, Nabel GJ. Expanded breadth of the T-cell response to mosaic human immunodeficiency virus type 1 envelope DNA vaccination. Journal of Virology (2009). 83, 5, 2201-15
44. Barouch DH, O'Brien KL, Simmons NL, King SL, Abbink P, Maxfield LF, Sun YH, La Porte A, Riggs AM, Lynch DM, Clark SL, Backus K, Perry JR, Seaman MS, Carville A, Mansfield KG, Szinger JJ, Fischer W, Muldoon M, Korber B. Mosaic HIV-1 vaccines expand the breadth and depth of cellular immune responses in rhesus monkeys. Nature Medicine (2010). 16, 3, 319-323
45. Santra S, Liao HX, Zhang R, Muldoon M, Watson S, Fischer W, Theiler J, Szinger J, Balachandran H, Buzby A, Quinn D, Parks RJ, Tsao CY, Carville A,Mansfield KG, Pavlakis GN, Felber BK, Haynes BF, Korber BT, Letvin NL. Mosaic vaccines elicit CD8+ T lymphocyte responses that confer enhanced immune coverage of diverse HIV strains in monkeys. Nature Medicine (2010). 16, 3, 324-328
46. Ndhlovu ZM, Piechocka-Trocha A, Vine S, McMullen A, Koofhethile KC, Goulder PJ, Ndung'u T, Barouch DH, Walker BD. Mosaic HIV-1 Gag antigens can be processed and presented to human HIV-specific CD8+ T cells. Journal of Immunology (2011). 186, 12, 6914-24
47. Halbur PG, Paul PS, Meng XJ, Lum MA, Andrews JJ, Rathje JA. Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. Journal of Veterinary Diagnostic Investigation (1996). 8, 1, 11-20
48. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods (2001) 25, 4, 402-408
49. Du Y, Lu Y, Wang X, Qi J, Liu J, Hu Y, Li F, Wu J, Guo L, Liu J, Tao H, Sun W, Chen L, Cong X, Ren S, Shi J, Li J, Wang J, Huang B, Wan R. Highly efficient expression of interleukin-2 under the control of rabbit beta-globin intron II gene enhances protective

immune responses of porcine reproductive and respiratory syndrome (PRRS) DNA vaccine in pigs. PLoS ONE (2014). 9, 3

50. Tang D, Liu J, Li C, Zhang H, Ma P, Luo X, Zeng Z, Hong N, Liu X, Wang B, Wang F, Gan Z, Hao F. Positive effects of porcine IL-2 and IL-4 on virus-specific immune responses induced by the porcine reproductive and respiratory syndrome virus (PRRSV) ORF5 DNA vaccine in swine. Journal of Veterinary Science (2014). 15, 1, 99-109
51. Pinelli-Saavedra A, Peralta-Quintana JR, Sosa-Castañeda J, Moya-Camarena SY, Burgara-Estrella A, Hernández J. Dietary conjugated linoleic acid and its effect on immune response in pigs infected with the porcine reproductive and respiratory syndrome virus. Research in Veterinary Science (2015). 98, 30-38
52. Patel D, Nan Y, Shen M, Ritthipichai K, Zhu X, Zhang YJ. Porcine reproductive and respiratory syndrome virus inhibits type I interferon signaling by blocking STAT1/STAT2 nuclear translocation. Journal of Virology (2010). 84, 21, 11045-11055
53. Chen Z, Lawson S, Sun Z, Zhou X, Guan X, Christopher-Hennings J, Nelson EA, Fang Y. Identification of two auto-cleavage products of nonstructural protein 1 (nsp1) in porcine reproductive and respiratory syndrome virus infected cells: nsp1 function as interferon antagonist. Virology (2010). 398, 1, 87-97
54. Renukaradhya GJ, Alekseev K, Jung K, Fang Y, Saif LJ. Porcine reproductive and respiratory syndrome virus-induced immunosuppression exacerbates the inflammatory response to porcine respiratory coronavirus in pigs. Viral Immunology (2010). 23, 5, 457-466
55. Meier WA, Galeota J, Osorio FA, Husmann RJ, Schnitzlein WM, Zuckermann FA. Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology (2003). 309, 18-31
56. Go N, Bidot C, Belloc C, Touzeau S. Integrative model of the immune response to a pulmonary macrophage infection: What determines the infection duration? PLoS ONE (2014). 9, 9
57. E. M. Bautista, T.W.Molitor. IFNγ inhibits porcine reproductive and respiratory syndrome virus replication in macrophages. Archives of Virology (1999) 144; 1191-1200
58. Rowland RR, Robinson B, Stefanick J, Kim TS, Guanghua L, Lawson SR, Benfield DA. Inhibition of porcine reproductive and respiratory syndrome virus by interferon-gamma

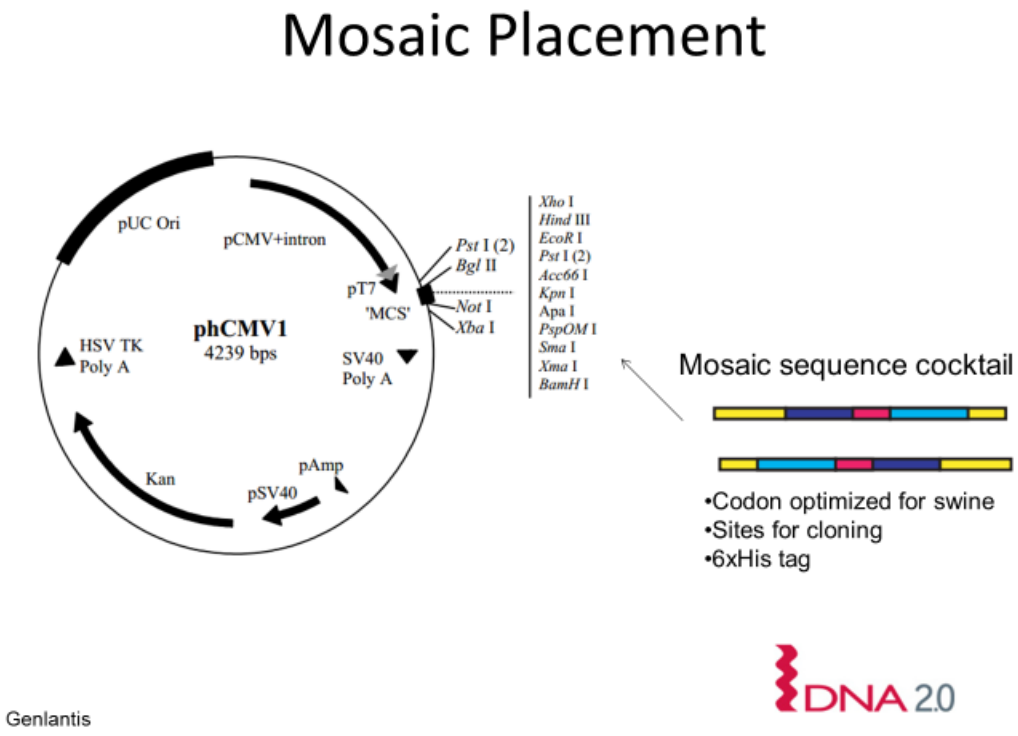
and recovery of virus replication with 2-aminopurine. Archives of Virology (2001). 146, 3, 539-555

59. Morgan SB, Graham SP, Salguero FJ, Sánchez Cordón PJ, Mokhtar H, Rebel JM, Weesendorp E, Bodman-Smith KB, Steinbach F, Frossard JP. Increased pathogenicity of European porcine reproductive and respiratory syndrome virus is associated with enhanced adaptive responses and viral clearance. Veterinary Microbiology (2013). 163, 1, 13-22
60. Díaz I, Darwich L, Pappaterra G, Pujols J, Mateu E. Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs. Virology (2006). 351, 2, 249-259
61. Luxembourg A, Hannaman D, Ellefsen B, Nakamura G, Bernard R. Enhancement of immune responses to an HBV DNA vaccine by electroporation. Vaccine. 2006 24(21):4490-3.
62. Ahlén G, Söderholm J, Tjelle T, Kjeken R, Frelin L, Höglund U, Blomberg P, Fons M, Mathiesen I, Sällberg M. In vivo electroporation enhances the immunogenicity of hepatitis C virus nonstructural 3/4A DNA by increased local DNA uptake, protein expression, inflammation, and infiltration of CD3+ T cells. J Immunol. 2007 179(7):4741-53.
63. Selby M, Goldbeck C, Pertile T, Walsh R, Ulmer J. Enhancement of DNA vaccine potency by electroporation in vivo. Journal of Biotechnology (2000). 83, 1-2, 147-152
64. K. Rossow. Porcine reproductive and respiratory syndrome. Veterinary Pathology (1998). 35, 1, 1-20
65. Rovira A, Balasch M, Segalés J, García L, Plana-Durán J, Rosell C, Ellerbrok H, Mankertz A, Domingo M. Experimental inoculation of conventional pigs with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. Journal of Virology (2002). 76, 7, 3232-9
66. Cho JG, Dee SA, Deen J, Guedes A, Trincado C, Fano E, Jiang Y, Faaberg K, Collins JE, Murtaugh MP, Joo HS. Evaluation of the effects of animal age, concurrent bacterial infection, and pathogenicity of porcine reproductive and respiratory syndrome virus on virus concentration in pigs. Am J Vet Res. (2006). 67, 3, 489-49

Table 2.1 GP5-Mosaic Sequence[a] Homologies to Selected Genotype II PRRSV Strains

	Mosaic 1	Mosaic 2	VR2332	NADC9	NADC30	SDSU73	MN184C
Mosaic 1	100	88	93	88	85	85	85
Mosaic 2	88	100	90.5	90	91	90	90
VR2332	93	90.5	100	89	84	87	85
NADC9	88	90	89	100	85	85	87
NADC30	85	91	84	85	100	86	93
SDSU73	85	90	87	85	86	100	84
MN184C	85	90	85	87	93	84	100

[a] PRRSV GP5 sequences were compared using BLAST (NCBI, Bethesda, MD)



Scheme 2.1. Construct of GP5-Mosaic vaccine. The mosaic sequences were separately inserted into pHCMV1 Title this diagram Mosaic DNA Vaccine Construction (courtesy of Caitlin O'Connell)

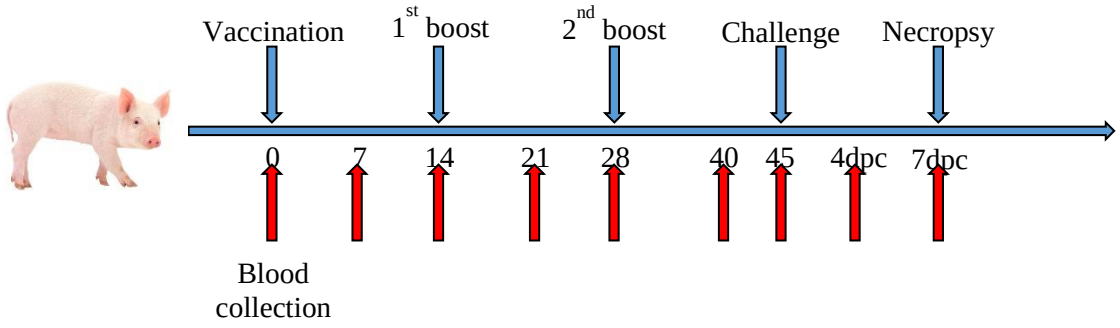
Table 2.2 GP5-Mosaic Vaccine[a] Immunogenicity Testing in Pigs

Gene gun[b] (Trial 1)		Electroporation[c] (Trial 2)	
Experimental Group	Vaccine Dose	Experimental Group	Vaccine Dose
Negative control (n=3)	100 µg empty phCMV1	Negative control (n=3)	Electroporation PBS buffer
GP5 control (n=3)	100 µg VR2332 phCMVORF5	Vector control (n=3)	1 mg empty phCMV1
Mosaic pool (n=4)	100 µg mosaic plasmid cocktail	Mosaic pool (n=4)	1 mg mosaic plasmid cocktail

[a]The GP5-Mosaic DNA vaccines were given as a cocktail

[b]The vaccines were delivered by Helios Gene Gun (Bio-Rad Hercules, CA) in the inner aspect of the thigh at 600 psi

[c] Vaccines were injected in the semimembranous muscle with a 1-1 ½", 21 gauge needles followed by electroporation with 0.5 A pulse width 52 milliseconds with second interval between pulses (3 pulses/pig) using Cellectra Electroporation Unit (Inovio Pharmaceuticals Inc. Blue Bell, PA)



Scheme 2.2. Vaccination/Challenge Design for Trial 1&2. Blue arrows indicate the major operations including vaccination, boost, challenge, and necropsy. Red arrows indicate blood sample collections.

A

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CLUSTAL O(1.2.1) multiple sequence alignment


Mosaic2         MLGKCLTAGCCSQLLFLWCIVPFCFAVLVNASNNSSSHLQLIYNLTICELNGTDWLANKF
VR2332          MLEKCLTAGCCSRLLSLWCIVPFCFAVLANASNDSSSHLQLIYNLTLCELNGTDWLANKF
Mosaic1         MLGRCLTAGCCSRLLSLWCIVPFWFAVLVNANSNSSSHFQLIYNLTLCELNGTDWLADKF
                ** :********:** ******* ****.**..:****:*******:**********:**

Mosaic2         DWAVESFVIFPVLTHIVSYGALTTSHFLDTVGLVTVSTAGYYHGRYVLSSVYAVCALAAL
VR2332          DWAVESFVIFPVLTHIVSYGALTTSHFLDTVALVTVSTAGFVHGRYVLSSIYAVCALAAL
Mosaic1         DWAVETFVIFPVLTHIVSYSALTTSHFLDTVALVTVSTAGFYHGRYVLSSIYAVCALAAL
                *****:*************.***********.********: ********:*********

Mosaic2         ICFVIRLAKNCMSWRYSCTRYTNFLLDTKGKLYRWRSPVIIEKGGKVEVEGHLIDLKRVV
VR2332          TCFVIRFAKNCMSWRYACTRYTNFLLDTKGRLYRWRSPVIIEKRGKVEVEGHLIDLKRVV
Mosaic1         TCFVIRFAKNCMSWRYACTRYTNFLLDTKGRLYRWRSPVIIEKRGKVEVEGHRIDLKRVV
                 *****:*********:*************:*********** ******** *******

Mosaic2         LDGSAATPVTKVSAEQWGRL
VR2332          LDGSVATPITRVSAEQWGRP
Mosaic1         LDGSVATPLTRVSAEQWGRP
                ****.***:*:********
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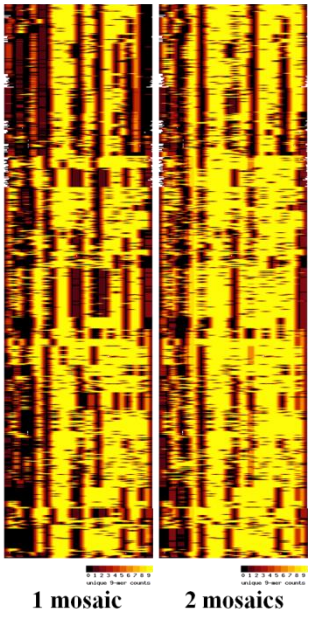
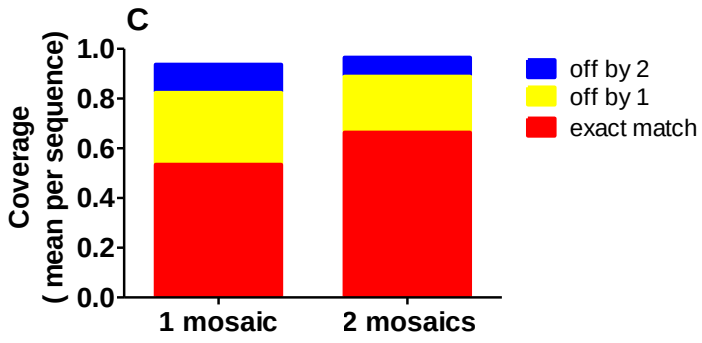
Asterisk (*) indicates a single, fully conserved residue. Colon (:) indicates strongly similar properties. Period (.) indicates weakly similar properties (Gonnet PAM 250 matrix).

B



C



D

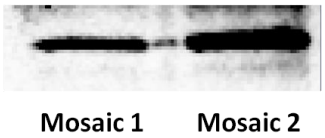


Figure 2.1 Characterization of GP5-Mosaic Sequences. A. GP5-Mosaic sequences aligned with VR2332 GP5 (GP5-WT). Mosaic1: Mosaic2 = 88% identical; Mosaic1: GP5-WT = 93% identical; Mosaic2: GP5-WT = 90.5% identical. **B**. Positional coverage over the 748 Genotype II sequences as obtained by the Mosaic Posicover Tool (Los Alamos National Laboratory). Each colored square represents an amino acid, each row is a sequence, and each column is an

alignment position. Each amino acid is colored according to the set of 9 amino acid strings that contain it: if all 9-mers that overlap with amino acids are perfectly matched in a peptide set, the amino acid is colored yellow; increasingly red values indicate fewer matches, and black indicates no matches. **C.** Comparison of one mosaic sequence and two mosaic sequences in epitope coverage over the 748 Genotype II sequences as obtained by the Mosaic Epicover Tool. Coverage denotes the proportion of 9-mers in each of the 748 PRRSV GP5 sequences that are present in that antigen set, averaged over the 748 sequences. 'Off-by-2' indicates coverage of 9-mers that match at 7 of 9 positions, and 'off-by-1' indicates coverage of 9-mers that match at 8 of 9 positions. **D**. *E. coli* BL21 cells used to test expression of GP5-Mosaic proteins. Lane 1 and 2 represent GP5-Mosaic protein 1 and 2.

