ABSTRACT

LEE, KANG MI. Antibody and Cellular Immune Responses of Swine Exposed to Porcine Reproductive and Respiratory Syndrome Virus or a GP5 Subunit Vaccine. (Under the direction of Dr Scott Laster.)

Developing effective vaccines against the porcine reproductive and respiratory syndrome virus (PRRSV) has proved difficult, highlighting the need for basic information on the nature of the immune response against this virus and the mechanisms of resistance that the virus employs. In this investigation our goal was to characterize the immune response against the major outer membrane protein of the virus, GP5, in pigs experimentally infected with a North Carolina isolate of PRRSV known as the NC Powell strain. In addition, we compared this response with the immune response seen after vaccination with purified recombinant GP5 (rGP5) protein. Humoral immune responses were monitored by western blot and immunofluorescence while T cell responses were monitored using proliferation assays and flow cytometry. Our results show strong humoral recognition of rGP5 protein during both natural and vaccine induced antibody responses. In addition, epitope mapping via western blot revealed that antibody responses were directed largely against the C-terminal endodomain of rGP5 protein in both experimentally infected and vaccinated pigs. We also investigated T cell responses to rGP5 protein. Our experiments revealed that T cells from vaccinated animals also responded to both rGP5 protein and inactivated NC Powell strain of PRRSV suggesting that T cells may play an important role in vaccine-induced resistance. Interestingly, we found that the inactivated NC Powell strain of PRRSV caused a strong proliferative response in naïve T cells from control animals, perhaps indicating the presence of a superantigen as a component of this highly virulent strain of PRRSV.

ANTIBODY AND CELLULAR IMMUNE RESPONSES OF SWINE EXPOSED TO PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS OR A GP5 SUBUNIT VACCINE

by

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DEDICATION

This dissertation is dedicated to my Dad, Sun Bong Lee in heaven and to our heavenly Father, who give us strength.

BIOGRAPHY

Kang Mi Lee was born in April of 1976 in Kangeong, South Korea as the daughter of Sun Bong Lee and Jong Yeob Kim. Kang Mi was raised in Wonju, South Korea, where she attended both elementary and junior high schools. Kang Mi Lee graduated high school in 1995 and moved to Seoul, the capital of South Korea, to pursue her interest in biological science at Hanyang University. As an undergraduate, Kang Mi became interested in the immune system after taking a course in immunology. Kang Mi received a Bachelor of Science in Biology in 1999. Later on, her interest in the life sciences led her to work as a research assistant at the school of medicine in Yonsei University, South Korea. While in undergraduate school, Kang Mi met her future husband, Eun Seok Gil and they had a vision to study further in America. After getting married, they moved to America in 2000. Kang Mi enrolled in the immunology program at North Carolina State University in 2001 where she began training under the direction of Dr. Scott Laster. Upon completion of her Ph.D. degree, she confirmed her future research to be in the field of immunotherapy. She wants to combine her experiences in scientific research with the health problems we face today and ultimately create solutions to aid patients at their bedside.

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Completing study for the doctoral degree in immunology has been one of the most challenging endeavors of my life. I had ups and downs and moments when I wasn't sure where I was heading. I struggled with what were right for me and my family. However, God gave me the heart to pursue a Ph.D. and fulfill it regardless of my weaknesses and lack of ability. For his grace, I am now who I am.

I would like to thank my family beginning with my husband, Eun Seok Gil and my son Daniel Gil for their patience, support, and love. Since I know they made too many sacrifices for me to achieve this goal, this degree is priceless. I would also like to acknowledge my mother, Jong Yeob Kim, and parents-in-law, Gi Hong Gil and Bang Ran Tak, for their unselfish support, confidence in my abilities, and endless prayer.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADE - antibody-dependent enhancement
APC - antigen presenting cell
CD - cluster of differentiation
DMSO - dimethy sulfoxide
DTH - delayed type hypersensitivity
EAV - equine arteritis virus
ER - endoplasmic reticulum
FACS - fluorescence activated cell sorting
FBS- fetal bovine serum
FSC - forward scatter
FITC - fluorescence isothiocyanate
GP - glycoprotein
HBSS - hank's balanced salt solution
HRP - horseradish peroxidase
LDV - lactate dehydrogenase-elevating virus
LN- lymph nodes
MHC - major histocompatibility complex
MLV - modified live vaccines
MOI - multiplicity of infection
NA - neutralizing antibodies
NK - natural killer
OAS - oligoadenylate synthetase
ORF - open reading frame
PADRE - pan-DR helper T cell epitope
PAM - porcine alveolar macrophages
PBMC - peripheral blood mononuclear cells
PCR - polymerase chain reaction
PEG - polyethylene glycol
PIM - pulmonary intravascular macrophages
PKR - double-stranded RNA-dependent protein kinase
Poly-ICLC - polylysine and carboxymethylcellulose
PRDC - porcine respiratory disease complex
PRRSV- porcine reproductive and respiratory syndrome virus
PVDF - polyvinylidene fluoride
RNase L - ribonuclease L
RT - reverse transcriptase
SDS - sodium dodecyl sulphate
SHFV - simian hemorrhagic fever virus
SI - stimulation index
SIV - simian immunodeficiency virus
SPF - specific pathogen free
SSC - side scatter
TCR - T cell receptor
TRS - transcriptional regulatory sequence

1

Literature Review

Porcine Reproductive and Respiratory Syndrome Virus

Porcine reproductive and respiratory syndrome virus (PRRSV) was first detected in North America in 1987 and continues to be major problem to the swine industry worldwide (1). PRRSV belongs to a member of the family *Arteriviridae* which includes equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) of mice, and simian hemorrhagic fever virus (SHFV) (2-4). PRRSV isolates can be divided into the European and the North American serotypes, which are genetically and antigenically different (5, 6). PRRS is known to induce reproductive failure in sows and respiratory distress in piglets and growing pigs (7). Although adult pigs usually show only mild signs of respiratory disease, the appearance of secondary infections suggests PRRSV can induce immunosuppression. Initially, prior to identification of the agent that causes PRRS, the syndrome was referred to as Mystery Swine Disease (8, 9). According to the report of USDA's National Animal Health Monitoring System in 2002, the percentages of PRRS in breeding females, nursery-age pigs, and grower/ finisher pigs were 21.4, 17.5, and 16.6 % of herd sites, respectively (Table 1), demonstrating that PRRS was the second most common disease in breeding females (10). These health problems have led to tremendous economic losses in the swine industry, which are estimated to cost approximately 560 million dollars annually in the USA (11). This amount is much larger than other economically important viral diseases of swine. For example, annual losses attributable to classical swine fever and pseudorabies were estimated at 364.09 million and 36.27 million dollars, respectively (12). Despite tremendous effort to control PRRSV infections, current strategies are not highly successful and therefore PRRS-associated losses will continue into the future. The inactivated or attenuated PRRS viruses used for a vaccine have provided limit in protecting pigs against PRRSV (13, 14). Moreover, severe forms of

PRRS have been reported as an atypical and acute PRRS has been identified in vaccinated herds since late 1996 suggesting that the vaccine strain can revert to virulent strain (15, 16). Beside insufficient vaccines, several characteristics of PRRSV should be considered as obstacles for effective prevention and eradication of PRRS. First, macrophage tropism can weaken the immunity of the exposed pigs. Second, the remarkable variation of field isolates can evade the immune system of pigs. Third, the poor host immunity caused by PRRSV can result in the ineffective elimination of the virus and the persistent infection in pigs for prolonged periods of time. Lastly, persistence can lead to the continuous circulation of the virus in a herd by transmission to naïve pigs. Therefore, alternative vaccine systems against PRRSV are necessary and will only likely appear through fundamental research to understand the biology and the mechanism of pathogenesis of PRRSV.

Molecular biology of PRRSV

PRRSV is an enveloped virus of 50-70 nm diameters. The virion of PRRSV consists of three morphologically distinct structural components (Figure 1). First is a linear single and positive-stranded RNA genome located in the central core. Second is an icosahedral nucleocapsid enclosing the viral genome. The third structure of the virus is an envelope which is a lipid membrane containing several viral glycoproteins and the matrix protein (17).

Full-length genomic sequences have been determined for several stains of PRRSV. The genome of PRRSV is approximately 15 kb in size and consists of 8 open reading frames (ORFs) flanked by 5′ and 3′ non-coding regions, and followed by a methyl-cap structure at its 5′ end and a poly (A) tail at its 3′ end (Figure 1) (17). In addition, a small internal ORF is found within ORF2 of PRRSV (18). The first two overlapping ORFs (ORF1a and ORF1b)

are located at the 5′ end of genome, comprising around 75% of viral genome and encode the non-structure proteins that are involved in viral replication (19, 20). ORFs 2-7 are located at the 3′ end of genome, and encode the structural proteins associated with the virion (19, 21).