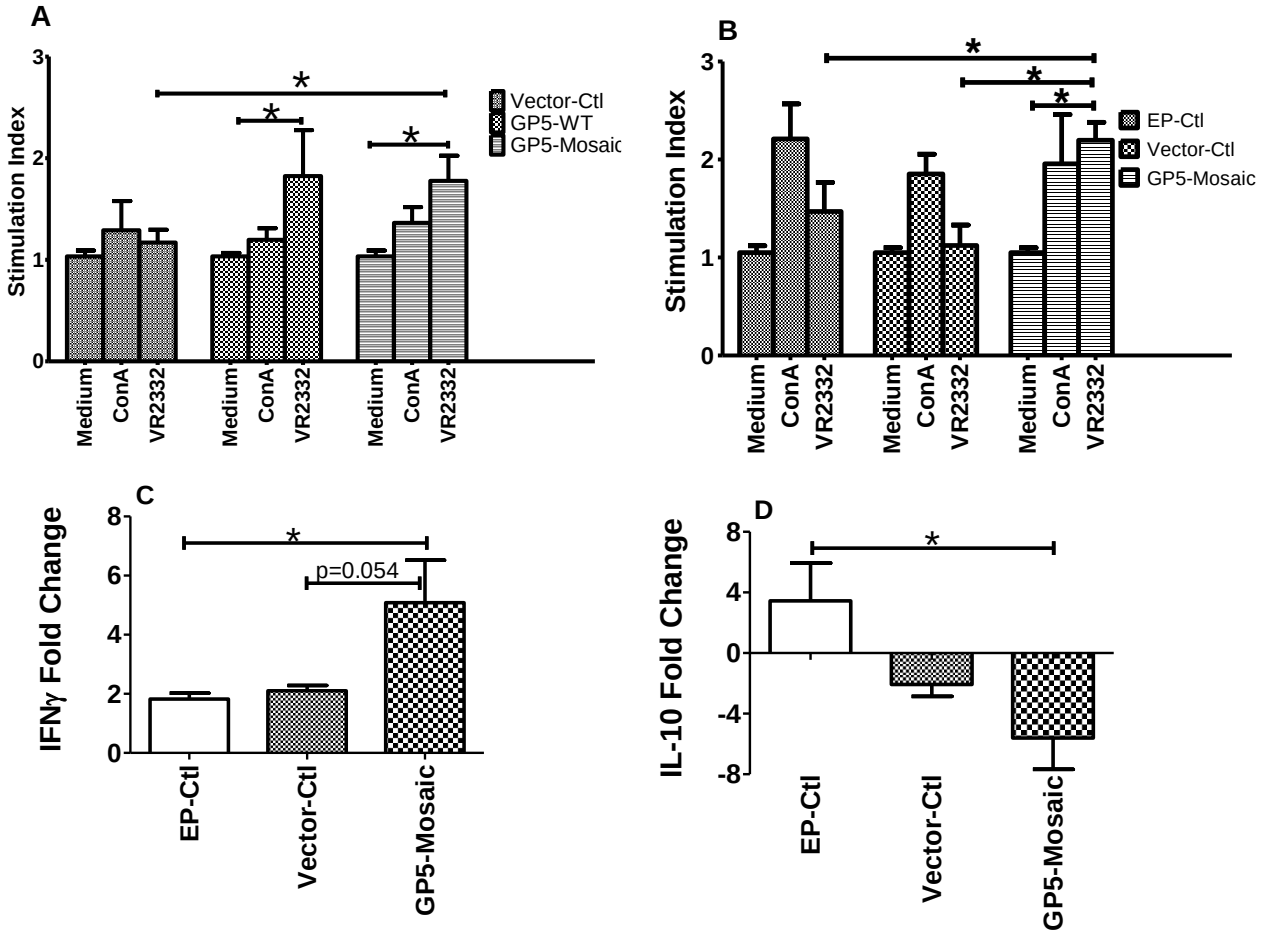


Figure 2.2 Vaccine-Induced Cellular Responses. **A**. Proliferative responses in PBMCs collected at day 38 detected by the MTT method after 48h of antigen stimulation in Trial 1. **B**. Proliferative responses in PBMCs collected at day 40 detected by the MTT method after 48h of antigen stimulation in Trial 2. **C**. IFN-γ mRNA fold changes of PBMCs at 5 days after challenge in Trial 2. **D**. IL-10 mRNA fold changes of PBMCs at 5 days after challenge in Trial 2. Each bar represents the mean value of each group. Variation expressed as standard error of the mean. Three experiments were performed independently. Significant difference is calculated using a repeated measure analysis (p<0.05*). (EP: electroporation)

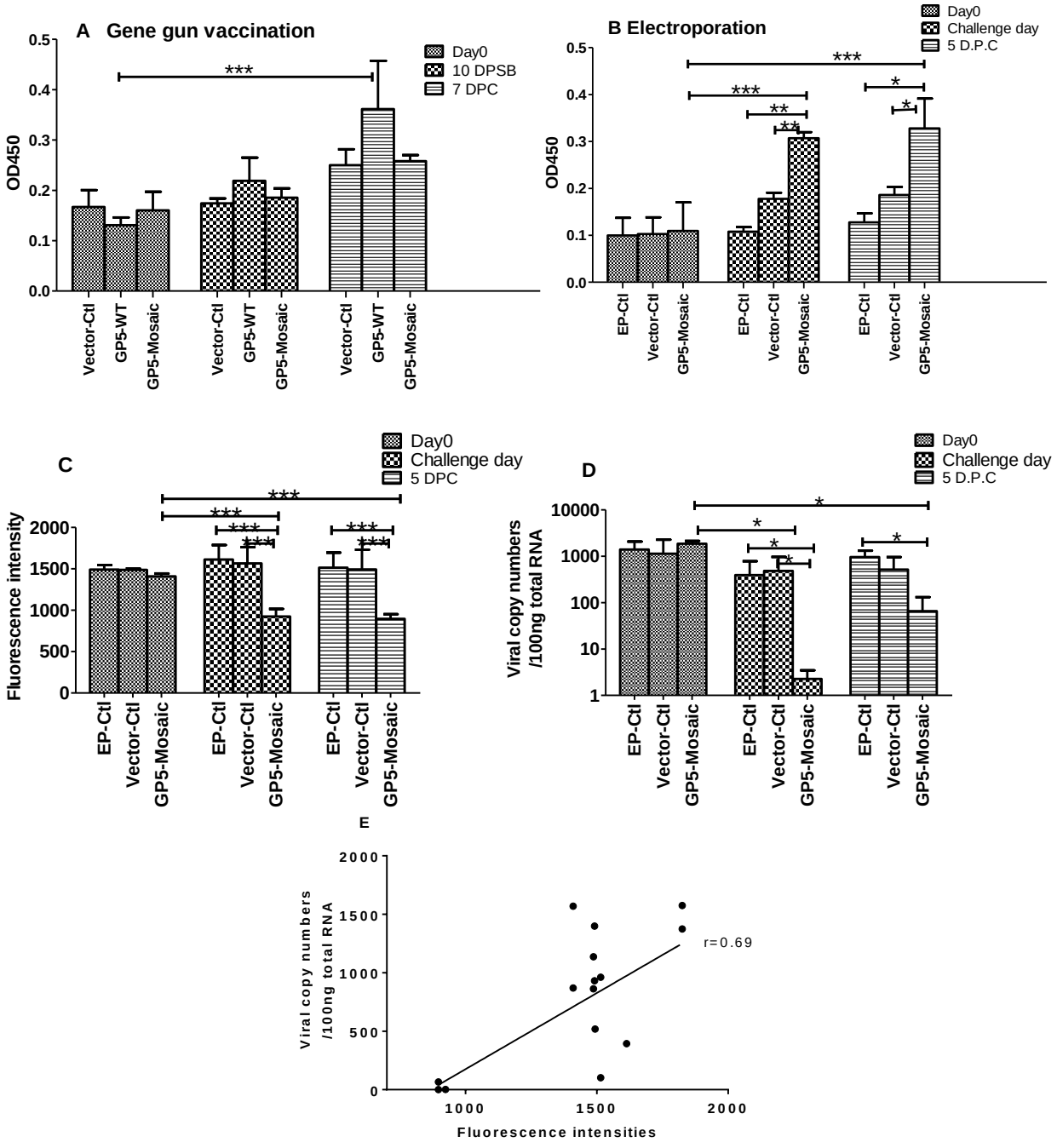


Figure 2.3 Vaccine-Induced Antibody Responses. A. Virus-specific antibodies detected by iELISA in Trial 1**. B.** Virus-specific antibodies detected by iELISA in Trial 2. **C.** Detection of neutralizing antibodies by FIR. **D.** Changes in viral copy numbers induced by serum antibodies detected in infected cell supernatants by qRT-PCR. **E.** Pearson correlation between **C&D**. Each bar represents the mean value of each group. Variation expressed as standard error of the mean. Three experiments were performed independently. Significant differences calculated by a Two-way ANOVA or student t test ($p<0.05$*, $p<0.01$** or $p<0.001$***) (DPC: days post challenge; DPSB: days post second boost; EP: electroporation; FIR: fluorescence intensity reduction).

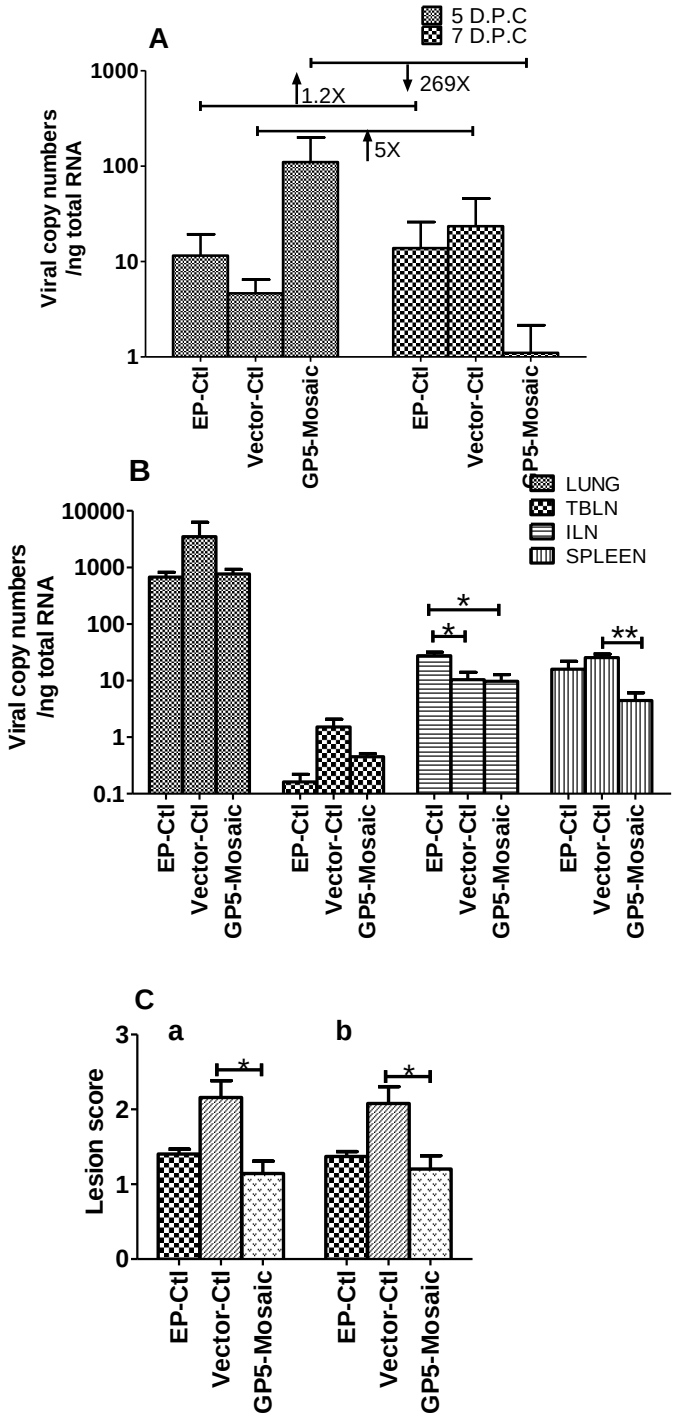


Figure 2.4 Virus Clearance and Lung Lesion Scores in Trial 2. A. Comparison of viral copy numbers in serum between 5 DPC and 7 DPC. **B.** Viral copy numbers in tissues at necropsy (7 DPC). **C**. Lung lesion scores. Scores were significantly lower (p<0.05) in GP5-Mosaic-vaccinated animals than those in vector-control animals. (a: score based on 7 lung sections or b: 9 lung sections;). (DPC: days post challenge; EP: electroporation).

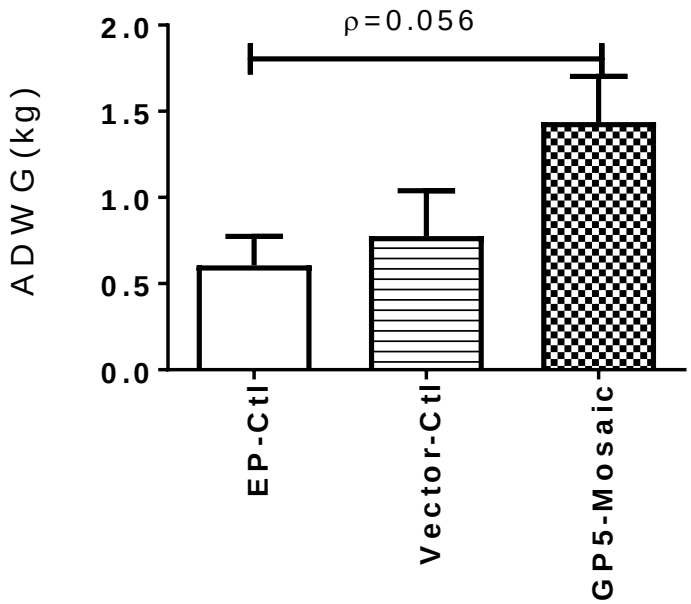


Figure 2.5 Average Daily Weight Gain (ADWG) within 14DPC in Trial 2. Each bar represents the mean value of each group. Variation expressed as standard error of the mean. (DPC: days post challenge; EP: electroporation).

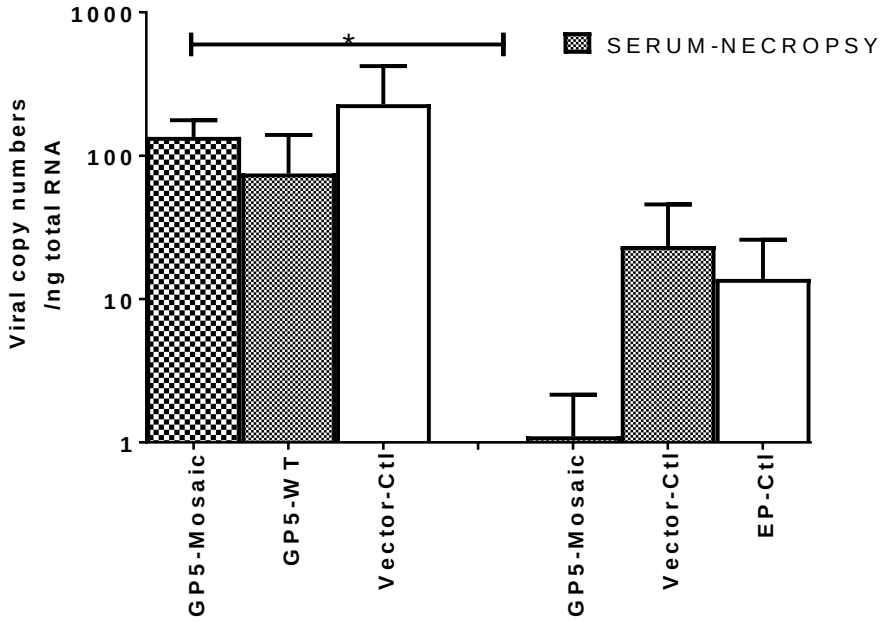


Figure 2.6 Comparison of Virus Clearance in Serum in Trial 1&2. Comparison of viral copy numbers in serum between Trial 1&2 at 7 DPC. Left panel showed viral copy numbers in Trial 1 and right panel showed viral copy numbers in Trial 2. Each bar represents the mean value of each group. Variation expressed as standard error of the mean. Three experiments were performed independently. Significant differences calculated by student t test (p<0.05*) (DPC: days post challenge; EP: electroporation;).

Chapter III GP5-Mosaic T-cell Vaccine Induces Broad Reactivity *ex-vivo* to Heterologous Type II PRRSV Strains and Confers Partial Protection in Pigs

Summary

A previous study showed that GP5-Mosaic T-cell vaccine, formulated in our laboratory as DNA vaccines, was immunogenic and induced partial protection in vaccinated pigs. In the present study, a vaccination/challenge trial was performed in pigs to test further whether the GP5-Mosaic vaccine had the capacity to induce cross-reactive cellular responses in swine. The GP5-Mosaic DNA vaccines complexed to cationic liposomes were administered to experimental pigs by intramuscular injection followed by three boosters at days 14, 28 and 42. Periodic sampling of blood and testing of vaccine-induced responses followed. Significantly higher levels of interferon-γ (IFN-γ) mRNA expression were detected in virus-stimulated peripheral blood mononuclear cells (PBMCs) of GP5-Mosaic-vaccinated pigs as compared to those detected in control pigs vaccinated with either GP5 wild type (GP5-WT) or empty vector at 21, 35 and 48 days after vaccination ($p<0.05$). Moreover, significantly higher levels of IFN-γ mRNA were detected in PBMCs from GP5-Mosaic-vaccinated pigs stimulated by four Genotype II PRRSV strains including VR2332, NADC9, NADC30 and SDSU73 which have at least 10% difference in GP5 amino acid sequence between them, while such responses were recorded only upon VR2332 stimulation in GP5-WT-vaccinated pigs. In addition, the levels of virus-specific antibodies were higher in GP5-Mosaic-vaccinated pigs than in control pigs. These antibodies were neutralizing. The experimental pigs that received the GP5-Mosaic vaccine were partially protected from challenge with VR2332 as indicated by significantly lower viral loads in sera, tissues and lower lung lesion scores ($p<0.05$). The data demonstrates that GP5-Mosaic vaccine

induced cross-reactive cellular responses to diverse strains, higher levels of neutralizing antibodies, and protection in pigs.

Key words: PRRS, PRRSV, mosaic T-cell vaccine, broad immune responses

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is the most important infectious disease affecting swine worldwide. PRRS is characterized by reproductive failure and respiratory disease (1)(2). PRRS causes a major negative economic impact to all major pork producing countries. For instance, the annual losses are more than 660 million dollars in the United States alone (3). The causative pathogen, PRRSV, is a positive-sense, single-stranded RNA virus with an enveloped virion and is classified as a member of the genus *Arterivirus*, family *Arteriviridae*, order *Nidovirales* (4)(5). The viral genome is approximately 15 kb, and encodes 8 structural proteins and 14 non-structural proteins (6)(7)(8)(9). The main feature of PRRSV is its great genetic and antigenic diversity. There are two main genotypes, Genotype I (European type) and Genotype II (North American type) (10), with up to 40% difference documented in the whole genome between them, and up to 20% divergence within the same genotype having been recorded (11).

The control of PRRS relies largely on vaccination with modified-live vaccines and killed-virus vaccines in the field. Additionally, autogenous vaccines are used (12). Unfortunately, vaccines used at present only confer protection against homologous strains and possess weak or no ability to provide cross-protection to heterologous strains which are circulating in the field and constantly mutating. Therefore, this high genetic and antigenic diversity is the major

obstacle for the control of PRRS. To address this diversity challenge, vaccines that can provide cross-protection are in urgent demand. Thus, the development of new vaccines is being intensively studied. There are several different approaches being attempted including consensus sequence vaccines (13)(14)(15), multi-subunit vaccines (16)(17)(18)(19), molecular breeding by DNA shuffling (20)(21)(22)(23) and mosaic T-cell epitope vaccines (24)(25)(26)(27)(28)(29)(30). These approaches are aimed at providing expanded vaccine breadth and depth of antigen for highly variable viruses. In our previous study, we have shown that a GP5-Mosaic vaccine designed using the Mosaic Vaccine Tool Suite originally developed for HIV at Los Alamos National Laboratory (Los Alamos, NM) (24) was immunogenic and conferred partial protection in pigs when delivered as a DNA vaccine (30).

To further characterize the vaccine performance in terms of breadth and depth of responses, we sought to enhance the immune response induced by our vaccine. Recent studies have shown that delivery of DNA vaccines in liposomes resulted in improved DNA uptake efficiency and higher levels of antigen expression in different animal models which range from mouse to non-human primate (31)(32)(33). Therefore, liposomes are a very attractive delivery system to improve our DNA vaccine efficiency. In this study, the GP5-Mosaic vaccine was complexed to cationic liposomes for delivery into pigs. The ability of the vaccine thus formulated to induce broad cross-reactive cellular responses was tested *ex-vivo* using four Genotype II PRRSV strains diverging with one another in amino acid sequence by at least 10%. The results of vaccine-induced responses and protection are discussed.

2. Materials and Methods

2.1. Viruses and Cells

NADC9, NADC30, SDSU73 were kindly provided by Drs. Kay Faaberg and Kelly Lager at USDA ARS. Viruses were propagated in MARC-145 cells. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U penicillin/mL, and 100 µg streptomycin/mL. The virus titers were calculated using the Reed and Muench method (34). The viruses were purified over continuous cesium chloride gradients, quantified using a spectrophotometer NanoDrop 1000 (Thermo Scientific) and stored at -80°C for future use. Purified VR2332 was used as antigen for indirect ELISA. Titrated viruses were used for neutralization assay, recall immune response assay and challenge.

2.2. Protein Sequence Alignment and Phylogenetic Analysis

The protein sequence alignments between GP5 sequences of VR2332, NADC9, NADC30, SDSU73, Mosaic 1 and Mosaic 2 and phylogenetic tree were evaluated using MEGA 7 (Molecular Evolutionary Genetics Analysis) (www.megasoftware.net).

2.3. Preparation of Liposomes and Formulation with Vaccine DNA

Liposomes were prepared by the thin-film hydration method (35). Briefly, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DPPG) and Cholesterol (8:1:1 molar ratio) (Avanti Polar Lipids, Inc. Alabaster, AL) were mixed and dissolved in chloroform (Fisher Scientific Pittsburgh, PA). The lipid/chloroform solution was evaporated using a Buchi Rotavapor followed by being placed under vacuum for 12 hours. Phosphate Buffer (pH 6.5), made isotonic with RNase-free Sucrose (MP Biomedicals), was then added to the dry lipid film. The lipid film was hydrated with the buffer at <10 degrees

Celsius with intermittent sonication (10-20 second durations) until all of the lipid was dissolved. The liposomes were then subjected to two freeze-thaw cycles (i.e. liquid nitrogen for 5 minutes followed by 55°C for 10 minutes). Lastly, the liposomes were extruded using a LIPEX™ extruder (Northern Lipids Inc., Canada) eight times through a 200-nm polycarbonate membrane (Whatman® Nuclepore Track-Etched Membranes) and one time through a stacked 150 nm and 50 nm polycarbonate membranes at 250 psi. Chitosan oligosaccharide solution (50 µM, MW 5k) was slowly added to the liposomal dispersion (250 µM) to form a diluted dispersion of liposomes coated with chitosan. The coated liposomes were then slowly mixed with pDNA (54 µg/mL) to form a liposome-pDNA complex. The complexes were then concentrated using a Vivaflow 50R crossflow filter (Sartorius). The final concentrations were 1 mM total lipid, 100 µM chitosan and 0.54 mg/mL pDNA. The particle size and zeta-potential were measured for each stage of the coating process using a Malvern Zetasizer.

The particle size and zeta-potential of the liposomes and liposome/pDNA complex were conducted using a Malvern Zetasizer Nano ZS90. The liposomal dispersions were diluted approximately 50 times prior to the measurement. All measurements were conducted at 25 °C in triplicate and were reported as mean ± StDev (Z-Ave ± distribution width for particle size) and mean ± StDev (zeta-potential ± mean of zeta-deviation).

2.4. Vaccination and Collection of Samples

Three to four-week-old, PRRSV-free, porcine circovirus-2-free, cross-bred piglets were used in this study. The GP5-Mosaic vaccines were delivered as liposome/pDNA complexes. Briefly, the liposome/DNA-GP5-Mosaic vaccine complexes containing 500 µg of DNA was injected intradermally (0.1 mL) on the back of ear and intramuscularly (0.9 mL) on the neck musculature at day 0 and boosts were given at days 14, 28 and 42. Virus challenge was given at

day 48 and necropsies were performed at day 62. Challenge was both via intranasal and intramuscular routes with a total of 10^6 $TCID_{50}$/pig of VR2332. Blood samples were collected at days 0, 7, 14, 21, 28, 35, 42, the challenge day, and 4, 7, 11 and 14 days post-challenge (shown in Scheme 3.2). At the end of the experiment, the pigs were euthanized and the lungs evaluated macroscopically, weighed, and bronchoalveolar lavages (BAL) performed. Tissue samples were collected from each lung lobe, tracheobronchial lymph nodes (TBLN), spleen, and inguinal lymph nodes (ILN) and then fixed in 10% neutral buffered formalin or kept frozen. Fixed tissues were sectioned, and stained with hematoxylin and eosin for histologic evaluation. Lung lesion scores were evaluated as previously reported (36). All the animal work was done under a protocol approved by the University of Connecticut Institutional Animal Care and Use Committee.

2.5. Recall Cellular Response to PRRSV

PBMCs collected at days 21, 28 and 35 seeded in 24-well flat-bottom plates ($5x10^5$ cells/well) in duplicate were stimulated with 200 $TCID_{50}$ VR2332/well or mock for 48 h at 37°C in a 5% CO_2 atmosphere. The cells were collected and total RNA was extracted for quantitative real-time PCR analysis. The same test was also done with PBMCs collected at days 0 and at challenge day. The cells were stimulated with 200 $TCID_{50}$ of VR2332, NADC9, NADC30 or SDSU73 /well or mock stimulated for 48 h at 37°C in a 5% CO_2 atmosphere.

2.6. Indirect Enzyme-Linked Immunosorbent Assay

ELISA was performed as described previously (30). Briefly, $CsCl_2$ purified VR2332 was coated onto 96-well plates at a concentration of 2.5 µg/mL overnight at 4°C, and the plates were

blocked with 10% dry milk in PBS-T (PBS containing 0.05% Tween-20) for 2 h at room temperature. The plates were washed twice with PBS-T and serum samples diluted 40-fold in PBS-T buffer containing 5% dry milk were added. After 60 min at room temperature, the plates were washed five times with PBS-T buffer and incubated with mouse HRP anti-pig IgG (The Jackson Laboratory, Bar Harbor, ME) for 60 min at room temperature. The plates were washed five times in PBS-T, substrate was added and incubated in the dark at room temperature for 15 min. The reactions were stopped with 1 M HCl solution. The plates were read at OD_{450} in a microplate reader (Biotek HTK model, Winooski, VT). Archival PRRSV positive and negative sera of pigs were used as controls in each run.

2.7. Serum Neutralization

Serum neutralization tests were performed using a previously reported method (30). Briefly, test sera were mixed with equal volumes of DMEM containing 100 $TCID_{50}$ of VR2332 (final serum dilution 1:4). After incubation at 37°C for 1 h, the sera-virus mixtures were added to MARC-145 monolayers in 96-well plates and incubated at 37°C in a 5% CO_2 atmosphere for 48 h. VR2332 virus plus negative serum and uninfected cells were used as virus and cell controls, respectively. Neutralization was quantified both by a fluorescence intensity reduction (FIR) test and qRT-PCR 48 h after infection. For FIR, cells 48 h after infection were washed three times with PBS and then fixed with 80% cold acetone for 15 min at 4°C. The cells were reacted with a protein-A purified PRRSV-positive swine serum for 1 h. After three washes with PBS, FITC-conjugated rabbit anti-pig IgG (Sigma, St. Louis, MO) was incubated for 1 h. After washing, fluorescence intensity was measured in a microplate reader (Biotek HTK model, Winooski, VT).

2.8. Quantitative Real-time PCR

Total RNA was extracted from 250 µL of serum or supernatants using TRIzol LS Reagent or from tissues using TRIzol Reagent (Invitrogen, Grand Island, NY). RNA was quantified using a spectrophotometer NanoDrop 1000 (Thermo Scientific). cDNA was synthesized using random primers (Invitrogen, Grand Island, NY) in a 20 µL reaction mixture. The reaction was run in a thermocycler (Applied Biosystems GeneAmp PCR System 2400) as follows: 26°C for 10 min, 42°C for 45 min and 75°C for 10 min. SYBR Green real-time PCR was then performed, SYBR qPCR Master Mix was purchased from Bimake (Bimake, Houston, TX) and the cDNA used as a template and 5'-ATG ATG RCC TGG CAT TCT- 3'and 5'-ACA CGG TCG CCC TAA TTG- 3' as the forward and reverse primers for ORF7, respectively. The PCR reaction was performed at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 61°C for 1 min using Bio-Rad CFX96 Touch System (Bio-Rad, Hercules, CA). For each assay, a standard curve was generated using serially diluted RNA which contained 10^2–10^7 copies/µL. In each run, positive and negative reference samples were run along with the test samples. The viral loads were determined by plotting the Ct values against the standard curve. Melting curves were analyzed to verify the specificity of the PCR.

To test for cytokine expression, total RNA was extracted from virus-stimulated or mock-treated PBMCs using TRIzol Reagent. cDNA synthesis and real-time PCR followed the same protocol described above. GAPDH (forward primer: 5'-CGT CCC TGA GAC ACG ATG GT-3'and reverse primer: 5'-CCC GAT GCG GCC AAA T -3') was used as internal control to calculate the changes of IFN-γ (forward primer: 5'-TGG TAG CTC TGG GAA ACT GAA TG -3'and reverse primer: 5'- GGC TTT GCG CTG GAT CTG -3') or Interleukin-10 (IL-10)

(forward primer: 5’- TGA GAA CAG CTG CAT CCA CTT C- 3’and reverse primer: 5’-TCT GGT CCT TCG TTT GAA AGA AA -3’) by the delta-delta method (37).

2.9. Lung Lesion Scoring

Lung lesion scoring was done by a board certified veterinary pathologist, blinded to the treatment groups as previously described (36).

2.10. Statistical Analysis

Student t-test or Two-way ANOVA was used to evaluate the differences between the samples within or between groups. The data were analyzed using GraphPad Prism (version 7.0).

3. Results

3.1. Sequence Alignment and Analysis of GP5

The GP5 amino acid (aa) sequences of Mosaic 1, Mosaic 2, NADC9, NADC30 and SDSU73 were of the same size and with no deletions or insertions, when compared to VR2332, the prototype of genotype II PRRSV strains. Sequence alignments showed that aa identities ranged from 84-89% (Scheme 3.1). To further investigate the genetic relationship of these strains, phylogenetic trees were built using neighbor-joining method based on the aa sequences of the two mosaics and four Type II PRRSV strains. The six sequences could be roughly categorized into two subgroups (Fig.3.1) with the two far ends being NADC30 and VR2332. Mosaic 1 and Mosaic 2 were closely related to VR2332 and NADC30, respectively. NADC30 shared high aa identity with Mosaic 2 and VR2332 shared high aa identity with Mosaic 1.

3.2. Size of Liposome/DNA Complexes Optimum for Processing and Activation of Cellular Immunity

Particle sizes were characterized, and the distribution was shown to be very narrow. The size of liposome only was 144.40±1.44 nm, the size of liposome+chitosan was 228.20±4.36 nm, and the size of liposome/pDNA complex was 380.93±6.66 nm (Table 3.1).

3.3. GP5-Mosaic Vaccine Activated Both Humoral and Cellular Responses

A higher IFN-γ mRNA expression was detected in PBMCs from pigs receiving the GP5-Mosaic vaccine collected at days 21, 35 and 48 compared to those detected in vector-control pigs ($p<0.05$). Similar differences were also observed between the pigs receiving GP5-WT vaccine and vector-control pigs. GP5-Mosaic vaccine induced significantly higher levels of antibodies, as detected in sera collected at days 21 ($p<0.05$) and 35($p<0.001$), (but not at day 14), than those of vector-control animals (Figure 3.2C). The positive control, GP5-WT vaccine induced significantly higher levels of antibodies as detected in sera collected at day 35 compared to those of vector-control animals ($p<0.01$). The sera collected at challenge day from GP5-Mosaic-vaccinated animals neutralized virus (Figs. 3.2B), and the levels were significantly higher ($p<0.01$) compared to those in sera from vector-control animals (Fig. 3.2B). As expected, the ability to induce neutralizing antibodies was also detected in GP5-WT-vaccinated animals.

3.4. GP5-Mosaic Vaccine Induced Broad Cellular Responses *ex vivo*

GP5-Mosaic vaccine induced broad recall cellular responses as detected *in vitro* by the significantly higher levels of IFN-γ mRNA relative fold-change detected upon stimulation of PBMCs of GP5-Mosaic-vaccinated pigs with four divergent Genotype II PRRS virus strains (VR2332, NADC9, NADC30 and SDSU73) compared to those in mock controls ($p<0.05$) shown in Fig.3.3. The response was shown as only significantly higher IFN-γ mRNA expression in VR2332-stimulated PBMCs in GP5-WT-vaccinated pigs at the same time points compared to those in mock controls. No such change was detected in those of vector-control pigs.

3.5. Rapid Clearance of Challenge Virus in GP5-Mosaic-vaccinated Pigs

Viral loads of sera in both GP5-WT and GP5-Mosaic-vaccinated pigs were significantly lower than those in vector-control pigs at 10 and 14 DPC ($p<0.05$). The GP5-Mosaic vaccine showed indistinguishable capability of reducing viral loads in serum from the GP5-WT vaccine. Furthermore, viral loads in sera decreased steadily in approximately 4 logs from 4 to 14 DPC in both GP5-WT and GP5-Mosaic-vaccinated pigs while viral loads remained about the same level in the vector-control pigs (Fig.3.4A). Viral loads in tissues including lung, TBLN, spleen and ILN of both GP5-WT and GP5-Mosaic-vaccinated pigs were significantly lower than those in vector-control pigs ($p<0.01$). A significant difference between GP5-Mosaic and GP5-WT group was undetectable (Fig. 3.4B).

3.6. Lower Lung Lesion Scores Detected in GP5-Mosaic-vaccinated Animals

Lung lesion scores were significantly lower in GP5-Mosaic-vaccinated animals than those in vector-control animals when evaluated with either 9 sections of lung (Fig. 3.4C, $p<0.05$). No differences were observed between GP5-WT and GP5-Mosaic-vaccinated groups.

4. Discussion

In order to address the extraordinary diversity of PRRSV, GP5-Mosaic vaccines were developed in our laboratory and their immunogenicity was confirmed (30). The potential of the GP5-Mosaic vaccines to induce broad cellular responses was further investigated in this study. Two GP5-Mosaic sequences and four Genotype II PRRSV strains including VR2332, NADC9, NADC30 and SDSU73 which showed at least 10% difference with one another in GP5 aa sequences were used to generate a phylogenetic tree using the neighbor-joining method to establish relationships. Interestingly, GP5-Mosaic 1 was the closest to VR2332 while GP5-Mosaic 2 was the closest to NADC30 among all the sequences, which indicated that the two GP5-Mosaic sequences showed broad coverage spanning strains belonging to different lineages. This is supported by the fact that VR2332 and NADC30 were used as the two far ends of the phylogenetic tree with only 84% aa identity between them (Fig.3.1). Therefore, altogether we could expect that GP5-Mosaic vaccine would induce broader cellular responses than GP5-WT when PBMCs are stimulated with the strains listed above.

The Mosaic vaccines were delivered using cationic liposome/pDNA complexes. Studies have shown that immunization with small liposomes (~100 nm) tended to induce a Th2 response, whereas large liposomes ($\geq$ 400 nm) tended to induce a Th1 response and higher IFN-γ levels (38). The size of our liposome/pDNA (Liposome/GP5-Mosaic, GP5-WT or vector-control) particles was characterized to be 380.93±6.66 nm (Table.3.1) which are approximately 400 nm and thus could be considered large liposomes. Therefore, the responses induced by the

liposome/pDNA complexes were consistent with Th1 responses with higher levels of IFN-γ, which reportedly play a significant role in PRRS control (39). Higher levels of IFN-γ mRNA expression were recorded at days 21, 35, and 48. Vaccination of pigs with the GP5-Mosaic vaccine also induced humoral responses as shown by a steady increase in antibodies over time, and higher levels of neutralizing antibodies before challenge. Interestingly, GP5-Mosaic vaccine induced even higher levels of antibodies than GP5-WT ($p<0.001$ vs $p<0.05$ at day 35, $p<0.05$ vs no significance at day 21) compared to vector-control pigs. The use of GP5-Mosaic vaccine as a cocktail of Mosaic 1 and Mosaic 2 may explain, in part, the improvement in vaccine coverage and the difference in responses.

Mosaic vaccines for HIV-1 have been shown to broaden epitope recognition and increase responses to high-frequency epitopic variants (27)(28). In the present study, GP5-Mosaic vaccine induced broader cellular responses compared to GP-WT vaccine, as shown by the significantly higher levels of IFN-γ mRNA expression in response to four divergent Genotype II strains tested in recall stimulation of PBMCs. In contrast, PBMCs from GP5-WT-vaccinated pigs only responded to homologous stimulation with VR2332. No recall responses were detected in the vector-control pigs. When comparing the conservation of GP5 T-cell epitopes with two reference ones, we found that GP5-Mosaic 1 had one perfectly matched T-cell epitope with VR2332, while Mosaic 2 had a perfect match with NADC9 T-cell epitope 1, and Mosaic 2 had a perfect match with NADC30 and SDSU73 T-cell epitope 2 (Table 3.2). These findings further supported the fact that GP5-Mosaic vaccine showed a broader T-cell epitope identity with all four Genotype II PRRSV strains than GP5-WT, which is homologous to VR2332. This could explain the broader recall immune responses recorded *ex vivo* with PBMCs from pigs vaccinated with the GP5-Mosaic vaccine. Compared with IFN-γ mRNA expression at challenge day in GP5-Mosaic-

vaccinated pigs, lower levels of IFN-γ mRNA were recorded at 4 DPC. This coincided with higher levels of IL-10 mRNA expression recorded at that time point which may have exerted temporary suppressive effects (data not shown). Nevertheless, the data demonstrates that the GP5-Mosaic vaccine expanded both the depth and breadth of antigen recognition, thus it induced much broader cellular responses than GP5-WT vaccine *ex-vivo*. This data supports the potential of the GP5-Mosaic vaccine in providing cross-protection in pigs. Additionally, investigations to identify new potential T-cell epitope(s) in GP5-Mosaic sequences need to be considered, as data of this study suggested their existence.

The rapid virus clearance upon vaccination with either GP5-Mosaic or GP5-WT supports that complexing pDNA to liposomes was an effective system to deliver DNA vaccines. The lower lung lesion scores in GP5-Mosaic-vaccinated animals is further evidence that a level of protection was achieved. Testing protection against divergent PRRSV strains will be essential and critical to further determine the value of the GP5-Mosaic vaccine to control PRRS.

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References

1. S H Done, D J Paton. Porcine reproductive and respiratory syndrome: clinical disease, pathology and immunosuppression. Vet Rec (1995) 136, 2, 32-35
2. Bøtner, A. Diagnosis of PRRS Veterinary. Microbiology (1997) 55, 1-4, 295-301
3. Holtkamp D.J., Kliebenstein J.B., Neumann E.J., J. J. Zimmerman, H.F. Rotto, T.K. Yoder, C. Wang, P.E. Yeske, C.L. Mowrer and C.A. Haley. Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. J Swine Health Prod. 2013; 21(2):72-84.
4. Karl-Klaus Conzelmann, Nico Visser, Peter Van Woensel, Heinz-Jürgen Thiel. Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group. Virology (1993) 193, 1, 329-339
5. E J Snijder, J.Meulenberg. The molecular biology of arteriviruses. The Journal of General Virology (1998). 79 (Pt 5), 5, 961-79
6. G. Wensvoort C. Terpstra, J.M.A. Pol, E.A. ter Laak, M. Bloemraad, E.P. de Kluyver, C. Kragten, L. van Buiten, A. den Besten, F. Wagenaar, J.M. Broekhuijsen, P.L.J.M. Moonen, T. Zetstra, E.A de Boer, H.J. Tibben, M.F. de Jong, P. van 't Veld, G.J.R. Greenland, J.A. van Gennep, M.Th. Voets, J.H.M. Verheijden & J. Braamskamp (1991) Mystery swine disease in the Netherlands: The isolation of Lelystad virus, Veterinary Quarterly, 13:3, 121-130
7. James E. Collins, David A. Benfield, William T. Christianson, Louis Harris, Jane C. Hennings, Daniel P. Shaw, Sagar M. Goyal, Sam McCullough, Robert B. Morrison, Han Soo Joo, David Gorcyca, Dan Chladek. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. Journal of Veterinary Diagnostic Investigation (1992). 4, 2, 117-126
8. Dea S, Gagnon CA, Mardassi H, Pirzadeh B, Rogan D. Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. Archives of Virology (2000).145, 4, 659-688
9. J J Meulenberg. PRRSV, the virus. Vet Res (2000). 31, 1, 11-21