Both ORF 1a and b are expressed from genomic RNA. The downstream ORFs encoding the structural proteins are translated from a set of 3′ co-terminal subgenomic mRNAs (sg mRNAs) (22). The sg mRNAs are produced through discontinuous mRNA transcription. A common leader sequence derived from the 5' end of the genomic RNA is joined to the 3' terminal body sequence which is, therefore, non-continuous in the genome (23). The connection between the leader and the body of sg mRNAs is formed by consensus "transcriptional regulatory sequence" (TRS) which is the key control element for structural gene expression. The sg mRNAs share their 3′ end, but only the first ORF at the 5′ end of each transcript is translated using TRS as a promoter. Recently, PRRSV defective RNAs, termed heteroclite sg mRNAs that have different size and sequence in 5'-3' junction, have been detected in some infected cells. Although the role of heteroclite sg mRNAs has not been determined, they have been implicated as important in PRRSV RNA package signal and to establish PRRSV persistence (24).

The products of each ORF and the relative locations of the viral proteins are illustrated in Figure 1. The products of ORF1 are found in virus-infected cells but not virions, and therefore are considered to be nonstructural proteins which function as a replicase complex. The purified virions of PRRSV are composed of seven proteins including four envelope glycoproteins, named GP2a (encoded by ORF2a), GP3 (ORF3), GP4 (ORF4), and GP5 (ORF5); non-glycosylated membrane protein M (ORF6); nucleocapsid protein N (ORF7); and non-glycosylated envelope protein E (ORF2b).

Major structural protein components in virions are N (14–15 kDa), M (18–19 kDa), and GP5 (24–26 kDa) proteins (21, 25). The N protein is highly antigenic and is the most abundant protein in virions, and has been useful for the diagnosis of PRRS (26-28). N protein bearing a RNA binding domain is suggested to play a role in viral replication (25). The GP5 is the most variable protein among PRRSV isolates, with the largest variation observed in the N-terminus. Despite these differences, the hydropathy profiles of the GP5 proteins of PRRSV isolates are similar (29). The N-terminus of the GP5 contains a predicted signal peptide, which is followed by a hydrophilic domain. This domain is presumed to be exposed on the outside of the virion and is therefore designated the ectodomain. This ectodomain contains two to four potential *N*-linked glycosylation sites. The ectodomain is followed by a hydrophobic region that is presumed to span the membrane either one or three times. The last C-terminal amino acids are thought to form the endodomain. The GP5 is presumed to play a role in attachment to host-cell receptors (30, 31). The GP5 and M proteins are closely associated and produced in heterodimeric complexes linked by disulfide bonds in virion particles (32). It has been suggested that formation of this heterodimer is necessary for PRRSV assembly because no particles are released when either the GP5 or M protein is absent (33, 34). The breaking of dimer structure between the GP5 and M proteins does not, however, reduce viral infectivity unlike LDV and EAV (33, 34). The M protein itself has been suggested to be critical for cellular immunity to PRRSV since it induces the strongest response of all PRRSV structural proteins in antigen-specific proliferation responses (35).

Minor structural protein components in PRRSV virions are GP2 (29–30 kDa), GP3 (41–50 kDa), and GP4 (31–35 kDa) proteins. The small envelope E protein (10 kDa) is

newly identified as another structural component (36). The GP2, GP3, and GP4 proteins are postulated to interact with each other and to be assembled into virions as a multimeric complex (33, 34). This complex structure was suggested to function in viral entry because the virus particles lacking the minor envelope proteins are not infectious (34). Lee *et al* (37) proposed that the E protein may form an ion-channel by becoming embedded in the viral envelope with other minor envelope proteins and facilitate uncoating of virus and releasing of genome in cytoplasm. The presence of the highly glycosylated GP3 protein in PRRSV particles is controversial, because the GP3 protein of European-type strains was found to be incorporated into virions, whereas the GP3 protein of North American strains was a soluble and secreted protein (38).

Pathogenesis of PRRSV

PRRSV spreads quickly in naïve swine populations; up to 95% of a herd became seropositive within 2-3 months after introduction of the virus (1). Direct contact mediated by body fluids between infected and naïve pigs is thought as a major route of PRRSV transmission, because PRRSV can be isolated from serum, semen, saliva, feces, urine, nasal swabs, and or oropharyngeal scrapings of infected pigs (39, 40). Additionally, aerosol exposure over short distance may be an infrequent but significant route of PRRSV transmission to naïve groups of pigs (41, 42). Lastly, flies appear to become contaminated with PRRSV from infected pigs and transport the virus suggesting a mechanism for PRRSV dispersal between farms (43).

PRRSV has a very selective tropism for monocyte/macrophage lineage cells such as porcine alveolar macrophages (PAMs), peripheral blood monocytes, and pulmonary

intravascular macrophages (PIMs) (44, 45). PRRSV can also replicate in established cell lines, including African green monkey kidney cells (MA-104) and their highly permissive clone MARC-145 (46). Although all the stages of PRRSV infection have not been clearly identified, it is predicted that viral particles precede through a multiple-step cycle including adsorption, penetration, uncoating, transcription, translation, replication, assembly, and release (47-49). PRRSV enters into a target cells by receptor-mediated endocytosis (50, 51). Uncoating occurs in endosomes where an acidic environment triggers the fusion between the viral envelop and endosomal membranes (50). In the process of internalization, the lipid envelope is shed and the viral capsid is disassembled to release the genome to initiate the replication cycle (47, 52). Each gene is expressed through sg mRNA as explained above. The membrane-associated proteins accumulate in the endoplasmic reticulum (ER) forming matrix-envelope complexes (32, 47). These complexes obtain envelopes by budding off the membrane of the smooth ER (53). PRRSV virions are assembled at the ER and maturation completed in the Golgi where glycosylation occurs (47). Progeny viruses are thought to be released from the cell initially by exocytosis and eventually by cell lysis (32, 47).

After infection and replication in the lung, PRRSV is transported to regional lymph nodes (LNs) where antigen presentation occurs and the virus can replicate again. Some PRRSV can escape the LN replicate in circulating monocyte or macrophage populations. Therefore, the first 2 weeks of infection, are considered as the ‘acute stage’ during which maximal viral titers can be recovered from all susceptible organs throughout the body. PRRSV is cleared from the circulatory system within 1 to 4 weeks. The period designated as the ‘persistent stage’ is characterized by no detectable viremia and lower levels of virus

replication within limited anatomical locations such as tonsil, spleen, and lymphoid tissues for several weeks to months (54).

Clinical presentation following PRRSV infection of naïve animals depends on the age of infected pigs and the pregnancy stage of infected sows. Prior studies indicate that young pigs (≤ 8 weeks old) are more susceptible to infection, produce higher titers of the virus, and excrete the virus at higher concentrations than older pigs (~ 6 months old) (55). The intensity of the disease appears to depend on the PRRSV isolate, suggesting PRRS viruses vary in virulence (56, 57). Studies found that pigs experimentally infected with nine different isolates of PRRSV had major differences in clinical disease, rectal temperatures, and gross and histological lung lesions (57). Reproductive failure has been demonstrated to occur predominantly in late term gestation sows resulting in transplacental infection. As a consequence of viral placentitis and transplacental transmission, sows often farrow prematurely with stillborn fetuses, partially autolyzed fetuses, and mummified fetuses as well as weak-born live viremic piglet (7). Clinical signs in infected sows or gilts vary from none to anorexia, fever, lethargy, rapid respirations, pneumonia, agalactia, red/blue discoloration of the ears and vulva, subcutaneous and hindlimb edema, a postweaning delayed return to estrus, and, less commonly, death (47). Clinical signs in PRRSV-infected neonatal pigs vary markedly in frequency and severity, with dyspnea and tachypnea being the most characteristic clinical signs (47). Mortality in neonatal pigs infected with PRRSV may approach 100% (47). PRRSV infection in weaned pigs is characterized by fever, pneumonia, lethargy, failure to thrive. A marked increase in mortality from single to multiple concurrent bacterial infections is commonly seen that also cause both the predominant clinical signs observed (54). PRRSV is considered as one of the most important etiological agent in multi-

factorial respiratory disease of swine. This phenomenon was firstly reported by Halbur et al., as Porcine Respiratory Disease Complex (PRDC); "Respiratory problems and poor growth in growing and finishing pigs with a multi-factorial etiology involving PRRSV, SIV, PRCV and a variety of bacteria" (58). Pathogens such as *Mycoplasma hyopneμMoniae* have been shown to increase the duration and severity of PRRSV-induced pneumonia and viremia (59). Dual infection of PRRSV with *streptococcus suis* was also found to increase mortality (60). PRRSV infection in finishing pigs, boars, and inbred gilts or sows is commonly characterized only by a transient fever and inappetence (39). PRRSV-infected boars demonstrate a loss of libido, while most appear clinically unaffected (61). The sequential events in pigs after PRRSV infection are summarized in Figure 2 (47).