10. Mateu E, Martín M, Vidal D. Genetic diversity and phylogenetic analysis of glycoprotein 5 of European-type porcine reproductive and respiratory virus strains in Spain. Journal of General Virology (2003). 84, 3, 529-534
11. Kim HK, Yang JS, Moon HJ, Park SJ, Luo Y, Lee CS, Song DS, Kang BK, Ann SK, Jun CH, Park BK. Genetic analysis of ORF5 of recent Korean porcine reproductive and respiratory syndrome viruses (PRRSVs) in viremic sera collected from MLV-vaccinating or non-vaccinating farms. Journal of Veterinary Science (2009).10, 2, 121-130
12. Kimman TG, Cornelissen LA, Moormann RJ, Rebel JM, Stockhofe-Zurwieden N. Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. Vaccine (2009). 27, 28, 3704-3718
13. Jian Y; Yoon, H; Kumar, S; Ramanathan, M.; Corbitt, N; Kutzler, M; Dai, A; Boyer, J; Weiner, D. Enhanced cellular immune responses elicited by an engineered HIV-1 subtype B consensus-based envelope DNA vaccine. Molecular Therapy (2007) 15, 411–421.
14. Santra S, Korber BT, Muldoon M, Barouch DH, Nabel GJ, Gao F, Hahn BH, Haynes BF, Letvin NL. A centralized gene-based HIV-1 vaccine elicits broad cross-clade cellular immune responses in rhesus monkeys. Proc Natl Acad Sci (2008). 105(30): 10489–10494.
15. Vu HL, Ma F, Laegreid WW, Pattnaik AK, Steffen D, Doster AR, Osorio FA. A Synthetic Porcine Reproductive and Respiratory Syndrome Virus Strain Confers Unprecedented Levels of Heterologous Protection. Journal of Virology (2015). 89, 23, 12070-12083
16. Jiang Y, Xiao S, Fang L, Yu X, Song Y, Niu C, Chen H. DNA vaccines co-expressing GP5 and M proteins of porcine reproductive and respiratory syndrome virus (PRRSV) display enhanced immunogenicity. Vaccine (2006). 24, 15, 2869-2879
17. Jiang Y, Fang L, Xiao S, Zhang H, Pan Y, Luo R, Li B, Chen H. Immunogenicity and protective efficacy of recombinant pseudorabies virus expressing the two major membrane-associated proteins of porcine reproductive and respiratory syndrome virus. Vaccine (2007). 25, 3, 547-560
18. Shen G, Jin N, Ma M, Jin K, Zheng M, Zhuang T, Lu H, Zhu G, Jin H, Jin M, Huo X, Qin X, Yin R, Li C, Li H, Li Y, Han Z, Chen Y, Jin M. Immune responses of pigs

inoculated with a recombinant fowlpox virus coexpressing GP5/GP3 of porcine reproductive and respiratory syndrome virus and swine IL-18. Vaccine (2007). 25, 21, 4193-4202

19. Jiang W, Jiang P, Wang X, Li Y, Du Y, Wang X. Enhanced immune responses of mice inoculated recombinant adenoviruses expressing GP5 by fusion with GP3 and/or GP4 of PRRS virus. Virus Research (2008). 136, 1-2, 50-57
20. Zhou L, Ni YY, Piñeyro P, Sanford BJ, Cossaboom CM, Dryman BA, Huang YW, Cao DJ, Meng XJ. DNA shuffling of the GP3 genes of porcine reproductive and respiratory syndrome virus (PRRSV) produces a chimeric virus with an improved cross-neutralizing ability against a heterologous PRRSV strain. Virology (2012). 434, 1, 96-109
21. Zhou L, Ni YY, Piñeyro P, Cossaboom CM, Subramaniam S, Sanford BJ, Dryman BA, Huang YW, Meng XJ. Broadening the heterologous cross-neutralizing antibody inducing ability of porcine reproductive and respiratory syndrome virus by breeding the GP4 or M genes. PLoS ONE (2013). 8, 6
22. Ni YY, Opriessnig T, Zhou L, Cao D, Huang YW, Halbur PG, Meng XJ. Attenuation of porcine reproductive and respiratory syndrome virus by molecular breeding of virus envelope genes from genetically divergent strains. J Virology (2013). 87, 1, 304-313
23. Tian D, Ni YY, Zhou L, Opriessnig T, Cao D, Piñeyro P, Yugo DM, Overend C, Cao Q, Lynn Heffron C, Halbur PG, Pearce DS, Calvert JG, Meng XJ.
Chimeric porcine reproductive and respiratory syndrome virus containing shuffled multiple envelope genes confers cross-protection in pigs. Virology. 2015 Nov; 485:402-13.
24. Fischer W, Perkins S, Theiler J, Bhattacharya T, Yusim K, Funkhouser R, Kuiken C, Haynes B, Letvin NL, Walker BD, Hahn BH, Korber BT. Polyvalent vaccines for optimal coverage of potential T-cell epitopes in global HIV-1 variants. Nature Medicine (2007). 13, 1, 100-106
25. Thurmond J, Yoon H, Kuiken C, Yusim K, Perkins S, Theiler J, Bhattacharya T, Korber B, Fischer W. Web-based design and evaluation of T-cell vaccine candidates. Bioinformatics (2008). 24, 14, 1639-1640
26. Kong WP, Wu L, Wallstrom TC, Fischer W, Yang ZY, Ko SY, Letvin NL, Haynes BF, Hahn BH, Korber B, Nabel GJ. Expanded breadth of the T-cell response to mosaic

human immunodeficiency virus type 1 envelope DNA vaccination. Journal of Virology (2009). 83, 5, 2201-15

27. Barouch DH, O'Brien KL, Simmons NL, King SL, Abbink P, Maxfield LF, Sun YH, La Porte A, Riggs AM, Lynch DM, Clark SL, Backus K, Perry JR, Seaman MS, Carville A, Mansfield KG, Szinger JJ, Fischer W, Muldoon M, Korber B. Mosaic HIV-1 vaccines expand the breadth and depth of cellular immune responses in rhesus monkeys. Nature Medicine (2010). 16, 3, 319-323
28. Santra S, Liao HX, Zhang R, Muldoon M, Watson S, Fischer W, Theiler J, Szinger J, Balachandran H, Buzby A, Quinn D, Parks RJ, Tsao CY, Carville A,Mansfield KG, Pavlakis GN, Felber BK, Haynes BF, Korber BT, Letvin NL. Mosaic vaccines elicit CD8+ T lymphocyte responses that confer enhanced immune coverage of diverse HIV strains in monkeys. Nature Medicine (2010). 16, 3, 324-328
29. Ndhlovu ZM, Piechocka-Trocha A, Vine S, McMullen A, Koofhethile KC, Goulder PJ, Ndung'u T, Barouch DH, Walker BD.. Mosaic HIV-1 Gag antigens can be processed and presented to human HIV-specific CD8+ T cells. Journal of Immunology (2011). 186, 12, 6914-24
30. J. Cui, C.M. O'Connell, J.D. Smith, J. Smyth, P. H. Verardi, Y. Pan and A. E. Garmendia. "A GP5 Mosaic T-cell Vaccine for Porcine Reproductive and Respiratory Syndrome Virus Is Immunogenic and Confers Partial Protection to Pigs" Vaccine Reports 2016, 6: 77-85
31. Bu J, Song Y, Rompato G, Burgess DJ, Garmendia AE. Co-delivery of IL-2 or liposomes augment the responses of mice to a DNA vaccine for pseudorabies virus IE180. Comparative Immunology, Microbiology and Infectious Diseases. 26: 175-87.
32. Kita Y, Tanaka T, Yoshida S, Ohara N, Kaneda Y, Kuwayama S, Muraki Y, Kanamaru N, Hashimoto S, Takai H, Okada C, Fukunaga Y, Sakaguchi Y, Furukawa I, Yamada K, Inoue Y, Takemoto Y, Naito M, Yamada T, Matsumoto M, McMurray DN, Cruz EC, Tan EV, Abalos RM, Burgos JA, Gelber R, Skeiky Y, Reed S, Sakatani M, Okada M. Novel recombinant BCG and DNA-vaccination against tuberculosis in a cynomolgus monkey model Vaccine. 2005 Mar 18;23(17-18):2132-5.
33. Okada M, Kita Y, Nakajima T, Kanamaru N, Hashimoto S, Nagasawa T, Kaneda Y, Yoshida S, Nishida Y, Fukamizu R, Tsunai Y, Inoue R, Nakatani H, Namie Y, Yamada J,

Takao K, Asai R, Asaki R, Matsumoto M, McMurray DN, Dela Cruz EC, Tan EV, Abalos RM, Burgos JA, Gelber R, Sakatani M. Evaluation of a novel vaccine (HVJ-liposome/HSP65 DNA+IL-12 DNA) against tuberculosis using the cynomolgus monkey model of TB Vaccine. 2007 Apr 20;25(16):2990-3. Epub 2007 Jan 22.

34. Reed, L.J.; Muench, H. (1938). "A simple method of estimating fifty percent endpoints". The American Journal of Hygiene. 27: 493–497.
35. Varona S, Martín A, Cocero M. Liposomal Incorporation of Lavandin Essential Oil by a Thin-Film Hydration Method and by Particles from Gas-Saturated Solutions. Industrial & Engineering Chemistry Research vol. 50 issue 4 (2011) pp: 2088-2097
36. Halbur PG, Paul PS, Meng XJ, Lum MA, Andrews JJ, Rathje JA. Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. Journal of Veterinary Diagnostic Investigation (1996). 8, 1, 11-20
37. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods (2001) 25, 4, 402-408
38. Ali Badiee, Ali Khamesipour, Afshin Samiei, Dina Soroush, Vahid Heravi Shargh, Masoumeh Tavassoti Kheiri, Farzaneh Barkhordari, W Robert Mc Master, Fereidoun Mahboudi, Mahmoud R Jaafari. The role of liposome size on the type of immune response induced in BALB/c mice against leishmaniasis: rgp63 as a model antigen. Experimental Parasitology 2012, 132 (4): 403-9
39. Díaz I, Pujols J, Ganges L, Gimeno M, Darwich L, Domingo M, Mateu E. In silico prediction and ex vivo evaluation of potential T-cell epitopes in glycoproteins 4 and 5 and nucleocapsid protein of genotype-I (European) of porcine reproductive and respiratory syndrome virus. Vaccine (2009). 27, 41, 5603-5611
40. Vashisht K, Goldberg TL, Husmann RJ, Schnitzlein W, Zuckermann FA. Identification of immunodominant T-cell epitopes present in glycoprotein 5 of the North American Genotype of porcine reproductive and respiratory syndrome virus. Vaccine (2008). 26, 36, 4747-4753

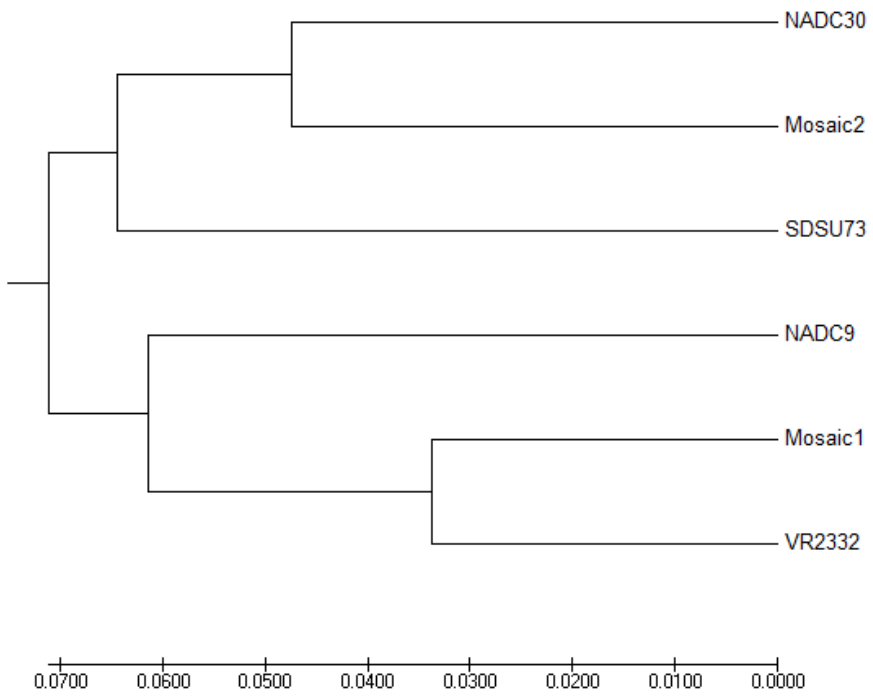
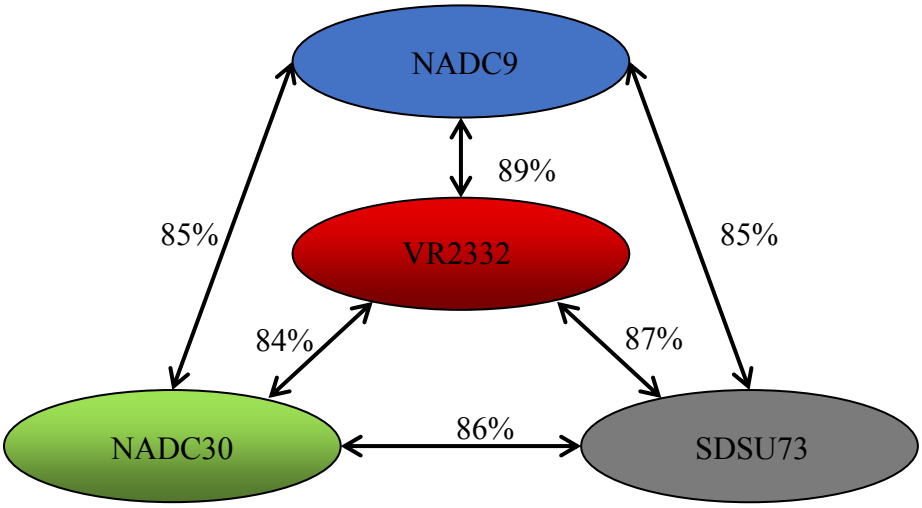


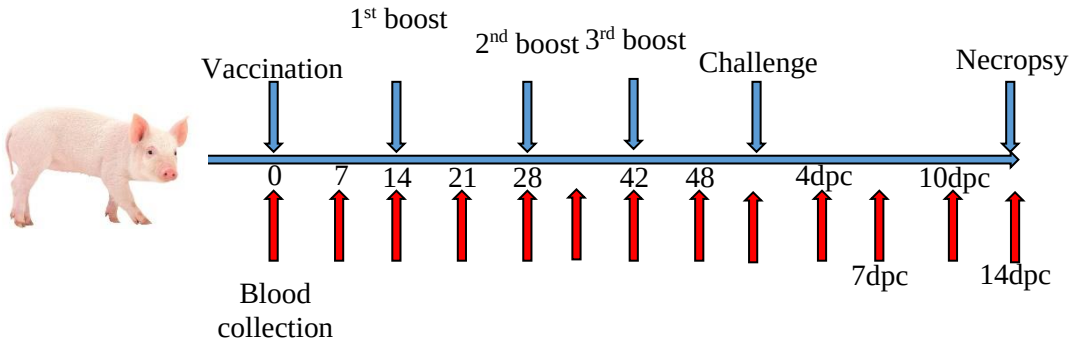
Figure 3.1. Phylogenetic Analysis Based on Amino Acid Sequences of GP5 of Two Mosaic Sequences and 4 Genotype II PRRSV Strains. The analysis was done using neighbor-joining method of MEGA7.0.



Scheme 3.1. Selection of Diverse PRRSV Strains. Percentage showing GP5 amino acid sequences identities between selected strains. Sequences were aligned using BLAST.

Table 3.1. Particle Size Results

	Size (d.nm)	StDev	PDI	StDev	Zeta Potential (mV)	StDev
Liposomes Only	144.40	1.44	0.15	0.02	-44.53	19.00
Liposomes+ Chitosan	228.20	4.36	0.10	0.03	16.67	4.69
Liposomes + Chitosan + pDNA	380.93	6.66	0.16	0.01	11.80	3.66



Scheme 3.2. Vaccination/Challenge Design. Blue arrows indicate the major operations including vaccination, boost, challenge and necropsy. Red arrows indicate blood sample collections.

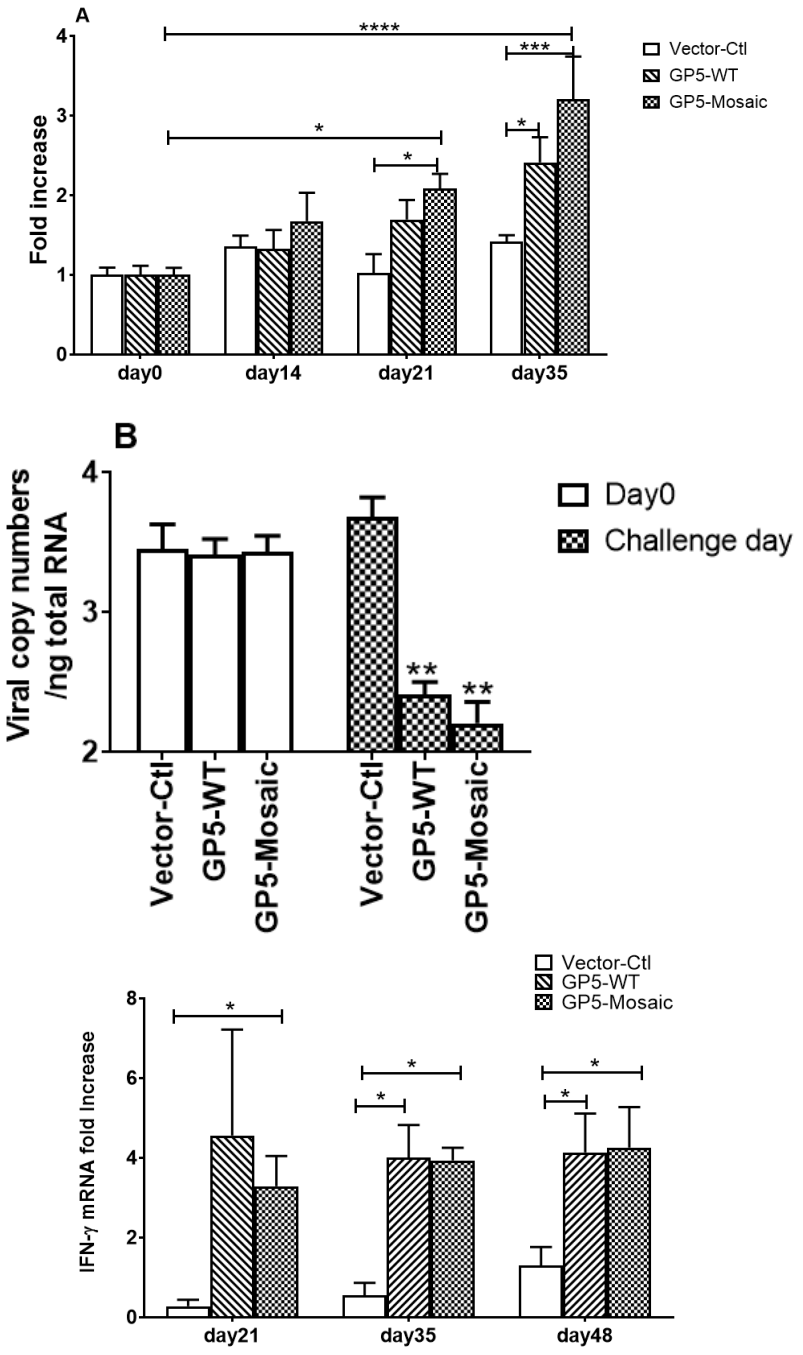


Figure 3.2. Vaccine-induced Humoral and Cellular Responses. A. Virus-specific antibodies detected by iELISA over time**. B.** Changes in viral copy numbers induced by serum antibodies detected in infected cell supernatants by qRT-PCR. **C**. IFN-γ mRNA fold changes of PBMCs at days 21, 35 and 48 post immunization. Each bar represents the mean value of each group. Variation expressed as standard error of the mean. Three experiments were performed independently. Significant differences calculated by a Two-way ANOVA or student t test (p<0.05*, p<0.01**, p<0.001*** or p<0.0001****).

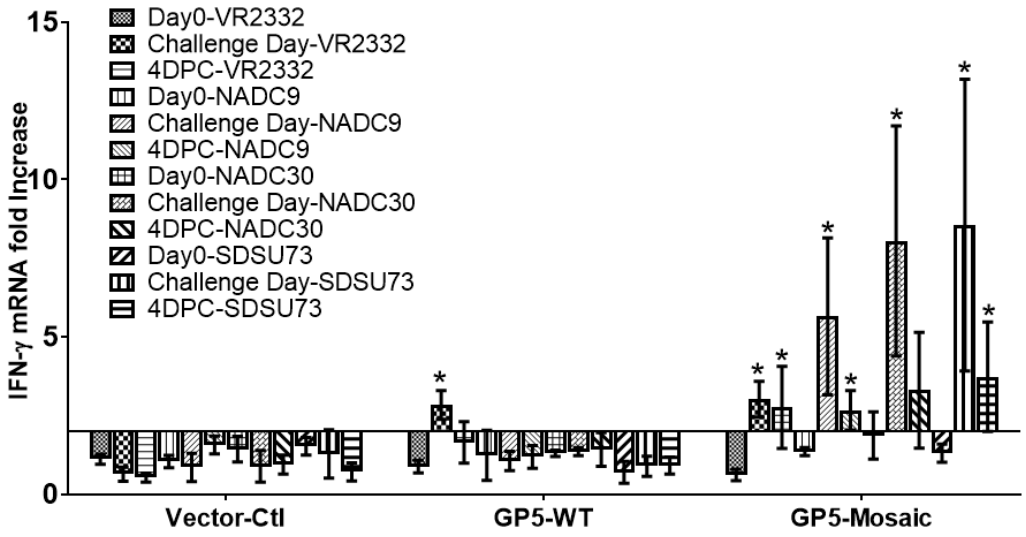


Figure 3.3. GP5-Mosaic Vaccine Induced a Broad Spectrum of Recall Cellular Responses ex vivo. IFN-γ mRNA fold changes of PBMCs at days 0, challenge and 4DPC in responding to VR2332, NADC9, NADC30 and SDSU73. Fold increase less than 2 was not considered as real change. Each bar represents the mean value of each group. Variation expressed as standard error of the mean. Three experiments were performed independently. Significant differences calculated by a student t test (p<0.05*).