Immune response to PRRSV infection

The immune response against PRRSV is characterized by the delay in the induction of protective humoral and cellular immune responses, which results in a persistent infection (Figure 3) (62). However, the mechanism underlying this affect is still largely unknown. Despite of intensive studies of convalescent animals and post-vaccination protection, little is known about the protective immune components against PRRSV infection in swine.

Innate immune responses Innate immunity is important to control the initial virus replication and spread via anti-viral activity, and to induce the adaptive immune response, even though it is neither a memory nor an antigen-specific response. The innate response to viruses is characterized by the release of antiviral and proinflammatory cytokines from the virus infected cells and the innate immune cells, but results with PRRSV show inherently weak innate immune responses. Therefore, it is possible that the weak innate response leads

to the suboptimal stimulation of the antigen-specific immune response and allows the persistent infection.

Proinflammatory cytokines such as IFN-α, IL-1, and TNF-α have been demonstrated as important mediators of viral respiratory diseases such as simian immunodeficiency virus (SIV) (63). Likewise, EAV-infected macrophages produce proinflammatory cytokines including IL-1β, IL-6, and IL-8 (64). In contrast, PRRSV fails to induce significant expression of inflammatory cytokines in macrophages (64).

Double stranded (ds) RNA, as a pathogen-associated molecular pattern, can be critical for the induction of type I interferon by infected cells (65). Virus-induced IFN-α/β activates several proteins including 2'5-oligoadenylate synthetase (OAS), double-stranded RNA-dependent protein kinase (PKR), and ribonuclease L (RNase L) and these molecules produce an anti-viral state by suppressing viral protein synthesis and degrading viral nucleic acid (66). Though PRRSV mRNA appears to have the necessary ds RNA structure, unprimed or resting PRRSV-infected macrophages do not degrade PRRSV nor inhibit synthesis of viral protein. Recombinant porcine IFN-α has been shown to inhibit the growth of PRRSV in alveolar macrophage cultures, but IFN-α was not detected in either alveolar fluid from virus infected pigs, or in the virus infected alveolar macrophage or peripheral blood mononuclear cell (PBMC) cultures (67, 68).

Natural killer (NK) cells are important in the innate cellular response to viral infection. They are activated by IFN-α/β secreted by virus infected cells, then produce cytotoxic proteins, and finally kill the virus infected cells (69). Swine possess a high proportion of NK cells, and the strong influx of NK cells ($CD3^-$, $CD8^+$ and $CD6^-$) is observed

in the lung during PRRSV infection (70). However, it is unclear if these NK cells kill PRRSV-infected cells and participate in the ant-PRRSV immune response (9,161).

Humoral immune responses Antibodies act against free virus or virus-infected cells, mediating several antiviral activities. Virus specific antibodies induce direct virus neutralization by binding to free virus. In addition, antibodies can initiate lysis of virus infected cells through the classical complement pathway or by antibody-dependent, cell-mediated cytotoxicity leading to a reduction in viral load (71).

During the course of disease, antibody responses are elicited against PRRSV infection. Anti-PRRSV IgM Abs can be detected in serum at 5-7 dpi (day post infection) and then decline quickly to be undetectable after 2-3 weeks. Anti-PRRSV IgG Abs are first detectable at 7-10 dpi, peak at 2-4 weeks post infection (PI), remain constant level for a period of months, and then decline to low levels (72).

The antibodies produced initially against PRRSV are non-neutralizing, and neutralizing antibodies (NAs) do not appear until 4 weeks PI and even then they can be detected only at low titers (2, 3). The primary neutralization determinants of PRRSV have been found to be located in the N-terminal portion of the GP5 (73, 74). Passive transfer of PRRSV hyperimmune serum fully protected pregnant sows against challenge with virulent PRRSV (75). However, NA generated by PRRSV infection are thought not to play a role in resistance since they develop slowly, at low levels, and viremia can occur in the presence of NAs (62).

Several mechanisms to account for inefficient production of neutralizing activity have been proposed. Selection of neutralization escape mutants might be one mechanism, but such mutants have not yet been observed. Glycosylation of neutralization epitopes may

play a role, since loss of glycan residues in the ectodomain of GP5 enhanced both the sensitivity of these viruses to *in vitro* neutralization and the immunogenicity of a nearby neutralization epitope (76). It is also possible that reduced neutralization and the prolonged duration of viremia might be associated with the phenomenon of antibody-dependent enhancement (ADE) (77). In ADE, low titers of NAs can increase the virus infectivity by facilitating uptake of the virus into permissive macrophages through binding of virus-antibody complex to the Fc receptor (78). Another explanation for the lack of NA to PRRSV concerns the presence of decoy epitopes. Ostrowski *et al* identified a non-neutralizing epitope (aa 27-30, epitope A) which is located near the neutralizing epitope (aa 37-45, epitope B) in the ectodomain of the GP5 protein (74). In fact, PRRSV-infected pigs first develop antibodies against epitope A and neutralizing antibodies against epitope B appear later suggesting that epitope A can impede and diminish humoral immunity against PRRSV (74, 79). Liurong *et al* tested whether enhanced NA develops when the decoy effect is minimized or eliminated using the modified GP5 of PRRSV by inserting a pan-DR helper T cell epitope (PADRE) between the neutralizing and decoy epitopes in order to allow efficient display of the neutralizing epitope (80). They demonstrated that the DNA vaccine encoding modified GP5 induced a significantly higher level of NA compared to naïve GP5 in vaccinated mice. Lastly, the role of PRRSV-specific antibodies in eliminating PRRSV-infected cells may be limited because viral proteins are not efficiently incorporated into the plasma membrane of PRRSV-infected macrophages, which make infected cells invisible to PRRSV-specific antibodies (81).

Cell-mediated immune responses T cell protective immunity to viral infections starts with an initial recognition of short antigenic peptides presented on the major-

histocompatibility complex (MHC), which are expressed on the surface of professional antigen presenting cells (APC). T cells are then stimulated to proliferate and develop more effective functions by the signals provided by the APCs. There are two T cell subtypes identified by $CD8^+$ or $CD4^+$ surface proteins. In addition to the known subpopulations, $CD4^+CD8^-$ T helper cells and $CD4^-CD8^+$ cytolytic T lymphocytes, extra-thymic $CD4^+CD8^+$ T lymphocytes lacking the thymus-specific CD1 antigen and a substantial proportion of $\gamma\delta^+$ T cells can be detectable in swine (82, 83). Functional analysis of porcine lymphocytes revealed that $CD4^+CD8^+$ T cells belong to the antigen-specific memory T-helper cell population (84). With regard to cytolytic activity, two subsets within the $CD4^-CD8^+$ T cells can be defined by expression of CD6. $CD6^-$ cells have spontaneous cytolytic activity and $CD6^+$ cells are MHC I-restricted cytolytic T cells (82). The porcine $\gamma\delta^+$ T cells have similar phenotype as APC and present soluble antigen to $CD4^+$ T cells via MHC class II (83).

Inadequate production of neutralizing antibody indicates a potential reliance on cell-mediated immunity to protect against PRRSV, however, cellular immunity has not been as well investigated. *In vitro*, PRRSV-specific lymphocyte proliferation can be detected at 4 weeks PI, peaks at 7 weeks PI and responses remain detectable for more than 3 months (85). Loperz-Fuertes *et al* reported that virus-specific proliferation is mainly mediated by $CD4^+$ cells by showing that addition of anti-CD4 antibody into the cultures blocked proliferative responses (86). Regarding the expression of CD markers of PBMC, numbers of $CD8^+$ and $CD4^+CD8^+$ PRRSV-specific T cells increase, whereas $CD4^+CD8^-$ cell numbers decline (86). The $CD4^+CD8^+$ lymphocytes of the porcine immune system have been considered to be associated with immunological memory due to their gradual increase with age, the expression of memory phenotype, and their ability to mount to recall response to viral

antigens (87, 88). The majority of CD8 cells activated by PRRSV indicated pigs expressed $\gamma\delta^+$ T cell receptors (86). Porcine $\gamma\delta^+$ T cells expressing CD3 and CD8 were shown to have non-specific constitutive natural killer activity (89). *In vivo*, DTH responses against PRRS viral antigens have also been detected, indicating that T helper cells may contribute protective immunity against PRRSV (85).

Host IFN-γ production is major mechanism of immune responses against various virus infection. In the case of pseudorabies virus vaccine, protective immunity is positively correlated with the generation of virus-specific IFN-γ producing cells rather than that of neutralizing antibody (9). IFN-γ is mainly produced by activated NK and T cells and plays a key role in cell-mediated immune responses (1-3). This cytokine plays a role in the development of Th1 cells from naive T helper cells, the generation of antigen specific memory T cells, and the production of an antiviral state in uninfected cells and tissues (90). It can also enhance MHC class I and II expression, nitric oxide production, and oxygen radical formation from APC. In this regard, the role of IFN-γ plays a role in protection against PRRSV in pigs was investigated. *In vivo*, IFN-γ mRNA has been detected in the lymph nodes, lungs and PBMCs of PRRSV infected pig (86, 91). *In vitro*, addition of IFN-γ blocks PRRSV replication by the inhibition of viral RNA synthesis with dsRNA inducible protein kinase (92). However, *in vivo* studies suggest that the level of the IFN-γ response is too weak to protect the pigs infected with PRRSV (93).

Control of PRRSV infection

Most herds infected with PRRSV suffer some level of loss in productivity. For this reason, prevention and control has been a topic of extreme interest. Vaccination has been the

major focus of attempts to control the spread of this virus. A limited number of PRRSV vaccines, which are either killed vaccines (CyBlue, Laboritorios Sobrino) or modified live vaccines (MLV) (RespPRRS/Repro™, Boehringer Ingelheim Inc; PrimePac PRRS, Schering-Plough Animal Health), are commercially available to protect pigs against PRRSV infection. In comparison with the killed vaccines, which only provide limited protection against PRRSV infection, the MLV induce longer and more effective immunity and shorten viremia in pigs (94-96). Therefore MLV is preferentially used for immunization of swine against PRRSV. However, while MLV have been shown to protect against clinical disease, they did not provide protection when pigs were challenged with a heterologous strain of the virus (13). The use of MLV in boars resulted in reduced or no shedding of wild-type PRRSV after challenge but reduced semen quality also resulted (97).

MLV have also been associated with introduction of pathogenic PRRS (15, 98), due to the intrinsic risk for reversion of MLV to a virulent phenotype. In general, RNA viruses have high rates of mutation resulting from imprecise replication of the genome due to a lack of proofreading by RNA replication enzyme (99). This replication infidelity results in a virus population having non-identical sequences called quasispecies (100, 101). Quasispecies evolution of PRRSV in response to positive or negative selection pressures may significantly change the genomic sequence of MLVs over time as the vaccine virus spreads among swine herds. Ultimately these genetic changes may revert MLVs to virulent phenotypes. It is also possible that virulent strains of PRRSV can be generated through RNA recombination between MLVs used in the vaccination and enzootic field strains of PRRSV. In experimental tests, using a restriction-site marker located in the vaccine virus, the restriction-site marker was detected in most field strains isolated after introduction of the vaccine, suggesting that

they are descendants of the vaccine virus (102). Moreover, genetic analysis indicated that the sequences of PRRSV isolates from pigs immunized with MLV were closely related to those of the vaccine virus (98). These data support the theory that attenuated PRRSV vaccine viruses can mutate during replication in swine and swine-passaged vaccine viruses have a substantial chance to regain virulence.

PRRSV isolates differ in virulence and are genetically and antigenically very heterogeneous (5, 13, 29, 57, 103, 104). Antigenic diversity observed among PRRSV strains is thought to interfere with effective control against antigenically different enzootic field strains of PRRSV. In fact, gilts inoculated with one strain of PRRSV are not completely protected against heterologous challenge (105). Serological differences among antigenically distinct PRRSV strains partially explain the incomplete immune protection against heterologous challenge (106, 107). Antigenic differences have been reported not only between the U.S. and European type isolates, but also within same genotype group (106-108). Antigenic variation is also found between an isolate and its progeny recovered after *in vivo* passage (109). In addition, it has been observed that more than one genotype or phenotype co-exists in the field at the same time (110, 111). The driving source for the high rate of variability among PRRSV isolates remains unknown. Neither accumulation of random neutral mutations or their recombination could explain the substantial nucleotide differences among PRRSVs (112-114). This antigenic diversity makes it difficult to dissect precise antigenic determinants (115).

A new generation of vaccines against PRRSV has been developed which emphasizes the following properties. Immunization should reduce clinical symptoms caused by a wild-type PRRSV infection. The vaccine itself should not cause disease. The vaccine, if a mutated

virus, should be stable and not revert to virulence. Finally the vaccine should prevent transmission of wild-type PRRSV. Many vaccines against PRRSV have been generated using recombinant DNA technology which has made it possible to manipulate viral genomes and to introduce specific modifications into target sequences. DNA immunizations, containing viral gene sequences, have been tested *in vivo* and show strong expression of viral proteins (116, 117). For example, DNA vaccination of pigs with ORFs 1-7 induced specific antibody responses against several viral proteins, however, no obvious positive effects of vaccination were observed with any of the ORFs following subsequent challenge with viable PRRSV (118). Tian *et al.* generated recombinant pseudorabies virus harboring GP5 gene of PRRSV and found that immunization with this recombinant virus induced an anamnestic immune response to PRRSV in inoculated pigs (115). Recently Jiang et al found that recombinant pseudorabies virus expressing the two major membrane-associated proteins (GP5 and M) of PRRSV induced more efficient protection against a PRRSV challenge compared with virus expressing either GP5 or M (119). This vaccine reduced body-temperature fluctuations, shortened viremia, lowered virus load in nasal and oropharyngeal scrapings and tissues, and produced mild lung lesions. Wenming *et al* also demonstrated that co-expression of the M and GP5 proteins in an adenovirus expression system enhanced the induction of neutralizing antibodies and cellular immune response against PRRSV (120). In an attempt to induce a highly robust immune response against PRRSV, various immunomodulating agents have also been tested. For instance, exogenous recombinant IL-12 was reported to enhance cellular immunity to PRRSV both *in vivo* and *in vitro* (121). IFN-α has also been shown to enhance anti-PRRSV immunity (122). Finally, Wasin *et al* evaluated the efficacy of an MLV plus various adjuvants and cytokines, including bacterial endotoxin,

porcine IFN-α, polyinosinic-polycyidylic acid stabilized with polylysine and carboxymethylcellulose, and porcine IL-12 (123). They found that a mixture of GP5 and IL-12 effectively enhanced cellular immunity when compared to MLV alone.

Significance of study

Even after 20 years of research on PRRSV, scientists are unable to conclusively determine the mechanisms of protective immunity against this virus. Our lack of understanding of this disease has hindered the pursuit for the cure of PRRS disease. The virus seems to suppress the immune response at the early critical time points when immunity is established. Therefore studies are needed to investigate the immune response to PRRSV as well as to identify the mechanisms used by the virus interferes with host defense. The goal of the present study is to characterize the specific immune response elicited in pigs experimentally infected with a North Carolina isolate of PRRSV known as the Powell strain or those immunized with recombinant GP5 of PRRSV derived from the Powell strain which has previously been shown to provide protection against disease. The GP5 is known as one of the key immunogenic proteins of PRRSV and is considered to be the leading target for a new generation of vaccines against PRRSV. The results of these studies should improve our understanding of this disease and help with the development of new and improved vaccines.

The research was undertaken with the following specific aims:

- **Specific aim 1.** To generate recombinant GP5 protein of PRRSV using a baculovirus expression system.
- **Specific aim 2**. To characterize the antibody response against the GP5 subunit vaccine and compare it to responses in PRRS virus-infected pigs.
- **Specific aim 3.** To identify the specific domain in the GP5 protein associated with positive antibody responses.
- **Specific aim 4**. To characterize the antigen-specific T cell proliferation responses in GP5 subunit vaccinated and PRRS virus-infected animals.
- **Specific aim 5**. To identify the phenotype of T cells responding *in vitro* antigen stimulation in GP5 subunit vaccinated and PRRS virus-infected animals.

Table 1. The diseases reported most commonly in breeding females, nursery-age and grow/ finisher pigs.

Breeding females	Nursery-age pigs	Grow/ finisher pigs
Roundworm (40.8)	*Streptoccus suis* Meningitis (31.6)	Ileitis (36.9)
PRRS (21.4)	Greasy Pig Disease (25.3)	Mycoplasma Pneumonia (29)
Mycoplasma (14.2)	*E.Coli* Diarrhea (24)	Roundworm (19.6)
Swine Flu (H1N1) (11.2)	Mycoplasma Pneumonia (19.6)	Gastric ulcers (19.3)
Gastric ulcers (10.7)	Roundworm (18)	Hemorrhagic Bowel Syndrome (18.4)
Erysipelas (6.8)	**PRRS** (17.5)	**PRRS** (16.6)
Swine Flu (H3N2) (5.3)	Glasser's Disease (7.3)	Atrophic Rhinitis (14)
Parovirus (4.4)	Swine Flu (H1N1) (7.2)	Swine Flu (H1N1) (12.3)
Glasser's disease (3.7)	Salmonella (6.6)	Salmonella (8.4)
APP*** (3.4)	APP*** (6.4)	APP*** (8.1)

* The numbers indicate the site percentile which data was collected from the 94% of the United States swine herd operations with 100 or more pigs in 2000.