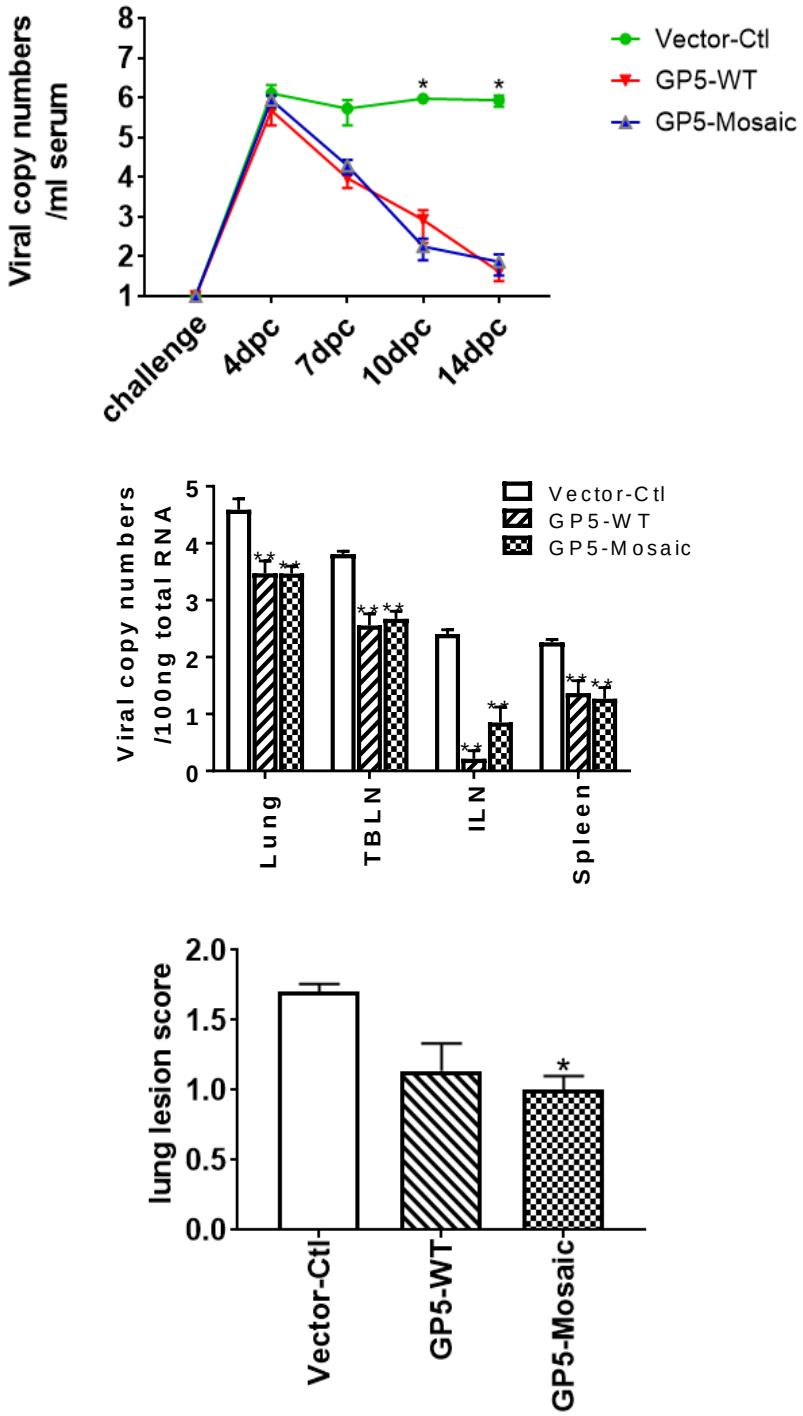


Figure 3.4. Virus Clearance and Lung Lesion Scores. A. Comparison of viral copy numbers in serum from 0 DPC to 14 DPC. **B.** Viral copy numbers in tissues at necropsy (14 DPC). **C**. Lung lesion scores. Scores were significantly lower (p<0.05) in GP5-Mosaic-vaccinated animals than those in vector-control animals. (DPC: days post challenge).

Table 3.2. Conservation of PRRSV GP5 B-cell/T-cell epitopes

GP5/PRRSV	B-cell epitope	T-cell epitope 1	T-cell epitope 2
reference	$_{37}$SHLQLIY	$_{117}$LAALICFVIRLAKNC	$_{149}$KGRLYRWRSPVIVEK
Mosaic 1	$_{37}$SHFQLIY	$_{117}$LAALTCFVIRFAKNC	$_{149}$KGRLYRWRSPVIIEK
Mosaic 2	$_{37}$SHLQLIY	$_{117}$LAALICFVIRLAKNC	$_{149}$KGKLYRWRSPVIIEK
VR2332	$_{37}$SHLQLIY	$_{117}$LAALTCFVIRFAKNC	$_{149}$KGRLYRWRSPVIIEK
NADC9	$_{37}$SNLQLIY	$_{117}$LAALICFVIRLAKNC	$_{149}$KGRLYRWRSPVIVEK
NADC30	$_{37}$SHLQLIY	$_{117}$LAALICFAIRLAKNC	$_{149}$KGKLYRWRSPVIIEK
SDSU73	$_{37}$SHFQSIY	$_{117}$LAALICFIIRLAKNC	$_{149}$KGKLYRWRSPVIIEK
Consensus	* _ _ * _* *	* * * *_ **_** _ _* * *	* * _ * * * * * * * ***_* *

Reference epitopes cited from studies (66)(65)

*means perfect match, _means substitution

Chapter IV Priming with DNA and Boosting with Vaccinia Virus (VACV) Expressing GP5-Mosaic Vaccine Confers Cross-Protection Against Heterologous PRRSV Strains in Pigs

Summary

A GP5-Mosaic T-cell vaccine, designed and developed in our laboratory as a DNA vaccine and recently tested, induced cross-reactivity *ex-vivo* in recall-stimulated peripheral blood mononuclear cells (PBMCs) and conferred partial protection in vaccinated pigs. The present study follows up on these results, where priming with an rDNA GP5-Mosaic and boosting with a recombinant Tet-inducible Vaccinia virus GP5-Mosaic (rVACV-GP5-Mosaic) followed by heterologous virus challenge was performed in pigs to test further whether the GP5-Mosaic vaccine cross-protected pigs. The rDNA GP5-Mosaic vaccines were administered by intramuscular (IM) injection along with Quil-A® as an adjuvant. The pigs received a boost of rVACV-GP5-Mosaic by IM injection three weeks later. Blood was collected periodically and tested for vaccine-induced responses. In samples collected at challenge day, the expression of interferon-γ (IFN-γ) mRNA in VR2332 or MN184C stimulated PBMNC of GP5-Mosaic-vaccinated pigs was significantly higher as compared to those of VR2332 GP5 (GP5-WT) or empty vector-vaccinated control pigs. In GP5-WT-vaccinated pigs, such responses were recorded only upon VR2332 stimulation ($p<0.05$). In addition, the levels of virus-specific antibodies were higher in GP5-Mosaic-vaccinated pigs than those of control pigs. Moreover, these antibodies were neutralizing. The GP5-Mosaic-vaccinated pigs were cross-protected from heterologous strains as demonstrated by significantly lower viral loads in sera, tissues, alveolar macrophages (PAMs), and bronchoalveolar lavage (BAL) fluids and lower lung lesion scores

(p<0.05) upon challenge with either MN184C or VR2332. Under the same criteria, the pigs receiving GP5-WT vaccination showed protection only against challenge with VR2332 when compared to the vector-control group. This data demonstrates that the GP5-Mosaic vaccine induced both humoral and cellular responses and conferred cross-protection against heterologous strains in pigs.

Key words: PRRS, PRRSV, mosaic T-cell vaccine, VACV, cross protection, heterologous challenge

1. Introduction

Porcine reproductive and respiratory syndrome viruses (PRRSV) rapidly spread across the world in all major pork-producing countries, causing significant economic losses to swine industries. For instance, in the United States alone, PRRS, costs more than $660 million to pork producers annually (1). PRRS is characterized clinically by reproductive failure in pregnant sows and respiratory diseases in pigs at all ages, especially the young. PRRSV is a positive-sense, single-stranded RNA virus with an enveloped virion that belongs to the family of *Arteriviridae* of the order *Nidovirales* (2)(3)(4). The viral genome is approximately 15 kb in length and encodes at least 22 different viral proteins including 14 non-structural and 8 structural proteins (5). Some viral proteins have been shown to induce humoral and/or cell-mediated immune responses in infected pigs, but none of those have been shown to provide complete immune protection (6)(7)(8)(9). Vaccines against PRRS including modified live virus (MLV) vaccines and killed virus (KV) vaccines have been licensed for more than two decades. Although the efficacy of MLV vaccines is generally better to that of KV vaccines (10)(11)(12)(13), it is very

unwise to ignore the fact that the levels of protection against heterologous PRRSV strains are highly variable and generally considered sub-optimal (10)(14)(15)(16)(17)(18). Additionally of great concern is the potential reversion of MLVs to virulence (19)(20)(21).

The extremely high genetic variation of PRRSV genome is the major challenge to the development of PRRS vaccines that confer adequate cross-protection. PRRSV is classified into 2 major genotypes, Genotype I (EU) and Genotype II (NA), which show up to 40% dissimilarity on sequence identity (22)(23). Moreover, Genotype I and Genotype II can be further sub-divided into 4 subgroups and 9 different lineages, respectively, based on phylogenetic analysis of the ORF 5 (24). The co-existence of multiple variants within one farm, among animals, or even within individuals possibly indicates a level of quasispecies variation of PRRSV (25). Numerous studies have been conducted to improve the vaccine capacity aiming at inducing effective cross-protection including multi-subunit vaccines (26), consensus vaccines (27), molecular breeding of different structural proteins (28), and mosaic T-cell vaccines (29). In addition to those mentioned above, a polyvalent vaccine containing five different live-attenuated PRRSV strains has been tested in pigs. However, this effort has not provided a significant improvement with regard to heterologous protection (30).

As clearly demonstrated in the field of human immune deficiency virus type 1 (HIV-1) vaccine research, the use of mosaic sequences that are made of natural peptide residues could effectively address the genetic diversity issue (31)(32). Mosaic vaccines have been shown to successfully elicit broader immune responses and confer cross-protection in non-human primates that eventually moved into human trials (33)(34)(35). We described previously the development and characterization of a GP5-Mosaic vaccine based on 748 Type II PRRSV strains (29). The GP5-Mosaic vaccine was immunogenic, induced cross-reactivity *ex-vivo* in PBMCs to 4

divergent Type II PRRSV strains with at least 10% difference in GP5 sequence and conferred protection in pigs (Cui et al. manuscript in preparation). Herein, we show that vaccination of pigs with the GP5-Mosaic vaccine confers heterologous protection against divergent PRRSV strains while the GP5-WT vaccine does not.

2. Materials and Methods

2.1. Viruses and Cells

MN184C was kindly provided by Drs. Kay Faaberg and Kelly Lager from USDA ARS. Viruses were propagated in MARC-145 cells (36). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U penicillin/mL, and 100 µg streptomycin/mL. Virus titers were calculated by the Reed and Muench method (37). The viruses were purified over continuous cesium chloride gradients, quantified using a spectrophotometer NanoDrop 1000 (Thermo Scientific) and stored at -80°C for future use. Purified VR2332 was used as antigen for indirect ELISA. Titrated viruses were used for neutralization assay, recall immune response assay and challenge.

2.2. Transfer Vector Construction

Three transfer vectors were generated by a combination of gene synthesis (DNA2.0, Menlo Park, CA) and standard subcloning. Each transfer vector contained a cassette consisting of the tetR gene (based on GenBank: X00694), as well as either a natural or mosaic version of PRRSV Genotype II ORF 5 (29), followed by the EMCV IRES (based on GenBank:

NC_001479.1) and the EGFP gene (based on plasmid pEGFP-1, GenBank: U55761). The tetR gene and the ORF 5–IRES-EGFP genetic segment were placed under back-to-back synthetic VACV early/late promoters (38). The tetO2 operator sequence was inserted after the putative VACV D6R promoter as described in (39). The cassettes were flanked by 600 bp of the VACV D5R gene to the left and 600 bp of the VACV D6R gene to the right (based on GenBank: NC_006998.1). Plasmid identity was confirmed by restriction endonuclease analysis.

2.3. Generation of Recombinant VACVs and Preparation of High-titer Stocks

Tetracycline-inducible recombinant VACVs were generated by standard homologous recombination after transfection of transfer vectors with FuGene HD Transfection Reagent (Promega, Madison, WI) into BS-C-1 cells infected 1 hour previously with an IPTG-inducible VACV strain (based on the WR clone 9.2.4.8.) in the presence of 0.1 mM IPTG and 1 µg/mL doxycycline. Recombinant EGFP-positive, tetracycline-inducible VACVs were plaque purified in the absence of IPTG and presence of 1 µg/mL doxycycline. Elimination of parental virus was confirmed by PCR analysis of viral DNA purified using a Nucleospin® Blood kit (Macherey-Nagel, Düren, Germany). High-titer stocks were generated by infecting HeLa S3 cells with recombinant virus at an MOI of 0.1 in the presence of 1 µg/mL doxycycline. Infected cells were harvested and homogenized 4 days post-infection. Homogenates were clarified by centrifugation at 750 x g for 10 min, purified by sucrose cushion (40), and resuspended in 1xPBS pH 7.3 (with no doxycycline).

2.4. Protein Sequence Alignment and Phylogenetic Analysis

The protein sequence alignments between GP5 sequences of VR2332, MN184C, Mosaic 1 and Mosaic 2 and phylogenetic tree were evaluated using MEGA 7 (Molecular Evolutionary Genetics Analysis) (www.megasoftware.net).

2.5. Vaccination and Collection of Samples

Three to four-week-old, PRRSV-free, porcine circovirus-2-free, cross-bred piglets were used in this study. The experimental design is shown as Table 4.1, and each pig is considered as one experimental unit. The vaccination scheme consisted of priming with rDNA vaccines previously constructed (29), followed by rVACV boosts. The GP5-Mosaic, GP5-WT, and vector-control vaccines were administered using both intradermal (ID) and intramuscular (IM) injection. Briefly, 0.1 mL GP5-Mosaic, GP5-WT, or vector-control vaccine containing 500 μg DNA/mL and 100 μg Quil-A®/mL as an adjuvant was injected ID on the back of the ear and 0.9 mL IM in the neck musculature at day 0 and boosted once at day 14. Then the animals were boosted via IM injection in the neck musculature with 1 mL solution containing 10^8 PFU rVACV- GP5-Mosaic, rVACV-GP5-WT, or VACV itself, and 100 μg Quil-A® as an adjuvant at day 28. Virus challenge was given at day 35 with VR2332 and day 37 with MN184C and necropsies were performed 14 days post-challenge. The challenge virus was given both intranasally and intramuscularly, with a total of 10^6 $TCID_{50}$/pig. Blood samples were collected at days 0, 7, 14, 21, 28, the challenge day, and 4, 7, 10, and 14 days post-challenge as shown in Scheme 4.1. At the end of the experiment, the pigs were euthanized, and the lungs were evaluated macroscopically, weighed, and bronchoalveolar lavages (BAL) were performed. Tissue samples were collected from each lung lobe, tracheobronchial lymph nodes (TBLN), spleen, inguinal lymph nodes (ILN) and tonsils and then fixed in 10% neutral buffered formalin

or kept frozen. Fixed tissues were sectioned, and stained with hematoxylin and eosin for histologic evaluation. Lung lesion scores were evaluated as previously reported (41). All the animal work was done under a protocol approved by the University of Connecticut Institutional Animal Care and Use Committee.

2.6. Cytokine Recall Cellular Response to PRRSV

PBMCs isolated at days 0 and challenge day were seeded in 24-well flat-bottom plates ($5x10^5$ cells/well) in duplicate and were stimulated with 200 $TCID_{50}$ VR2332 or MN184C /well or mock control for 48 h at 37°C in a 5% CO_2 atmosphere. The cells were collected, and total RNA was extracted for quantitative real-time PCR analysis of IFN-γ.

2.7. Indirect Enzyme-Linked Immunosorbent Assay

ELISA was performed as described previously (29). Briefly, purified VR2332 was coated overnight at 4°C onto 96-well plates at a concentration of 2.5 µg/mL, and the plates were blocked with 10% dry milk in PBS-T (PBS containing 0.05% Tween-20) for 2 h at room temperature. The plates were washed with PBS-T twice and sera samples diluted 40-fold in PBS-T buffer containing 5% dry milk was added. After 60 min at room temperature, the plates were washed five times with PBS-T buffer and incubated with mouse HRP anti-pig IgG (The Jackson Laboratory, Bar Harbor, ME) for 60 min at room temperature. The plates were washed five times in PBS-T, substrate was added, and the plates were incubated in the dark at room temperature for 15 min. The reactions were stopped with 1 M HCl solution. The plates were read at OD_{450} in a

microplate reader (Biotek HTK model, Winooski, VT). Archival PRRSV positive and negative sera of pigs were used as controls in each run.

2.8. Serum Neutralization

Serum neutralization tests were performed as reported previously (29). Briefly, test sera were mixed with equal volumes of DMEM containing 100 $TCID_{50}$ of VR2332 or MN184C (final serum dilution 1:4). After incubation at 37°C for 1 h, the sera-virus mixtures were added to MARC-145 monolayers in 96-well plates, and incubated at 37°C in a 5% CO_2 atmosphere for 48 h. VR2332 or MN184C virus plus negative serum and uninfected cells were used as virus and cell controls, respectively. Neutralization was quantified both by a fluorescence intensity reduction (FIR) test and qRT-PCR 48 h after infection. For FIR, cells 48 h after infection were washed three times with PBS and then fixed with 80% cold acetone for 15 min at 4°C. The cells were reacted with a protein-A purified PRRSV-positive swine serum for 1 h. After three washes with PBS, FITC-conjugated rabbit anti-pig IgG (Sigma, St. Louis, MO) was incubated for 1 h. After washing, fluorescence intensity was measured in a microplate reader (Biotek HTK model, Winooski, VT).

2.9. Quantitative Real-time PCR

Total RNA was extracted from 250 µL of serum or supernatants using TRIzol LS Reagent or from tissues using TRIzol Reagent (Invitrogen, Grand Island, NY). RNA was quantified using a spectrophotometer NanoDrop 1000 (Thermo Scientific). cDNA was

synthesized using random primers (Invitrogen, Grand Island, NY) in a 20 μL reaction mixture. The reaction was run in a thermocycler (Applied Biosystems GeneAmp PCR System 2400) as follows: 26°C for 10 min, 42°C for 45 min and 75°C for 10 min. SYBR Green real-time PCR was then performed, SYBR qPCR Master Mix was purchased from Bimake (Bimake, Houston, TX), and the cDNA was used as a template with 5'-ATG ATG RCC TGG CAT TCT- 3'and 5'-ACA CGG TCG CCC TAA TTG- 3' as the forward and reverse primers for ORF7, respectively. The PCR reaction was performed at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 61°C for 1 min using Bio-Rad CFX96 Touch System (Bio-Rad, Hercules, CA). For each assay, a standard curve was generated using serially diluted RNA which contained 10^2–10^7 copies/μL. In each run, positive and negative reference samples were run along with the test samples. The viral loads were determined by plotting the Ct values against the standard curve. Melting curves were analyzed to verify the specificity of the PCR.