** These data were derived from *USDA, Swine 2002 Part II: Reference of swine health and health management in the United States, 2000* and the swine 2006 study will update the information and the results will be published in 2007.

*** APP is *Actinobacillus pleuropneumoniae, Haemophilus.*

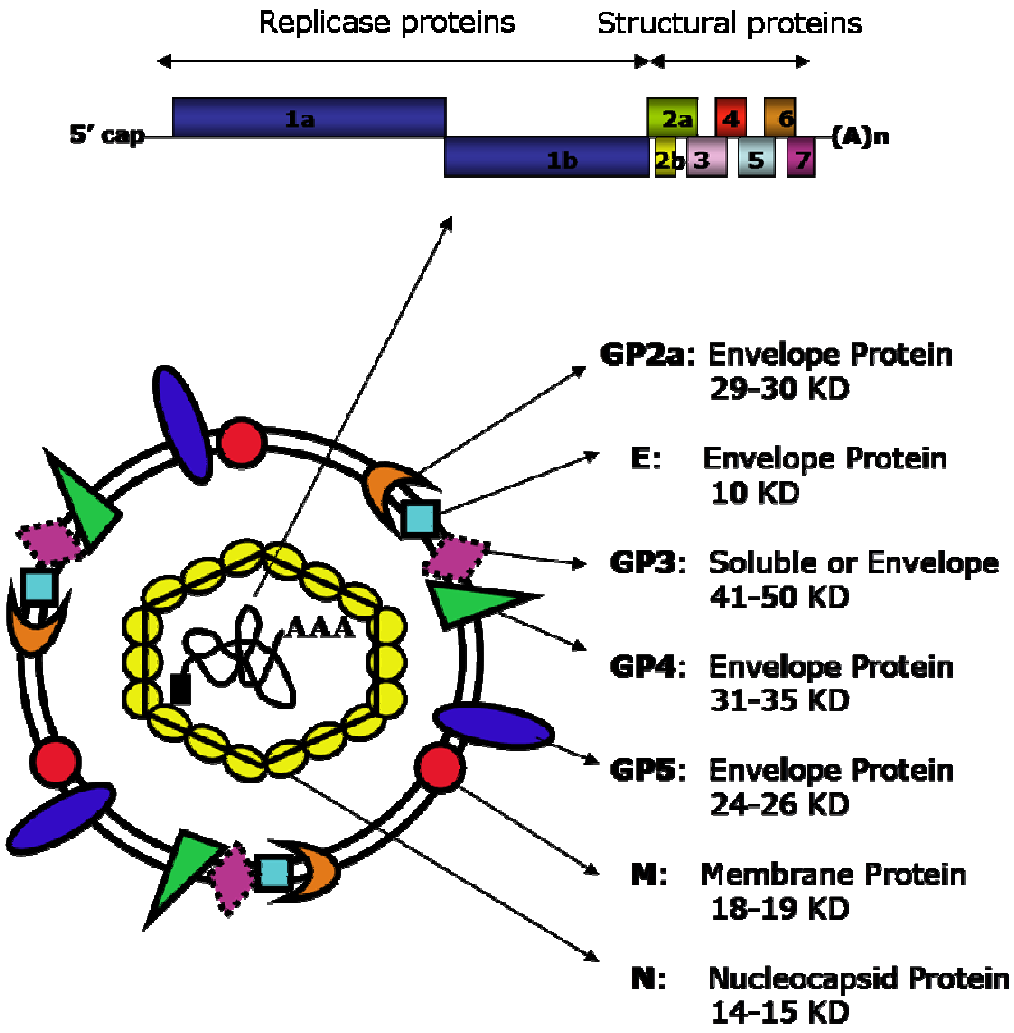

Figure 1. PRRS Virion. PRRSV is a small enveloped virus, 50-70 nm in diameter. PRRSV consists of a linear single strand RNA, 15 kb in size and an isomeric nucleocapsid, 35 nm in diameter, surrounded by a closely adherent envelope. The genome of PRRRSV encodes non-structural proteins such as replicase proteins at 5' end and structural proteins at 3' end. The location of the relative viral proteins is indicated.

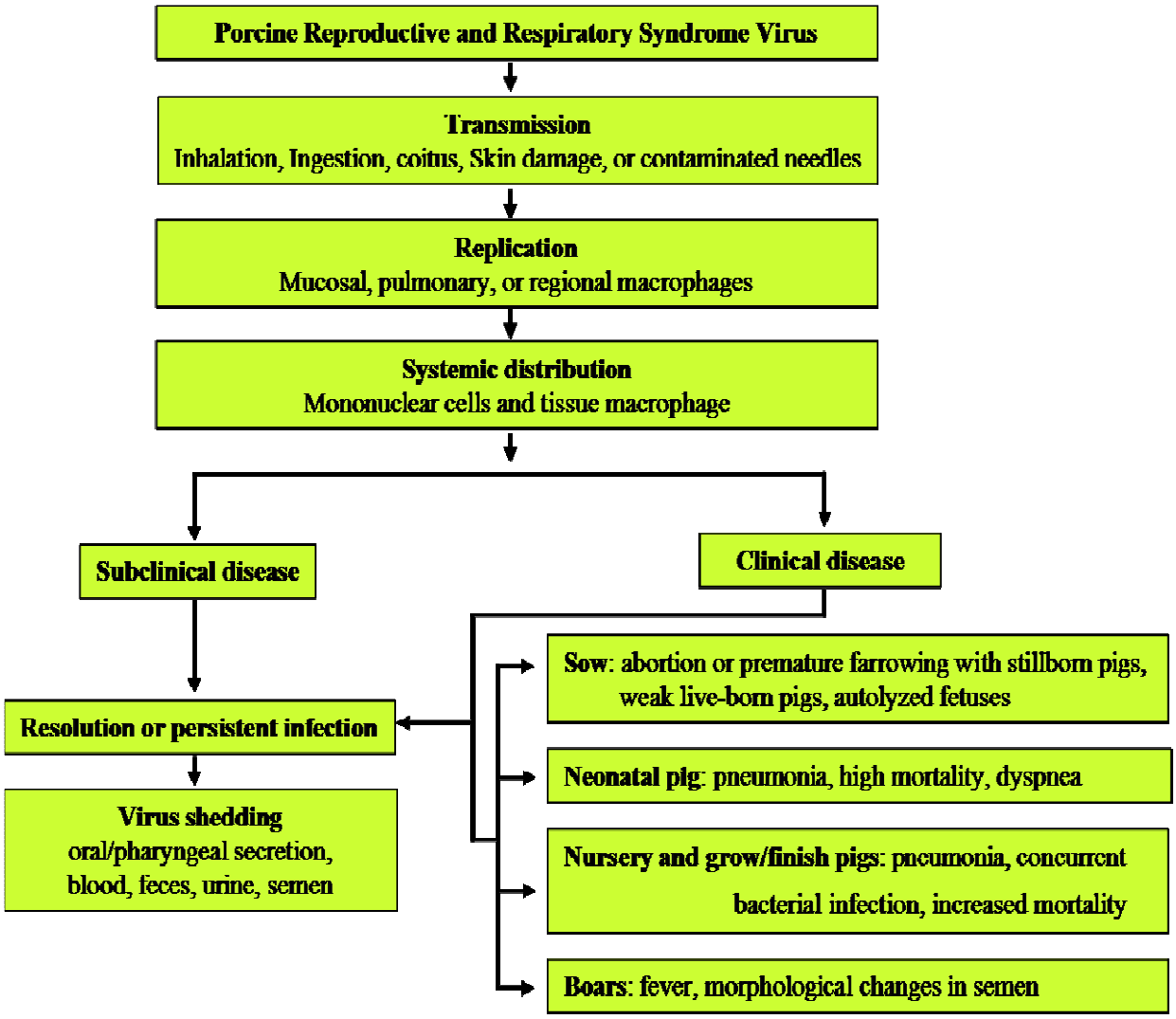

Figure 2. Pathogenesis of PRRSV. PRRSV is transmitted by direct contact or indirect aerosolization over short distances. Initial replication in macrophages from lungs and regional lymph nodes is followed by viremia and systemic distribution of virus to other macrophage populations. Virus spread may result in clinical or subclinical disease. The clinical symptoms are usually revealed in young pigs and pregnant sows. Derived from Rossow, *Vet. Pathol*. 35:1-20 (1998).