For cytokine evaluation, total RNA was extracted from virus-stimulated or mock-treated PBMCs using TRIzol Reagent. cDNA synthesis and real-time PCR followed the same protocol described above. GAPDH (forward primer: 5'-CGT CCC TGA GAC ACG ATG GT- 3'and reverse primer: 5'-CCC GAT GCG GCC AAA T -3') was used as internal control to calculate the changes of IFN-γ (forward primer: 5'-TGG TAG CTC TGG GAA ACT GAA TG - 3'and reverse primer: 5'- GGC TTT GCG CTG GAT CTG -3') or Interleukin-10 (IL-10) (forward primer: 5'- TGA GAA CAG CTG CAT CCA CTT C- 3'and reverse primer: 5'-TCT GGT CCT TCG TTT GAA AGA AA -3') by the delta-delta method (42).

2.10. Lung Lesion Scoring

Lung lesion scoring was done by a board certified veterinary pathologist, blinded to the treatment groups as previously described (41).

2.11. Statistical Analysis

Student t-test or Two-way ANOVA was used to evaluate the differences between the samples within or between groups. The data were analyzed using GraphPad Prism (version 7.0).

3. Results

3.1. Sequence Alignment and Analysis of GP5

The GP5 amino acid (aa) sequences of Mosaic 1, Mosaic 2, MN184C, and VR2332, the prototype of Type II PRRSV strains, were of the same size and with no deletions or insertions. Sequence alignments showed that aa identities ranged from 85 to 93%. To further investigate the antigenic relationship of these strains, phylogenetic trees were built using neighbor-joining method based on the aa sequences of the two mosaics, VR2332 and MN184C. The four sequences could be roughly categorized into two subgroups (Fig. 4.1), and the two far ends were represented by Mosaic 1 and MN184C. Mosaic 1 and Mosaic 2 were closely related to VR2332 and MN184C respectively. VR2332 shared high aa identity with Mosaic 1 and MN184C shared high aa identity with Mosaic 2.

3.2. GP5-Mosaic Vaccines Activated Both Humoral and Cellular Responses

GP5-Mosaic vaccines induced broad recall cellular responses as detected by the significantly higher levels of IFN-γ mRNA relative fold-change shown in Fig.4.2. Significantly higher levels of IFN-γ mRNA expression were detected upon stimulation of PBMCs of GP5-Mosaic-vaccinated pigs with both VR2332 and MN184C as compared to those in mock controls ($p<0.05$). The response was only significantly higher in IFN-γ mRNA expression in VR2332-stimulated PBMCs of pigs vaccinated with GP5-WT- at the same time points compared to those in mock controls. No such change was detected in those of vector-control pigs.

The vaccination with the GP5-Mosaic vaccine induced significantly higher levels of antibodies, as detected in sera collected at challenge day ($p<0.05$) than those of vector-control animals. Such effects were detected in sera of GP5-WT-vaccinated pigs at the same time points compared to those of vector-control animals ($p<0.05$). Furthermore, sera collected at challenge day from GP5-Mosaic-vaccinated pigs neutralized virus (Figs. 4.2C). The levels of neutralizing antibodies in sera from GP5-Mosaic-vaccinated pigs were higher compared to those in sera from vector-control animal (Fig. 4.2C) as shown by significantly lower viral copy numbers in sera-VR2332 mixture ($p<0.0001$) and srea-MN184C mixture ($p<0.05$), compared to those pigs receiving vector-control. The ability to induce neutralizing antibodies was detected in GP5-WT-vaccinated animals only mixed with VR2332 but not with MN184C.

3.3. Vaccination Did Not Significantly Affect Rectal Temperatures of Experimental Pigs

Rectal temperatures showed a very similar pattern in all groups with VR2332 challenge; there were two peaks at 7DPC and 11DPC in vector-control pigs (Fig. 4.3. top). Rectal temperatures of GP5-Mosaic-vaccinated pigs were generally lower than those in vector-control

pigs and no peak observed while there were two peaks observed at 4DPC and 8DPC with MN184C challenge (Fig. 4.3. bottom).

3.4. GP5-Mosaic Vaccination Induced Cross-protection in Pigs

Viral loads in sera of both GP5-WT and GP5-Mosaic-vaccinated pigs were significantly lower than those of vector-control pigs at 7, 10 and 14 DPC with VR2332 ($p<0.05$*, $p<0.01$**). The GP5-Mosaic vaccine showed comparable capability of reducing viral loads in sera compared to the GP5-WT vaccine. Viral loads in sera of pigs challenged with VR2332 steadily decreased from 4 to 14 DPC in both GP5-WT and GP5-Mosaic-vaccinated pigs while viral loads increased after challenge and reached its peak at 10 DPC, then slightly declined at 14 DPC in sera of vector-control pigs (Fig.4.4A). In contrast, viral loads in sera of GP5-Mosaic-vaccinated pigs were significantly lower than those in both GP5-WT and vector-control pigs at 7 and 14 DPC with MN184C ($p<0.05$*). Viral loads in sera in pigs vaccinated with GP5-WT showed a similar pattern as the vector-control pigs but in lower levels, they increased during the infection and reached peak at 10DPC with MN184C, compared to the vector-control pigs. The viral loads in pigs vaccinated with GP5-Mosaic-vaccine and challenged with MN184C steadily decreased from 4 to 10 DPC, then stayed with no changes up to 14 DPC (Fig.4.4B).

Viral loads in tissues including lung, TBLN, spleen, ILN, and tonsils of GP5-Mosaic-vaccinated pigs were significantly lower than those of vector-control pigs ($p<0.05$) after challenge with MN184C, while those of GP5-WT-vaccinated pigs were similar or slightly lower compared to those of vector-control pigs (Fig.4.5A). Viral loads in tissues including lung, TBLN, and tonsil of pigs vaccinated with GP5-Mosaic-vaccine were significantly lower than those of

vector-control pigs ($p<0.05$) after challenge with VR2332, while those of GP5-WT-vaccinated pigs were considerably lower in lung, spleen, and ILN compared to vector-control pigs ($p<0.05$). No significant differences in tissue virus loads between GP5-Mosaic and GP5-WT vaccination in pigs were found (Fig. 4.5B).

Viral loads in PAMs and BAL fluids showed very a similar pattern in which only those of GP5-Mosaic-vaccinated pigs were significantly lower than those of vector-control pigs with MN184C challenge ($p<0.05$* and $p<0.01$**), while there was no difference between those of GP5-WT and vector-control pigs (Fig. 4.6B, 4.7B). The viral loads in PAMs or BAL of both GP5-Mosaic and GP5-WT-vaccinated pigs were lower than those of vector-control pigs after VR2332 challenge (Fig. 4.6A, 4.7A).

3.5. Lower Lung Lesion Scores Detected in GP5-Mosaic-vaccinated Animals

Lung lesion scores were significantly lower in GP5-Mosaic-vaccinated animals than those in both GP5-WT and vector-control animals when 9 sections of lung were scored after MN184C challenge (Fig. 4.8. right panel, $p<0.05$). The lung lesion scores were lower but not statistically significant in both GP5-Mosaic and GP5-WT-vaccinated pigs, compared to those of vector-control animals after VR2332 challenge. No differences were observed between GP5-WT and GP5-Mosaic-vaccinated pigs (Fig. 4.8, left panel).

4. Discussion

In attempts to effectively address the extraordinary diversity of PRRSV, numerous studies have been reported (29)(28)(43)(44). Recently, several studies including a synthetic

consensus PRRSV strain (27), chimeric PRRSV strain containing shuffled multiple ORFs (45), and an intranasal live virus vaccine with adjuvant (46) showed the capacity of inducing certain levels of heterologous protection in pigs. However, these vaccines are modified live virus vaccines. In addition to the safety concerns, the major drawback of the currently available MLV vaccines is that they are unable to address the great diversity issue of PRRSV and thus cannot provide adequate levels of cross-protection (47). An alternative approach holding great promise is the mosaic T-cell epitope vaccine strategy (31). There is mounting evidence with HIV, another hyper-variable RNA virus, that mosaic T-cell epitope-based vaccines increase the vaccine's breadth and depth and thus broaden the coverage (34)(35) that provides a great potential to effectively deal with the problem of genetic diversity. We have previously demonstrated that GP5-Mosaic vaccine was immunogenic, induced cross-reactivity *ex-vivo,* and conferred partial protection in pigs (29)(Cui et al. manuscript in preparation). The potential of the GP5-Mosaic vaccine to provide cross-protection against heterologous PRRSV strains was further investigated and proven in the present study.

To test the capacity of GP5-Mosaic vaccine to induce cross-protection in pigs, two PRRSV strains were comprehensively compared and meticulously selected as challenge viruses. VR2332, the prototype of Genotype II PRRSV that is also the parental strain for several commercial MLVs, belongs to lineage 5 and MN184C, a strain with high virulence, belongs to lineage 1 (24). These two strains show 15% difference in GP5 aa sequence. Therefore, VR2332 and MN184C were very representative for Type II PRRSV and used in this heterologous protection study. Computer-based assessments are commonly used to predict epitopes, evaluate epitopic coverage or genetic/ antigenic relationships in vaccine development or virus evolution (48)(31)(27)(24). Under such circumstance, two GP5-Mosaic sequences, VR2332 and MN184C

were used to generate a phylogenetic tree using the neighbor-joining method to establish their relationship with regard to genetic diversity. Interestingly, GP5-Mosaic 1 was closer to VR2332 (93% aa identity), while GP5-Mosaic 2 was closer to MN184C (90% aa identity). This indicates that the two GP5-Mosaic sequences showed broader coverage spanning strains belonging to different lineages. Therefore, broader immune responses and cross-protection could be expected when pigs vaccinated with GP5-Mosaic vaccine were challenged with either VR2332 or MN184C. The VR2332 and MN184C share only 85% in GP5 aa identity between them making this challenge heterologous (Fig. 4.1).

In order to enhance vaccine efficacy, Quil-A® was used as adjuvant in this study to increase the host's immune responses as reported before (49)(50)(51). Furthermore, the DNA prime/vaccinia boost regimen has already been shown to greatly increase the vaccination efficacy (52)(53). In addition, VACV vector is very attractive for the development of vaccines including animal vaccines, human vaccines and cancer immunotherapy (54). Thus, in this study, a GP5-Mosaic DNA prime/ VACV-GP5-Mosaic boost vaccination regimen was an appropriate choice to test the vaccine efficacy, in terms of cross-protection. The immune responses induced by GP5-Mosaic vaccine were consistent with Th1 responses with higher levels of IFN-γ, which were reported to play a critical role in the control of PRRSV infection (55). Moreover, evidence was recorded such as both humoral and cellular responses were activated in GP5-Mosaic-vaccinated pigs in this study as demonstrated by steadily increasing antibodies after immunization (Fig. 4.2B) and higher levels of neutralizing antibodies before challenge (Fig. 4.2C). Vaccination induced higher levels of IFN-γ mRNA expression upon stimulation with either VR2332 or MN184C at challenge day (Fig. 4.2A). This demonstrated that GP5-Mosaic vaccine-induced cross-reactivity was consistent with a previous study (Cui et al, manuscript in

preparation) which predicted the ability to induce cross-protection in pigs. In contrast, PBMCs isolated from pigs vaccinated with GP5-WT only responded to stimulation with VR2332. The use of GP5-Mosaic vaccine as a cocktail of Mosaic 1 and Mosaic 2 may explain, in part, the broadening in epitope coverage of Type II GP5 sequences.

Body temperature, lung lesion scores, and the PRRSV viral loads in sera, lung and other tissues such as TBLN, ILN, spleen, and tonsils (after challenge) are the most frequently used assessments for evaluating PRRS vaccine protective efficacy (56)(10)(57)(58). In general, higher body temperature was expected in the control pigs compared to the vaccinated/challenged pigs. As expected when challenged with MN184C virus, GP5-Mosaic-vaccinated pigs had a lower body temperature mean, while vector-control pigs developed higher body temperature in the same period with two peaks at 4DPC and 8DPC. The positive control, GP5-WT-vaccinated pigs, displayed a body temperature curve between those of GP5-Mosaic and vector-control pigs with smaller peaks at 6DPC and 10DPC (Fig. 4.3B). When challenged with VR2332, both GP5-Mosaic and GP5-WT-vaccinated pigs showed similar body temperature curves with a peak at 9DPC while two peaks at 7DPC and 11DPC were recorded in vector-control pigs (Fig. 4.3A). A possible explanation is that MN184C is more virulent than VR2332. Therefore, pigs with MN184C challenge developed higher and earlier peak temperature with around 104°F at 4DPC and 8DPC while pigs with VR2332 challenge displayed lower and later peak temperature with around 103°F at 7DPC and 11DPC in vector-control pigs.

As an important protection measure, viral loads in sera of both GP5-Mosaic and GP5-WT-vaccinated pigs showed significantly lower levels at 7, 10 and 14DPC than those of vector-control pigs after VR2332 challenge. In contrast, after MN184C challenge only pigs vaccinated with the GP5-Mosaic-vaccine had significantly lower viral loads in sera at 7DPC and 14DPC

than the vector-control pigs (Fig. 4.4). This piece of evidence demonstrated that the GP5-Mosaic vaccine was able to clean circulating viruses in pigs in either challenge while the GP5-WT vaccine was only able to clean homologous virus circulating in pigs. Furthermore, a similar pattern was recorded in tissues where GP5-Mosaic-vaccinated pigs displayed significantly lower viral loads in lung, TBLN, spleen, ILN, and tonsils, compared to those of vector-control pigs with MN184C challenge, while GP5-WT-vaccinated pigs only showed slightly lower but not significantly lower viral loads. Additionally, lower viral loads in PAMs or BAL fluid were recorded in GP5-Mosaic-vaccinated pigs. Lung lesion score is an essential and crucial method to evaluate the effects of PRRSV infection, including enhancing the susceptibility to coinfection with other pathogens namely, viral or bacterial pathogens (59)(60)(61). GP5-Mosaic-vaccinated pigs, not surprisingly, showed significantly lower lung lesion scores than both the GP5-WT and vector-control pigs after challenge with the MN184C strain. In contrast, pigs vaccinated with either GP5-Mosaic or GP5-WT showed lower, although not significant, lung lesion scores compared to the vector-control pigs after challenge with VR2332. Moreover, atrophic rhinitis (AR), a common respiratory infection of piglets, was recorded in some of the vector-control pigs but not in any of the GP5-Mosaic-vaccinated pigs. Together, our results strongly demonstrate that GP5-Mosaic vaccine was able to clean both circulating and local heterologous viruses, thus clearly providing cross-protection against challenge with two heterologous PRRSV strains including VR2332, the prototype Genotype II strain, and MN184C, a highly virulent strain.

Over the course of this study, we successfully demonstrated that the GP5-Mosaic vaccine was able to provide cross-protection against heterologous PRRSV strains in pigs with the contributions from both arms of the immune system, humoral and cell-mediated immune responses. Additionally, we demonstrated that the GP5-Mosaic vaccine is a viable approach to

generate novel vaccines with enhanced cross-protection abilities against diverse PRRSV strains. In the future, components such as other PRRSV structural and non-structural proteins containing T-cell epitopes will be incorporated into multivalent, multiepitopic mosaic vaccine to further enhance efficacy and improve cross-protection.

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References

1. Holtkamp D.J., Kliebenstein J.B., Neumann E.J., J. J. Zimmerman, H.F. Rotto, T.K. Yoder, C. Wang, P.E. Yeske, C.L. Mowrer and C.A. Haley. Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. J Swine Health Prod. 2013; 21(2):72-84.
2. Collins JE, Benfield DA, Christianson WT, Harris L, Hennings JC, Shaw DP, Goyal SM, McCullough S, Morrison RB, Joo HS, Gorcyca D, Chladek D. 1992. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J Vet Diagn Invest 4:117–126. doi:10.1177/104063879200400201
3. Wensvoort G, Terpstra C, Pol JMA, ter Laak EA, Bloemraad M, de Kluyver EP, Kragten C, van Buiten L, den Besten A, Wagenaar F, Moonen PLJM, Zetstra T, de Boer EA, Tibben HJ, de Jong MF, Van't Veld P, Greenland GJR, van Gennep JA, Voets MT, Verheijden JHM, Braamskamp J. 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet Q 13:121–130.
4. Cavanagh D. 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch Virol142:629–633
5. Lunney JK, Fang Y, Ladinig A, Chen N, Li Y, Rowland B, et al. Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction with the Immune System. Annu Rev Anim Biosci. 2016; 4: 129–154. pmid:26646630
6. Mokhtar H, Eck M, Morgan SB, Essler SE, Frossard JP, Ruggli N, Graham SP. 2014. Proteome-wide screening of the European porcine reproductive and respiratory syndrome virus reveals a broad range of T cell antigen reactivity. Vaccine 32:6828–6837.
7. Parida R, Choi IS, Peterson DA, Pattnaik AK, Laegreid W, Zuckermann FA, Osorio FA. 2012. Location of T-cell epitopes in nonstructural proteins 9 and 10 of type-II porcine reproductive and respiratory syndrome virus. Virus Res 169:13–21.
8. Brown E, Lawson S, Welbon C, Gnanandarajah J, Li J, Murtaugh MP, Nelson EA, Molina RM, Zimmerman JJ, Rowland RR, Fang Y. 2009. Antibody response of nonstructural proteins: implications for diagnostic detection and differentiation of type I and type II porcine reproductive and respiratory syndrome viruses. Clin Vaccine Immunol 16:628–635.

9. Vanhee M, Van Breedam W, Costers S, Geldhof M, Noppe Y, Nauwynck H. 2011. Characterization of antigenic regions in the porcine reproductive and respiratory syndrome virus by the use of peptide-specific serum antibodies. Vaccine 29:4794–4804.
10. Zuckermann FA, Garcia EA, Luque ID, Christopher-Hennings J, Doster A, Brito M, Osorio F. 2007.Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. Vet Microbiol 123:69–85.
11. Osorio FA, Zuckermann F, Wills R, Meier W, Christian S, Galeota J, Doster A. 1998. PRRSV: comparison of commercial vaccines in their ability to induce protection against current PRRSV strains of high virulence, p 176–182. *In* Proceedings of the 25th Allen D. Leman Swine Conference. University of Minnesota, Minneapolis, MN.
12. Scortti M, Prieto C, Alvarez E, Simarro I, Castro JM. 2007. Failure of an inactivated vaccine against porcine reproductive and respiratory syndrome to protect gilts against a heterologous challenge with PRRSV. Vet Rec 161:809–813.
13. Geldhof MF, Vanhee M, Van Breedam W, Van Doorsselaere J, Karniychuk UU, Nauwynck HJ. 2012.Comparison of the efficacy of autogenous inactivated porcine reproductive and respiratory syndrome virus (PRRSV) vaccines with that of commercial vaccines against homologous and heterologous challenges. BMC Vet Res 8:182.
14. Labarque G, Reeth KV, Nauwynck H, Drexler C, Van Gucht S, Pensaert M. 2004. Impact of genetic diversity of European-type porcine reproductive and respiratory syndrome virus strains on vaccine efficacy. Vaccine 22:4183–4190.
15. Okuda Y, Kuroda M, Ono M, Chikata S, Shibata I. 2008. Efficacy of vaccination with porcine reproductive and respiratory syndrome virus following challenges with field isolates in Japan. J Vet Med Sci70:1017–1025.
16. Han K, Seo HW, Park C, Chae C. 2014. Vaccination of sows against type 2 porcine reproductive and respiratory syndrome virus (PRRSV) before artificial insemination protects against type 2 PRRSV challenge but does not protect against type 1 PRRSV challenge in late gestation. Vet Res 45:12.
17. Kim T, Park C, Choi K, Jeong J, Kang I, Park SJ, Chae C. 2015. Comparison of two commercial type 1 porcine reproductive and respiratory syndrome virus (PRRSV) modified

live vaccines against heterologous type 1 and type 2 PRRSV challenge in growing pigs. Clin Vaccine Immunol 22:631–640.