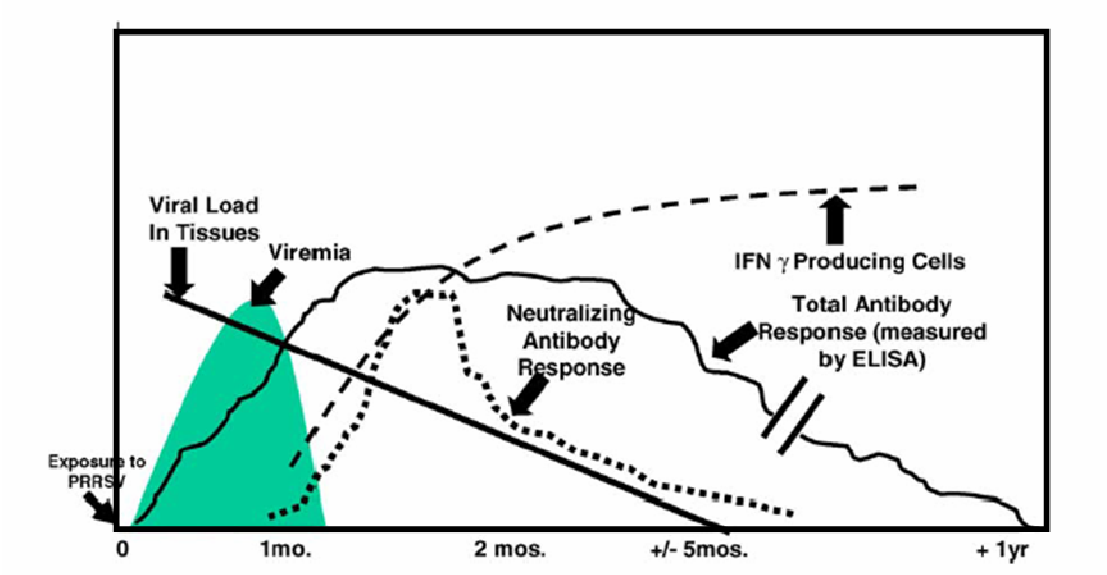

Figure 3. Infection of PRRSV and subsequent events. In the early post-infection period, virus replicates in target cells, typically macrophages and spreads to blood and tissues. The acute stage is representative with high viral load in tissues and serum associated viremia which may last up to 1 month. In late infection phase, virus persists at lower load level in several organs but is eventually cleared from the body after several months post-infection. Infected pigs develop immune response against PRRSV infection. Antibodies are generated in the early post infection period, but they do not neutralize PRRSV. Neutralizing antibody responses appears only at later post infection times. IFN-γ producing T cells as indicators of T cell cytotoxicity show a delay in the response as neutralizing antibodies do. Derived from O. J. Loperz *et al., Veterinary immunology and immuopathology* 10:155-163 (2004).

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2

Antibody and Cellular Immune Responses of Swine Exposed to Porcine Reproductive and Respiratory Syndrome Virus or a GP5 Subunit Vaccine

ABSTRACT

Developing effective vaccines against the porcine reproductive and respiratory syndrome virus (PRRSV) has proved difficult, highlighting the need for basic information on the nature of the immune response against this virus and the mechanisms of resistance that the virus employs. In this investigation our goal was to characterize the immune response against the major outer membrane protein of the virus, GP5, in pigs experimentally infected with a North Carolina isolate of PRRSV known as the NC Powell strain. In addition, we compared this response with the immune response seen after vaccination with purified recombinant GP5 (rGP5) protein. Humoral immune responses were monitored by western blot and immunofluorescence while T cell responses were monitored using proliferation assays and flow cytometry. Our results show strong humoral recognition of rGP5 protein during both natural and vaccine induced Antibody responses. In addition, epitope mapping via western blot revealed that Antibody responses were directed largely against the C-terminal endodomain of rGP5 protein in both experimentally infected and vaccinated pigs. We also investigated T cell responses to rGP5 protein. Our experiments revealed that T cells from vaccinated animals also responded to both rGP5 protein and inactivated NC Powell strain of PRRSV suggesting that T cells may play an important role in vaccine-induced resistance. Interestingly, we found that the inactivated NC Powell strain of PRRSV caused a strong proliferative response in naïve T cells from control animals, perhaps indicating the presence of a superantigen as a component of this highly virulent strain of PRRSV.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, single-stranded positive sense RNA virus belonging to the family *Arteriviridae* (1). PRRS is characterized by reproductive failure in sows and respiratory complications in pigs of all ages (2). The disease tends to be more severe in young pigs, although adult pigs are not safe from this disease as the virus is often associated with recurrent of secondary infections (3, 4). PRRSV is also thought to have an important role in multi-factorial respiratory disease (5, 6). PRRSV exerts a major impact on the swine industry worldwide and has been a major focus for research for the last two decades (7).

PRRSV infects swine predominantly through the mucosal surface of the respiratory tract (8). PRRSV replication initially occurs in macrophages in lungs and regional lymph nodes followed by viremia and systemic distribution of the virus to additional peripheral macrophage/monocyte cell populations (9). PRRSV tends to form a persistent state, with replication readily detected for well over a month after initial infection (10, 11). Overall, the immune response against PRRSV is characterized by a weak inflammatory or innate immune response and a delay in the induction of the adaptive immune response (6, 12-14). Virus-specific, IFN-γ secreting T cells, remain at low levels for several weeks post-infection and increase only gradually thereafter (15) while the antibody (Ab) response that is induced by PRRSV is comprised predominantly by non-neutralizing Abs (16). Neutralizing Abs (NA), which can confer protection when administered passively in a large bolus, may actually enhance the pathogenicity of the virus under natural conditions (17, 18). In addition, Ab-dependent enhancement (ADE) of virus replication in macrophages, which occurs through Fc

receptor-mediated endocytosis, has been reported for PRRSV (19-21). The mechanisms that cause the weakened and delayed immune response and the viral gene products that produce these effects are not known.

Current strategies to control PRRSV depend on immunization with either modified live or killed vaccines. Immunization has been shown to reduce fever, duration and level of viremia, lung lesions, and transmission but vaccines are not fully protective and generally do not prevent disease (15, 22-27). Outbreaks of atypical or acute PRRS have been reported in immunized herds (28, 29). The failure of PRRSV vaccines may be due to minimal cross-reactivity between vaccine and field strains. Alternatively, vaccine failure may arise from the induction of an ineffective immune response. These situations have raised concern about the efficacy of current vaccines and stimulated research into a new generation of PRRSV vaccines. Recombinant live vector vaccines against PRRSV have been developed using pseudorabies virus, baculovirus, or adenovirus as vectors (30-34). In addition, novel strategies have been created to increase vaccine efficiency. For instance, a combined multi-strain vaccine was developed in an effort to combat the heterogeneity seen in different PRRSV isolates (25). Various adjuvants have also been tested; including, an endotoxin-derived adjuvant, porcine IFN-α, porcine IL-12, or polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose (poly-ICLC) (23, 26).

Our laboratory has previously developed a subunit vaccine for PRRS (35). The structural genes from an acute-type PRRSV were cloned and expressed in baculovirus. Lysates were subjected to SDS-PAGE and the individual proteins purified by electroelution from gel slices. We found that recombinant GP4 and GP5 proteins could induce strong protection when used as vaccines, inhibiting virus replication and the appearance of clinical

symptoms. In current studies, our goal was to further characterize the immune responses directed at the GP5 subunit vaccine and compare them to those following wild type PRRSV infections. We investigated several aspects of anti-GP5 humoral and cellular immunity since it has been previously reported to be associated with the development of neutralizing antibody and protection (32, 33, 36-40). The results of our investigation revealed differences in the T cell response between animals vaccinated with a subunit GP5 and animals infected with the parental wild type virus. Improved vaccines for PRRSV may therefore arise from a better understanding of the mechanisms that control T cell responses to PRRSV proteins.

METERIALS AND METHODS

1. Generation of recombinant proteins

The NC Powell strain of PRRSV was obtained from clinically affected piglets in a North Carolina herd experiencing an acute outbreak of PRRS. The full-length cDNA of the native ORF5 gene of the Powell strain had been cloned previously in our laboratory (35). This DNA was modified and used for expression of GP5 protein. Full-length and sub-domains of NC Powell PRRSV strain GP5 protein were produced using a baculovirus. Bac-to-Bac expression system was purchased from Invitrogen (San Diego, CA). The overall steps are shown in Figure 1. Reagents used in all experiments, unless otherwise indicated, were purchased from Sigma (St. Louis, MO).

1.1 Insect cell culture

The *Spodoptera frugiperda* (Sf9, Invitrogen) cell line was maintained in SF-900 serum-free medium (SFM, Gibco-BRL, Grand Island, NY). The *Trichoplusia ni* (Hi-5, Invitrogen) cell line was maintained in Express Five SFM medium (Gibco-BRL, Grand Island, NY) supplemented with 20 mM L-glutamine. Gentamycin (10 µg/ml) was added to media to avoid contamination. These insect cells were cultured at 27°C.