18. Trus I, Bonckaert C, van der Meulen K, Nauwynck HJ. 2014. Efficacy of an attenuated European subtype 1 porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs upon challenge with the East European subtype 3 PRRSV strain Lena. Vaccine 32:2995–3003.
19. Bøtner, A., Strandbygaard, B., Sørensen, KJ., Have, P., Madsen, KG., Madsen, ES., Alexandersen, S. (1997) Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine Veterinary Record 141, 497-499.
20. Madsen, K. G., Hansen, C. M., Madsen, E. S., Strandbygaard, B., Bøtner, A., & Sørensen, K. J. (1998). Sequence analysis of porcine reproductive and respiratory syndrome virus of the American type collected from Danish swine herds. Archives of Virology, 143(9), 1683–1700.
21. Nielsen, H. S., Oleksiewicz, M. B., Forsberg, R., Stadejek, T., Bøtner, A., & Storgaard, T. (2001). Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. Journal of General Virology, 82(6), 1263–1272.
22. Nelsen CJ, Murtaugh MP, Faaberg KS. 1999. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J Virol 73:270–280.
23. H.K. Kim, J.S. Yang, H.J. Moon, S.J. Park, Y. Luo, C.S. Lee, D.S. Song, B.K. Kang, S.K. Ann, C.H. Jun, B.K. Park. Genetic analysis of ORF5 of recent Korean porcine reproductive and respiratory syndrome viruses (PRRSVs) in viremic sera collected from MLV-vaccinating or non-vaccinating farms. J. Vet. Sci., 10 (2) (2009), pp. 121–130
24. Shi M, Lam TT, Hon CC, Murtaugh MP, Davies PR, Hui RK, Li J, Wong LT, Yip CW, Jiang JW, Leung FC. 2010. Phylogeny-based evolutionary, demographical, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses. J Virol 84:8700–8711.
25. Goldberg TL, Lowe JF, Milburn SM, Firkins LD. 2003. Quasispecies variation of porcine reproductive and respiratory syndrome virus during natural infection. Virology 317:197–207.

26. Jiang Y, Xiao S, Fang L, Yu X, Song Y, Niu C, Chen H. DNA vaccines co-expressing GP5 and M proteins of porcine reproductive and respiratory syndrome virus (PRRSV) display enhanced immunogenicity. Vaccine (2006). 24, 15, 2869-2879
27. Vu HL, Ma F, Laegreid WW, Pattnaik AK, Steffen D, Doster AR, Osorio FA. A Synthetic Porcine Reproductive and Respiratory Syndrome Virus Strain Confers Unprecedented Levels of Heterologous Protection. Journal of Virology (2015). 89, 23, 12070-12083
28. Zhou L, Ni YY, Piñeyro P, Sanford BJ, Cossaboom CM, Dryman BA, Huang YW, Cao DJ, Meng XJ. DNA shuffling of the GP3 genes of porcine reproductive and respiratory syndrome virus (PRRSV) produces a chimeric virus with an improved cross-neutralizing ability against a heterologous PRRSV strain. Virology (2012). 434, 1, 96-109
29. J. Cui, C.M. O'Connell, J.D. Smith, J. Smyth, P. H. Verardi, Y. Pan and A. E. Garmendia. A GP5 Mosaic T-cell Vaccine for Porcine Reproductive and Respiratory Syndrome Virus Is Immunogenic and Confers Partial Protection to Pigs. Vaccine Reports 2016, 6: 77-85
30. Mengeling WL, Lager KM, Vorwald AC, Clouser DF. 2003. Comparative safety and efficacy of attenuated single-strain and multi-strain vaccines for porcine reproductive and respiratory syndrome. Vet Microbiol 93:25–38.
31. Fischer W, Perkins S, Theiler J, Bhattacharya T, Yusim K, Funkhouser R, Kuiken C, Haynes B, Letvin NL, Walker BD, Hahn BH, Korber BT. Polyvalent vaccines for optimal coverage of potential T-cell epitopes in global HIV-1 variants. Nature Medicine (2007). 13, 1, 100-106
32. Thurmond J, Yoon H, Kuiken C, Yusim K, Perkins S, Theiler J, Bhattacharya T, Korber B, Fischer W. Web-based design and evaluation of T-cell vaccine candidates. Bioinformatics (2008). 24, 14, 1639-1640
33. Kong WP, Wu L, Wallstrom TC, Fischer W, Yang ZY, Ko SY, Letvin NL, Haynes BF, Hahn BH, Korber B, Nabel GJ. Expanded breadth of the T-cell response to mosaic human immunodeficiency virus type 1 envelope DNA vaccination. Journal of Virology (2009). 83, 5, 2201-15
34. Barouch DH, O'Brien KL, Simmons NL, King SL, Abbink P, Maxfield LF, Sun YH, La Porte A, Riggs AM, Lynch DM, Clark SL, Backus K, Perry JR, Seaman MS, Carville A, Mansfield KG, Szinger JJ, Fischer W, Muldoon M, Korber B. Mosaic HIV-1 vaccines expand the breadth and depth of cellular immune responses in rhesus monkeys. Nature Medicine (2010). 16, 3, 319-323

35. Santra S, Liao HX, Zhang R, Muldoon M, Watson S, Fischer W, Theiler J, Szinger J, Balachandran H, Buzby A, Quinn D, Parks RJ, Tsao CY, Carville A,Mansfield KG, Pavlakis GN, Felber BK, Haynes BF, Korber BT, Letvin NL. Mosaic vaccines elicit CD8+ T lymphocyte responses that confer enhanced immune coverage of diverse HIV strains in monkeys. Nature Medicine (2010). 16, 3, 324-328
36. H.S. Kim, J. Kwang, I.J. Yoon, H.S. Joo, M.L. Frey. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. Arch. Virol., 133 (3–4) (1993), pp. 477–483
37. Reed, L.J.; Muench, H. (1938). A simple method of estimating fifty percent endpoints. The American Journal of Hygiene. 27: 493–497.
38. Chakrabarti, S., Siler, J.R., Moss, B., 1997. Compact, synthetic, vaccinia virus early/late promoter for protein expression. BioTechniques 23 (6), 1094-1097.
39. Hagen, C.J., Titong, A., Sarnoski, E.A., Verardi, P.H. 2014. Antibiotic-dependent expression of early transcription factor subunits leads to stringent control of vaccinia virus replication. Virus Research 181: 43-52.
40. Cotter, C.A., Earl, P.L., Wyatt, L.S., and Moss, B. 2017. Preparation of cell cultures and vaccinia virus stocks. Curr. Protoc. Mol. Biol. 117:16.16.1-16.16.18.
41. Halbur PG, Paul PS, Meng XJ, Lum MA, Andrews JJ, Rathje JA. Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. Journal of Veterinary Diagnostic Investigation (1996). 8, 1, 11-20
42. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods (2001) 25, 4, 402-408
43. Ni YY, Opriessnig T, Zhou L, Cao D, Huang YW, Halbur PG, Meng XJ. Attenuation of porcine reproductive and respiratory syndrome virus by molecular breeding of virus envelope genes from genetically divergent strains. J Virology (2013). 87, 1, 304-313
44. Zhou L, Ni YY, Piñeyro P, Cossaboom CM, Subramaniam S, Sanford BJ, Dryman BA, Huang YW, Meng XJ. Broadening the heterologous cross-neutralizing antibody inducing ability of porcine reproductive and respiratory syndrome virus by breeding the GP4 or M genes. PLoS ONE (2013). 8, 6

45. Tian D, Ni YY, Zhou L, Opriessnig T, Cao D, Piñeyro P, Yugo DM, Overend C, Cao Q, Lynn Heffron C, Halbur PG, Pearce DS, Calvert JG, Meng XJ.
Chimeric porcine reproductive and respiratory syndrome virus containing shuffled multiple envelope genes confers cross-protection in pigs. Virology. 2015 Nov; 485:402-13.
46. Dwivedi, V., Manickam, C., Patterson, R., Dodson, K., Murtaugh, M., Torrelles, J. B. Renukaradhya, G. J. (2011). Cross-protective immunity to porcine reproductive and respiratory syndrome virus by intranasal delivery of a live virus vaccine with a potent adjuvant. Vaccine, 29(23), 4058–4066.
47. Vu H, Pattnaik A, Osorio F. Strategies to broaden the cross-protective efficacy of vaccines against porcine reproductive and respiratory syndrome virus. Veterinary Microbiology (2016) Published by Elsevier B.V.
48. Lampe, K., Gottstein, B., Becker, T., Stahl-Hennig, C., Kaup, F. J., & Mätz-Rensing, K. (2017). Immunization of rhesus macaques with Echinococcus multilocularis recombinant 14-3-3 antigen leads to specific antibody response. Parasitology Research, 116(1), 435–439.
49. Díaz, I., Pujols, J., Ganges, L., Gimeno, M., Darwich, L., Domingo, M., Mateu, E., 2009. In silico prediction and ex vivo evaluation of potential T-cell epitopes in glycoproteins 4 and 5 and nucleocapsid protein of genotype-I (European) of porcine reproductive and respiratory syndrome virus. Vaccine 27, 5603–5611.
50. Tahoun, A., Jensen, K., Corripio-Miyar, Y., McAteer, S. P., Corbishley, A., Mahajan, A, Gally, D. L. (2015). Functional analysis of bovine TLR5 and association with IgA responses of cattle following systemic immunisation with H7 flagella. Veterinary Research, 46(1).
51. Gómez-Gascón, L., Cardoso-Toset, F., Tarradas, C., Gómez-Laguna, J., Maldonado, A., Nielsen, J. Luque, I. (2016). Characterization of the immune response and evaluation of the protective capacity of rSsnA against Streptococcus suis infection in pigs. Comparative Immunology, Microbiology and Infectious Diseases, 47, 52–59.
52. Chapman, R., Jongwe, T. I., Douglass, N., Chege, G., & Williamson, A. L. (2017). Heterologous prime-boost vaccination with DNA and MVA vaccines, expressing HIV-1 subtype C mosaic Gag virus-like particles, is highly immunogenic in mice. PLoS ONE, 12(3).

53. Deng, Y., Chuai, X., Chen, P., Chen, H., Wang, W., Ruan, L., Tan, W. (2017). Recombinant vaccinia vector-based vaccine (Tiantan) boosting a novel HBV subunit vaccine induced more robust and lasting immunity in rhesus macaques. Vaccine, 1–7.
54. Verardi, P. H., Titong, A., & Hagen, C. J. (2012, July). A vaccinia virus renaissance: new vaccine and immunotherapeutic uses after smallpox eradication. Human Vaccines & Immunotherapeutics.
55. Morgan, S.B., Graham, S.P., Salguero, F.J., Sánchez Cordón, P.J., Mokhtar, H., Rebel, J.M.J., Weesendorp, E., Bodman-Smith, K.B., Steinbach, F., Frossard, J.P., 2013. Increased pathogenicity of European porcine reproductive and respiratory syndrome virus is associated with enhanced adaptive responses and viral clearance. Veterinary Microbiology 163, 13–22.
56. Kimman TG, Cornelissen LA, Moormann RJ, Rebel JM, Stockhofe-Zurwieden N. Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. Vaccine (2009). 27, 28, 3704-3718
57. Martelli, P., Gozio, S., Ferrari, L., Rosina, S., De Angelis, E., Quintavalla, C., Bottarelli, E., Borghetti, P., 2009. Efficacy of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs naturally exposed to a heterologous European (Italian cluster) field strain: Clinical protection and cell-mediated immunity. Vaccine 27, 3788–3799.
58. Wang R, Xiao Y, Opriessnig T, Ding Y, Yu Y, Nan Y, et al. Enhancing neutralizing antibody production by an interferon-inducing porcine reproductive and respiratory syndrome virus strain. Vaccine. 2013, 31(47):5537–43.
59. Thanawongnuwech, R., Brown, G.B., Halbur, P.G., Roth, J.A., Royer, R.L., Thacker, B.J., 2000. Pathogenesis of Porcine Reproductive and Respiratory Syndrome Virus-induced Increase in Susceptibility to Streptococcus suis Infection. Veterinary Pathology 37, 143–152.
60. Van Reeth, K., Nauwynck, H., Pensaert, M., 1996. Dual infections of feeder pigs with porcine reproductive and respiratory syndrome virus followed by porcine respiratory coronavirus or swine influenza virus: A clinical and virological study. Veterinary Microbiology 48, 325–335.
61. Tsai, Y.-C., Chang, H.-W., Jeng, C.-R., Lin, T.-L., Lin, C.-M., Wan, C.-H., Pang, V.F., 2012. The effect of infection order of porcine circovirus type 2 and porcine reproductive and

respiratory syndrome virus on dually infected swine alveolar macrophages. BMC Veterinary Research 8, 174.

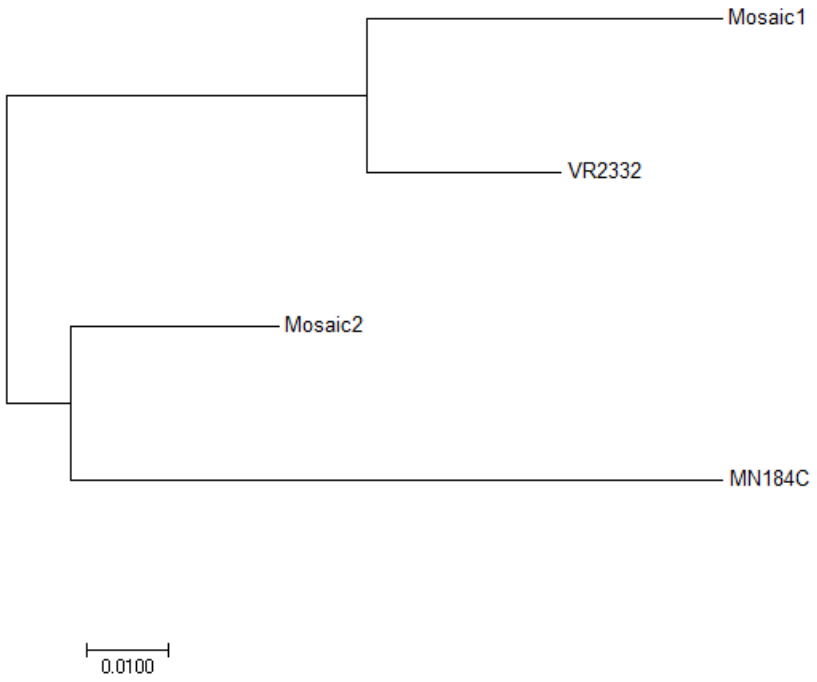


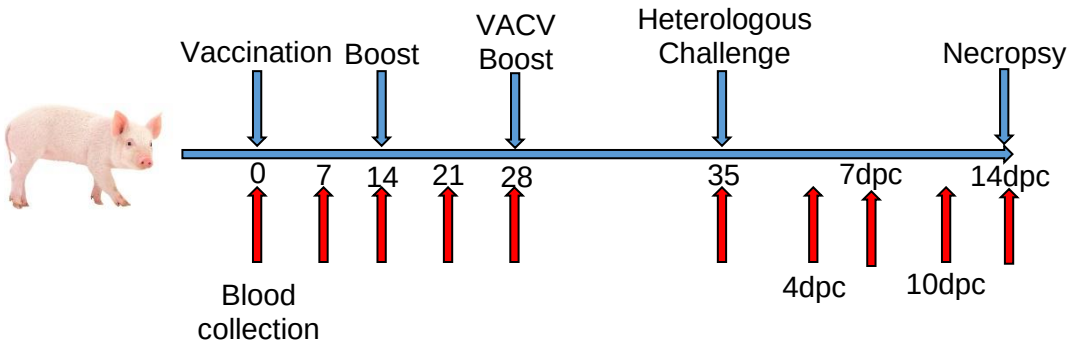
Figure 4.1. Phylogenetic Analysis Based on Amino Acid Sequences of GP5 of Two Mosaic Sequences, MN184C and VR2332. The analysis was done using neighbor-joining method of MEGA7.0.

Table 4.1 Experimental Design

Group	Immunization[a]	Challenge[b]
A(n=8)	Vector-control	VR2332
		MN184C
B(n=8)	GP5-WT	VR2332
		MN184C
C(n=8)	GP5-Mosaic	VR2332
		MN184C

[a] 1 mL vaccine containing 500 μg DNA and 100 μg Quil-A® as adjuvant was injected intradermally (0.1 mL) on the back of ear and intramuscularly (0.9 mL) on the neck musculature at day 0 and boosted once at days 14. 10E8 PFU VACV and 100 μg Quil-A® as an adjuvant boosted at day 28.

[b] Pigs were challenged at day 35 with 10^6TCID$_{50}$ VR2332 and day 37 with 10^6TCID$_{50}$ MN184C via both intranasal and intramuscularly. All groups had equal numbers of male and female pigs



Scheme 4.1. Vaccination/Challenge Design. Blue arrows indicate the major operations including vaccination, boost, challenge, and necropsy. Red arrows indicate blood sample collections.

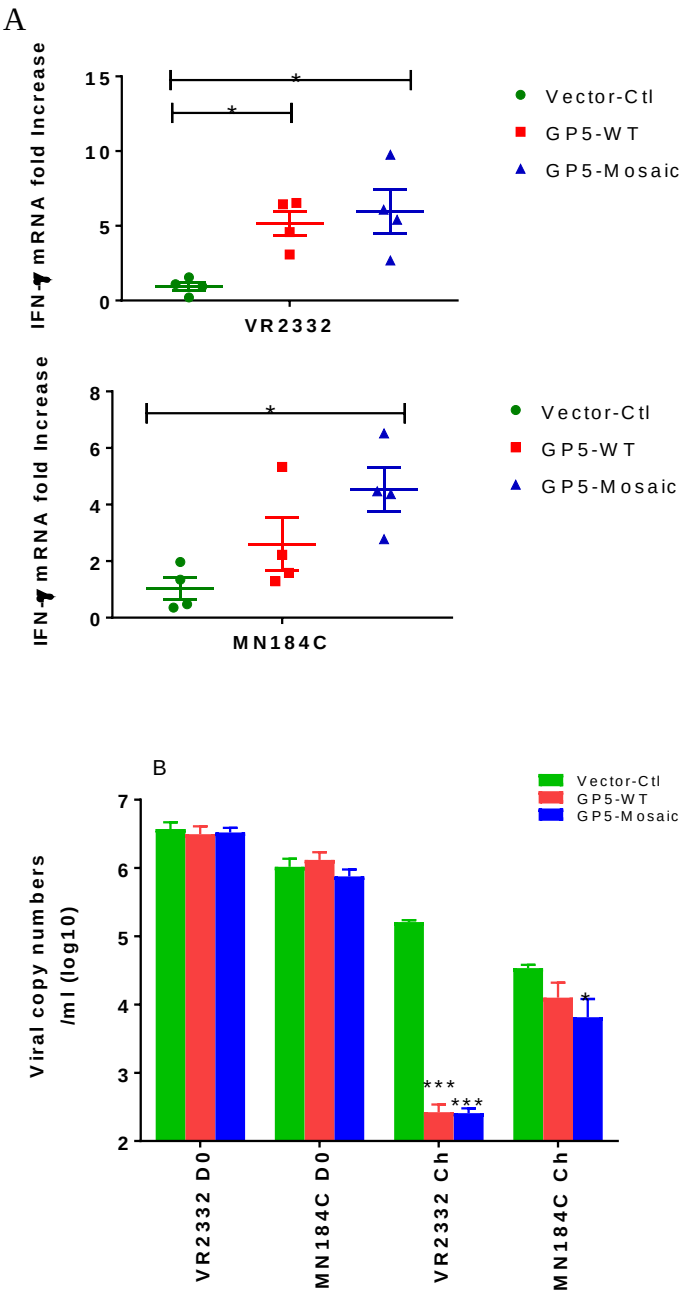


Figure 4.2. Vaccine-induced Humoral and Cellular Responses. A. Virus-specific antibodies detected by iELISA over time**. B.** Changes in viral copy numbers induced by serum antibodies detected in infected cell supernatants by qRT-PCR. Each dot represents the mean value of each animal. Each bar represents the mean value of each group. Variation expressed as standard error of the mean. Three experiments were performed independently. Significant differences calculated by a Two-way ANOVA or student t-test ($p<0.05$* or $p<0.001$***).