1.2 Construction of baculovirus entry vector

Baculovirus entry vector was generated by inserting the gene of interest into the pFastBac donor plasmid. The insertion DNA included ORF 5 cDNA with a penta-His tag was prepared by excising the pBlueBac4.5/V5-His/ORF5 using a BamHI and Sal I restriction

enzymes (Promega, Madision, WI). The pBlueBac4.5/V5-His/ORF5 had been constructed previously by ligation of the ORF5 viral cDNA into the TOPO cloning site of the transfer vector pBlueBac (35). The recombinant pFastBac donor plasmid was generated by ligating the insertion DNA into the BamHI-Sal I site on the pFastBac vector, creating recombinant pFastBac-His/ORF5 plasmid (Figure 2). This recombinant plasmid was amplified in Top10 chemically competent *E. coli* (Invitrogen) following the manufacturer's instructions. Insertion was verified by sequencing using the universal pFastBac primers (MCLAB, San Francisco, CA).

pFastBac forward 5' – GGATTATTCATACCGTCCCA - 3'

pFastBac reverse 5' – CAAATGTGGTATGGCTGATT - 3'

1.3 Construction of recombinant baculovirus

The pFastBac-His/ORF5 donor plasmids were transformed into competent DH10Bac *E. coli* cells (Invitrogen) according to the manufacturer's protocols. Transformed cells grew on LB plates with antibiotics (7 µg/ml gentamycin, 10 µg/ml tetracycline, and 50 µg/ml kanamycin). Cells were overlaid with 40 µg/ml X-gal and the inducer 40 µg/ml IPTG (provided from Dr E. Miller, Department of Microbiology, NC State University) for 48 hrs before use. The correct colonies containing recombinant bacmids were identified by color screening. The transposition was confirmed by PCR analysis using M-13 universal primers which was kindly provided by Dr. J. Brown, Department of Microbiology, NC State University.

M13 forward 5' – GTTTTCCCAGTCACGAC - 3'

M13 reverse 5' – CAGGAAACAGCTATGAC - 3'

The recombinant bacmid DNA was transfected into Sf9 cells by a lipid mediated mechanism to produce recombinant baculovirus, which was designated as rBac-GP5. Briefly, the recombinant bacmid DNA was incubated with Cellfectin (Invitrogen) at room temperature for 45 minutes. This transfected mixture was added to the 9×10^5 Sf9 cells prepared in six-well plates and then incubated at 27°C for 5 h. After the transfected mixture was removed, the Sf9 cells were incubated in fresh growth media at 27°C for 72 h, and the culture medium was harvested and designated as P1 viral stock. Since P1 stock was a small-scale and low-titer stock, the viral stock was amplified by infecting Sf9 cells at 0.1 multiplicity of infection (MOI). According to manufacturer's guideline, the titer of P1, P2 and P3 viral stocks are generally accepted as 10^6, 10^7 and 10^8 plaque forming units (pfu)/ml respectively. Viral stocks were stored at 4°C in SFM supplemented with 2% fetal bovine serum (FBS) which acts as substrate for protease and shielded from exposure to light.

1.4 Production and analysis of recombinant protein

For production of recombinant proteins, Hi-5 cells were cultured to density 4.5×10^5 cells in 24 well plates and infected with recombinant baculovirus at 5 MOI for 4 days. After harvesting infected cells, the cells were washed with phosphate buffered saline (PBS), suspended in 400 µl lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and protease inhibitor) and lysed at 4 °C for 1 h. Total cell lysate protein concentration was determined by the BCA protein assay kit (Pierce, Rockford, IL). The expression of recombinant protein was detected by western blot using anti-His Abs (Qiagen, Valercia, CA).

1.5 Purification of recombinant proteins

Purification of sufficient amounts of the recombinant GP5 (rGP5) protein, approximately 10 mg of the lysates were loaded onto large preparative SDS-PAGE gels. The rGP5 protein band corresponding to the predicted molecular weight was excised from the gel. The sealed tubing bag including the sliced gel was put in a tank filled with SDS Running buffer (25 mM Tris, 250 mM Glycine pH 8.3, 1% SDS). The rGP5 protein was electroeluted overnight at 20V and dialyzed overnight against PBS. To concentrate the protein, the solution containing rGP5 protein was dehydrated with polyethylene glycol (PEG) 8000 (Fisher Scientific, Pittsburg, PA). The final rGP5 protein concentration was determined by the BCA protein assay kit (Pierce). Aliquots of the purified rGP5 preparation were stored at -80°C until use.

1.6 Production of recombinant protein domain of GP5 protein

Each domain of GP5 protein was produced using the Bac-to-Bac baculovirus expression system as described above. The hydropilicity profile of GP5 protein was analyzed by the method of Kyte and Doolittle (41) using Mac Vector software. The GP5 protein was divided into a signal sequence (aa 1-32), an ectodomain (aa 33-63), a transmembrane region (aa 64-134), and an endodomain (aa 135-201). Insertion cDNAs encoding each domain were prepared by PCR amplification. Based on the sequence of pFastBac-His/ORF5, primers were designed as shown in Figure 19 and synthesized (Integrated DNA Technology, Coralville, IA). The amplification was performed in a 50 µl reaction mixture containing 1.5 mM $MgCl_2$, 1 × PCR buffer, 0.2 mM of each dNTP, 20 pmol of each primer, 1.5 U of TaqDNA polymerase (Promega) and 1 µl of pFlueBac-His/ORF5

plasmid as template. The reaction was run in a thermocycler (PTC-150) with the following program: initial denaturation at 94°C for 3 min, 30 cycles composed of denaturation at 94°C for 40 s, annealing at 60°C for 40 s and extension at 72°C for 1 min, and was ended with a final extension step of 10 min at 72 °C. PCR products were adjusted to 5mM EDTA, 10mM Tris (pH 8.0) and 0.5 % SDS. Proteinase K was added to a final concentration of 50 µg/ml. The mixture was incubated for 30 min at 37 °C to promote the enzyme reaction and then for 10 min at 68 °C to stop the reaction. The Proteinase K reactions were subsequently purified with QIAquick PCR purification kit (Qiagen), digested with BamHI and Xba I, and subjected to electrophoresis using 1% agarose. The PCR products were recovered from the gel using the QIAquick Gel Extraction kit (Qiagen) following the supplier's instructions. These synthetic nucleotide sequences were inserted into BamHI - Xba I site on the pFastBacHTb vector by ligation using T4 DNA ligase (Bio-Rad, Hercules, CA). These recombinant plasmids were used as baculovirus entry vectors.

2. Vaccine and challenge virus

2.1 Virus and cells

PRRSV Powell strain, which was isolated from clinically affected piglets in a North Carolina herd experiencing an acute-type PRRS, was used throughout this research. The virus was propagated once by coculture of viremic serum with porcine alveolar macrophages (PAMs) until the virus reached a 70-80% cytophatic effect. PRRSV stocks were obtained from virus infected cell lysates by twice freezing and thawing the cell monolayer. Cell lysates were centrifuged at 1000 × g for 10 min at 4°C. Virus-containing supernatants were

titrated on PAM culture and 50% tissue culture infectivity doses ($TCID_{50}$) were calculated. Stock virus solution was stored in aliquots of 1 ml at -80°C until use.

PAMs were harvested via lung lavage of PRRSV-free piglets as previously described (42). Isolated PAMs were maintained in RPMI 1640 medium supplemented with 10% heat inactivated FBS (HIFBS), and an antibiotic-antimycotic solution containing 100 IU/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin, and 25 µg/ml fungizone at 37 °C and 5% CO_2. Overall cell viability was determined by the trypan blue dye exclusion assay. Viability of cells harvested by lavage was greater than 95%. The cells were adjusted to 2×10^7 cells/ml in a pre-cooled medium containing 60% RPMI-1640, 20% HIFBS, and 20% dimethylsulfoxide (DMSO) and stored in liquid nitrogen until used.

2.2 Animals and experimental design

Six week old Yorkshire pigs were purchased from a specific pathogen free (SPF) herd and housed in isolation at the Laboratory Animal Resource Facility at the College of Veterinary Medicine, North Carolina State University. Weaned pigs were allowed 2 weeks to adapt and then were randomly assigned to one of four different groups. Three pigs were assigned to each group, as follows: group A; control pigs, group B; rGP5 vaccinated and non-challenged pigs, group C; unvaccinated and challenged pigs, and group D; rGP5 vaccinated and homologous virus challenged pigs. Pigs in groups B and D were injected intramuscularly with 0.5 mg recombinant protein twice at interval of three weeks. Five weeks after the second injection, pigs in groups C and D were challenged intramuscularly with 1ml of 10^3 $TCID_{50}$/ml of the Powell isolate. The pigs in group A were neither

vaccinated nor challenged served as control pigs. After challenge, all pigs were monitored clinically by visual inspection, and rectal body temperature was measured for 10 days.