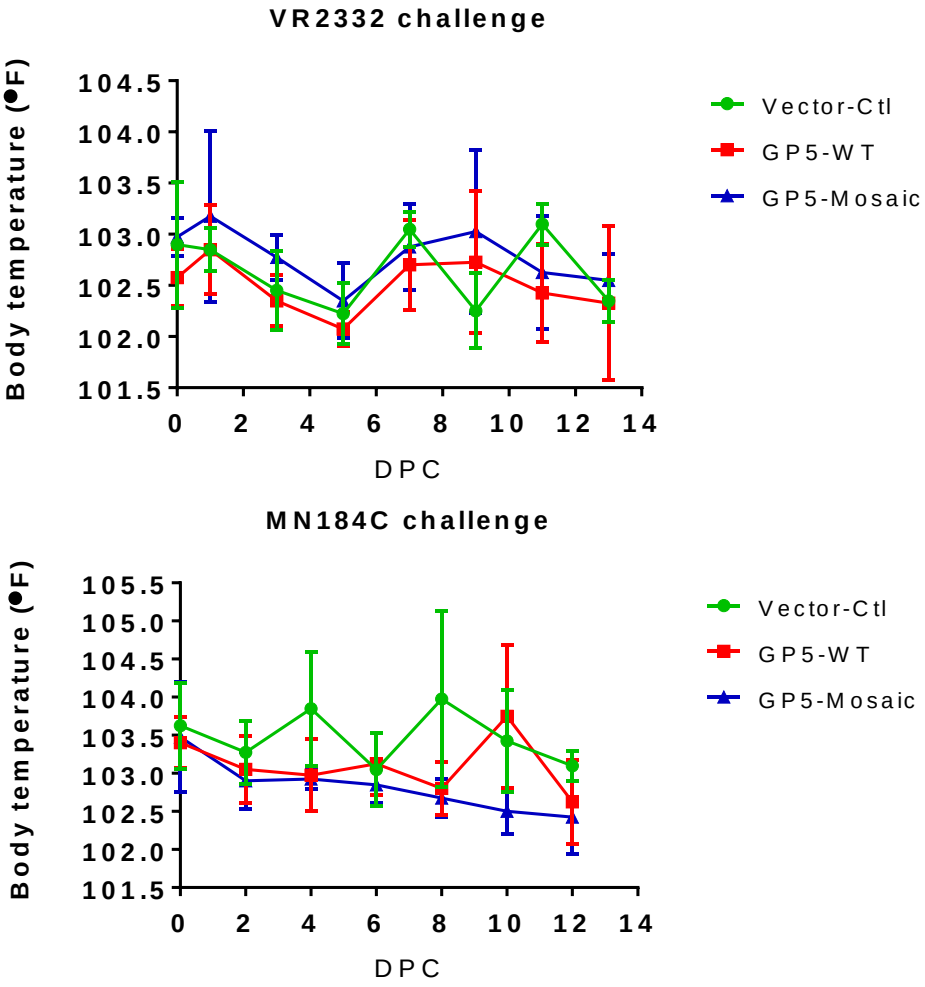


Figure 4.3. Rectal Temperature of Pigs Comparison between Immunization Groups after Heterologous Challenge. Top. Rectal temperatures changes post VR2332 challenge. **Bottom.** Rectal temperatures changes post MN184C challenge. Each dot represents the mean value of each group. Variation expressed as standard error of the mean. Experiments were performed independently. Significant differences calculated by a student t-test (DPC: days post challenge)

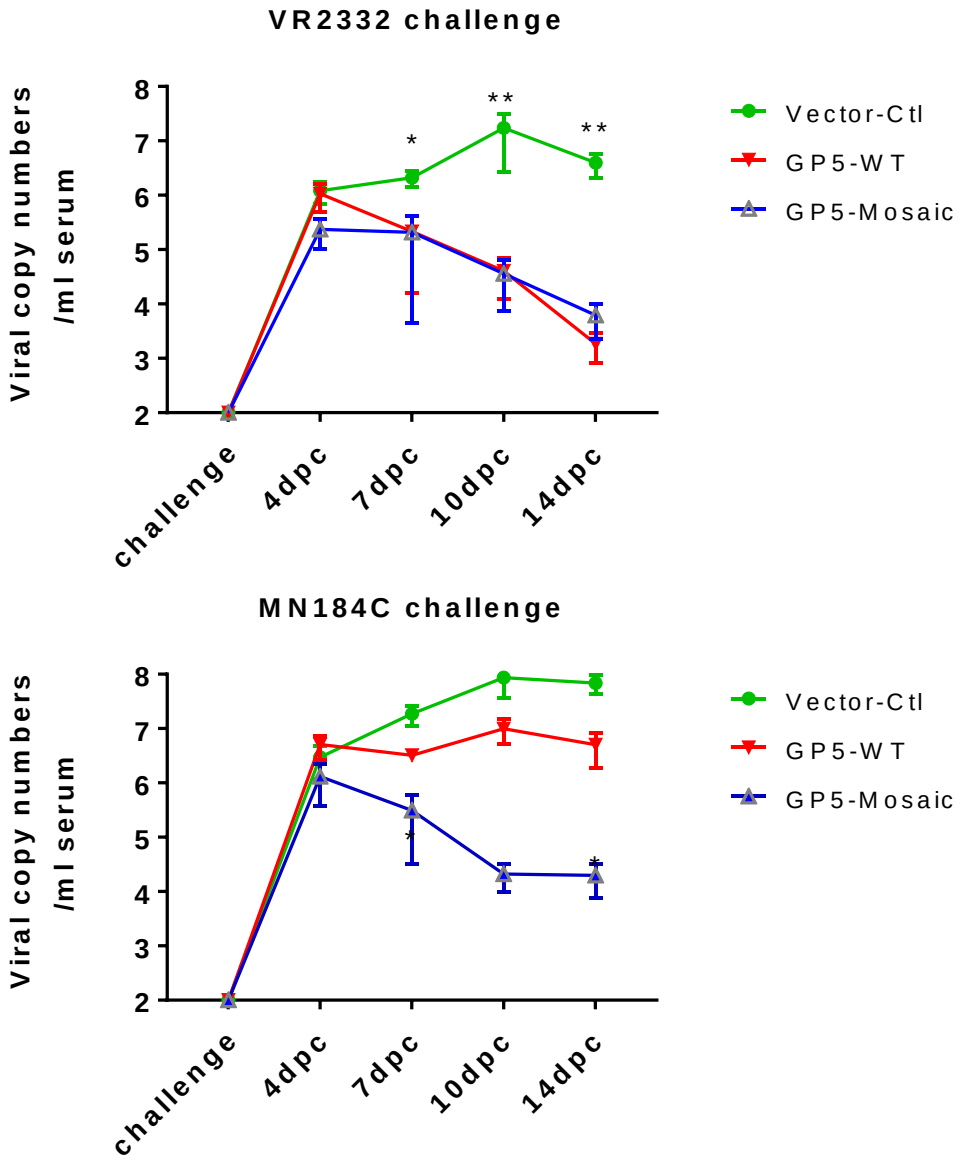


Figure 4.4. Virus Clearance in Sera. A. Comparison of viral copy numbers in serum from 0 DPC to 14 DPC upon VR2332 challenge. **B.** Comparison of viral copy numbers in serum from 0 DPC to 14 DPC upon MN184C challenge. Each dot represents the mean value of each group. Variation expressed as standard error of the mean. Three experiments were performed independently. Significant differences calculated by a Two-way ANOVA or student t-test (p<0.05*, p<0.01**, DPC: days post challenge).

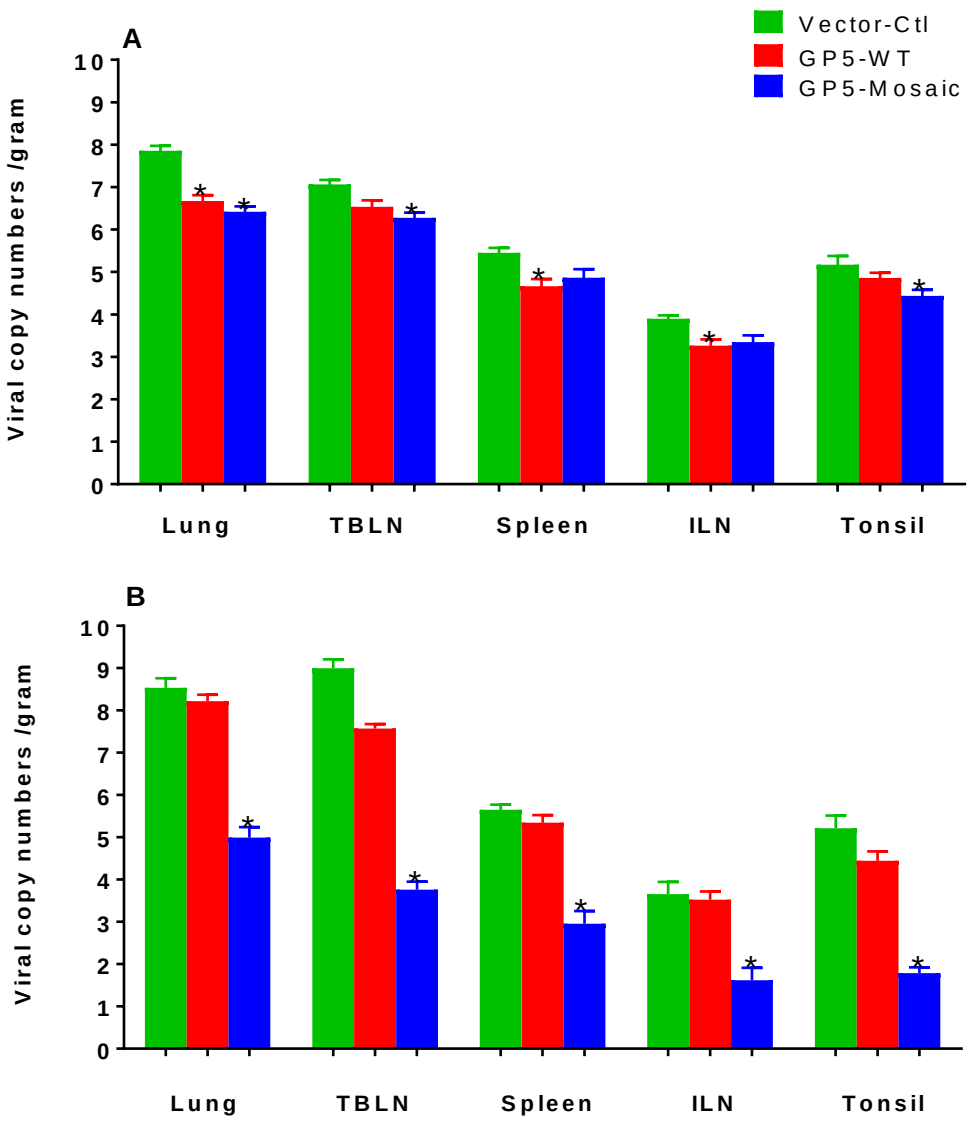


Figure 4.5. Virus Clearance in Tissues. A. Viral copy numbers in tissues at necropsy upon VR2332 challenge. **B.** Viral copy numbers in tissues at necropsy upon MN184C challenge. Each bar represents the mean value of each group. Variation expressed as standard error of the mean. Three experiments were performed independently. Significant differences calculated by student t-test (p<0.05*).

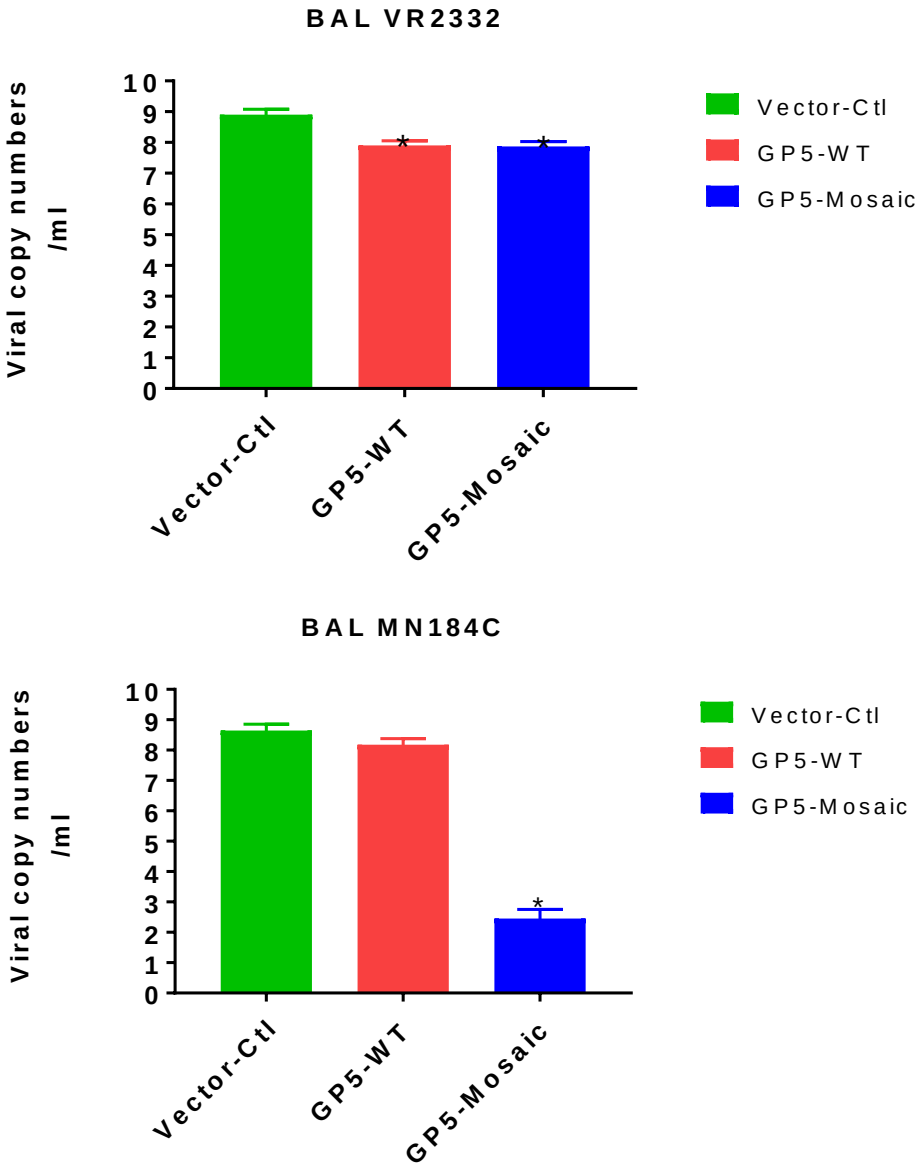


Figure 4.6. Virus Clearance and Lung Lesion Scores. A. Viral copy numbers in BAL fluids at necropsy upon VR2332 challenge. **B.** Viral copy numbers in BAL fluids at necropsy upon MN184C challenge. Each bar represents the mean value of each group. Variation expressed as standard error of the mean. Three experiments were performed independently. Significant differences calculated by a student t-test ($p<0.05$*).

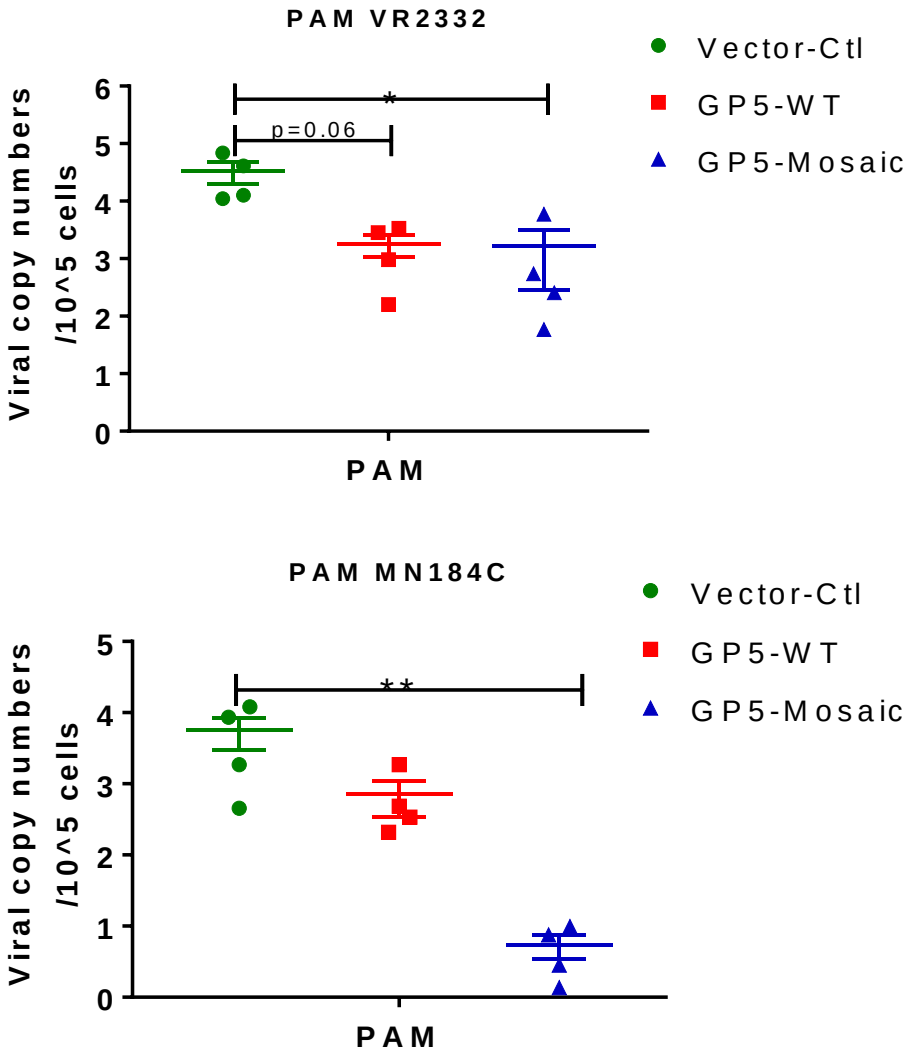


Figure 4.7. Virus Clearance in PAMs. A. Viral copy numbers in PAMs at necropsy upon VR2332 challenge. **B.** Viral copy numbers in PAMs at necropsy upon MN184C challenge. Each dot represents the mean value of each animal. Variation expressed as standard error of the mean. Three experiments were performed independently. Significant differences calculated by a student t-test ($p<0.05$*, $p<0.01$**).

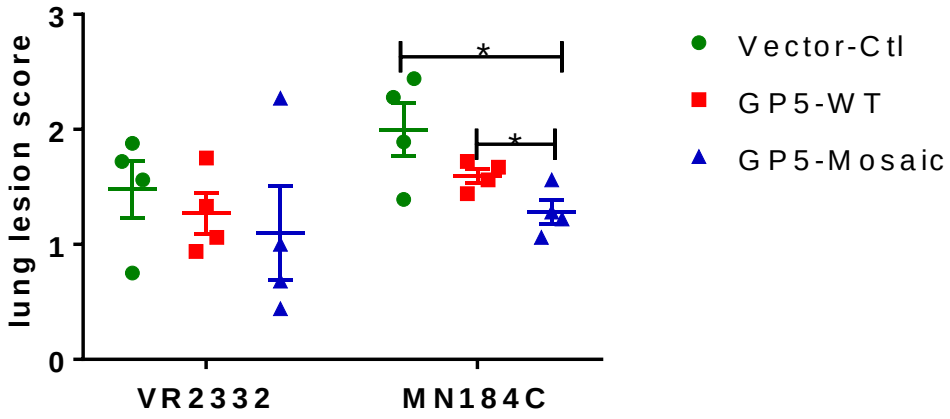


Figure 4.8. Lung Lesion Scores. Lung lesion scores. Scores were significantly lower (p<0.05) in GP5-Mosaic-vaccinated animals than those in vector-control animals. Each dot represents the mean value of each individual. Variation expressed as standard error of the mean. Three experiments were performed independently. Significant differences calculated by a student t-test (DPC: days post challenge).