2.3 Blood samples

All animals were bled immediately before the vaccination (Pre-IMM), 3 weeks after primary and second vaccinations (Post-IMMs) and 0, 3, 7, 10, 17 and 24 days post infection (dpi). Sera were used for PRRSV-specific Real-Time RT-PCR and determination of humoral immune responses. Peripheral blood mononuclear cells (PBMC) isolated with Ficoll-hypaque density-gradient centrifugation were used for flow cytometry assays to determine cellular immune responses.

2.4 Real-Time quantitative RT-PCR

Viral RNA was manually extracted from serum samples using QIAamp viral RNA purification kits (Qiagen) and dissolved in 60 µl of water, aliquoted (5 µl), and stored at −80 °C until used. Real-Time quantitative RT-PCR using a iCycler (Bio-Rad) and a QuantiTect Probe RT-PCR kit (Qiagen) was performed using 5 µl RNA according to the manufacturer's protocol. The three primers in 0.4 µM and one probe in 0.2 µM final concentrations were used. The conditions for the PCR were as follow: 50°C for 30 min for reverse transcription and 95°C for 15 min to activate HotStar Taq DNA polymerase followed by 40 cycles of 94°C for 15 s to denature and 60°C for 60 s for annealing/ extension. To quantify viral RNA copies, the purified viral RNA and a series of 10-fold dilution corresponding to concentration 1 to 10^8 copies used as standard samples.

Forward 1; 5'-ATGATGRGCTGGCATTCT-3'

Forward 2; 5'-ATRATGRGCTGGCATTCC-3'

Reverse; 5'-ACACGGTCGCCCTAATTG-3'

Probe; 5'HEX-TGTGGTGAATGGCACTGATTGACA-BHQ2-3'

3. Analysis of humoral immune response

3.1 Western Blot analysis

The loading protein samples were prepared from the purified rGP5 protein, crude lysates of rBac-GP5 infected Hi-5 cells at 5 MOI, or NC Powell PRRSV infected PAM at 0.1 $TCID_{50}$/cell. Hi-5 cells were resuspended in loading buffer (2% SDS). PAMs were resuspended in lysis buffer (50 mM Hepes pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.5% SDS, 200 nM Sodium Orthovanadate, and freshly added protease inhibitors) and this crude protein extract was centrifuged at 14,000 × g to pellet sticky genomic DNA. Total protein concentrations were determined using the BCA protein assay kit (Pierce) as described by the manufacture's instructions. The loading proteins (250 ng of purified rGP5 protein and 20 µg of crude lysates) were subjected to 12% SDS-PAGE, and blotted onto nitrocellulose or polyvinylidene fluoride (PVDF, Millipore, Bedford, MA) membrane using the NOVEX system (Invitrogen). After membranes were blocked in 3% milk solution overnight, proteins were probed for 1.5 hr with sera diluted 1:500 in PBS-T (PBS + 0.1% Tween 20 + 0.1% dry milk), followed by incubation for 1 hr with HRP-labeled rabbit anti-swine IgG diluted 1:10,000 in PBS-T. To detect His-tagged protein, recombinant proteins were primarily incubated for 1 hr with mouse anti-His IgG at a 1:5,000 dilution in PBS-T and then for 1 hr with HRP-labeled rabbit anti-mouse IgG in PBS-T at a 1:20,000 dilution. Detection was performed using the SuperSignal chemiluminescence kit (Pierce).

3.2 Immunofluorescent antibody test (IFA)

The Baculovirus-infected Hi-5 cells presenting the GP5 antigen and PRRSV-infected PAM presenting whole virus were used for IFA. The Baculovirus-infected Hi-5 cells were plated on a 8-well chamber slide (Nalgene Nunc International, Rochester, NY) with 2×10^4 cells/well and incubated for 2 hours for attachment on bottom. PAM were seeded in a 8-well chamber slide with 2×10^5 cells/well and incubated overnight. The next day, each well was inoculated with the NC Powell strain of PRRSV at 0.1 $TCID_{50}$/cell for 1 hour and replaced with fresh growth media at 37°C and 5% CO_2 for 2 days. For immunofluorescence cells were gently washed with Hank's balanced salt solution (HBSS) because virus-infected cells could be easily detached from chamber slides and fixed in cold acetone and methanol solution (1:1) for 15min at room temperature. After air-drying, cells were primarily incubated with appropriate serum at a 1:500 dilution at 37°C for 30 min and then stained with rhodamine-conjugated anti-porcine IgG at 1:2,000 for 30 min. Virus infection was confirmed by a fluorescien isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) against PRRSV nucleoprotein, SDOW 17. Finally, the cells mounted in 10% glycerol were observed under a Zeiss Axiovert microscope (Zeiss, Oberkochen, Germany).

4. Analysis of cellular immune response

4.1 Isolation and culture of PBMC

Swine PBMC were isolated by density-gradient centrifugation. Briefly, whole blood was diluted in HBSS (1:3), overlaid on the Ficoll-Histopaque 1077 carefully, and centrifuged at 400 × *g* for 30 min. Buffy coat containing PBMC in the Ficoll/plasma interface were collected, washed twice with HBSS and suspended in RPMI-1640 with L-glutamine

supplemented with 25 mM HEPES, 5×10^{-5} M 2-mercaptoethanol, 10% HIFBS, and an antibiotic-antimycotic solution.

4.2 *In vitro* antigen stimulation

Swine PBMC were stimulated with either purified rGP5 protein (5 µg/ml) or UV-inactivated NC Powell strain of PRRSV (10 µl/ml of 10^3 $TCID_{50}$/ml), or culture media alone (no stimulation). Plates were then incubated for 7 days at 37°C in 5% CO_2. Cells from replicate wells of each treatment group were analyzed by flow cytometry for PKH67 staining intensity as well as cell surface marker expression.

4.3 Lymphocytes proliferation assay

Cell proliferation was evaluated using PKH67 green fluorescent cell linker kit. 2×10^7 PBMC were wash with serum-free RPMI-1640 and resuspended in 1ml of Diluent C and then mixed with PKH67 green fluorescent dye (4×10^{-6} M) resuspended in 1ml of Diluent C. Cells were incubated in the dye solution for 5 min followed by 1 min incubation with an equal volume (2 ml) of FBS to stop the reaction. The stained cells were washed three times with RPMI 1640 culture media. The PKH67 stained cells were cultured with appropriate antigen at 37 °C in 5% CO_2 for 5 days and then proliferation responses were measured using flow cytometry. The stimulated index (SI) represents the proportion of cells proliferating in response to rGP5 protein or NC Powell strain of PRRSV divided by the proportion of cells undergoing background proliferation in absence of stimulator.

4.4 Cell surface antigen staining

Phenotypic analysis of PBMC subsets was done by flow cytometry using the following mAbs: FITC-anti-porcine CD4 (PIGCD4A-4FT, isotype IgG2b, RDI, Concord, MA), PE-anti-porcine CD8 (PIGCD8A-11PE, isotype IgG2a, RDI), mouse anti-porcine CD25 (PGBL25A, isotype IgG1, VMRD, Pullman, WA) and secondary antibody comprised PE-conjugated goat anti-mouse IgG (A-11030, Molecular Probe) for CD25 staining. At the end of the culture period, the culture plates were chilled on ice for 15 min. PBMC were mixed gently with a micropipette and transferred to 5 ml polystyrene round-bottom tubes, centrifuged at 1,200 rpm and 4 °C for 1min, and washed with cold PBS with 1% BSA and 0.1% NaN_3 (FACS buffer). For surface CD4 and CD8 staining, 50 µl of mAbs at 1:400 and 1:100 dilutions, respectively was added to each tube and incubated in the dark at 4°C for 30 min. For surface CD25 staining, 50 µl of mAbs at 1:200 was added to each tube and incubated in the dark at 4 °C for 30 min. PBMC were then centrifuged at 1,200 rpm and 4°C for 1min. The supernatants were discarded and PBMC were washed three times with FACS buffer and incubated with 50 µl of FITC or PE conjugated secondary antibody at 1:400 dilution in the dark at 4 °C for 30 min. Finally PBMC were washed twice with FACS buffer. Stained cells were analyzed with a FACSCaliber flow cytometer (BD Biosciences, Mountain View, CA) and the data were analyzed with CELLQuest software.

5. Statistical analysis

Prism software (GraphPad) was used for statistical analysis of data and figure preparation. Comparison of data was performed using a nonparametic, two-tailed *t*-test. A probability (P) value less than 0.05 was considered statistically significant.

RESULTS

1. Vaccine Generation

1.1 Construction of recombinant transfer vector and generation of recombinant baculovirus

Figure 1 shows a schematic diagram of the approach used to generate a recombinant baculovirus expressing the PRRSV outer membrane protein, GP5. PRRSV ORF5 gene was excised from the recombinant plasmid encoding ORF5 (pBlueBac4.5/V5-His/ORF5) by digesting with Bam HI and Sal I. A fragment of 684 bp containing the ORF5 gene, 6 × His tag and stop codon was cloned into Bam HI-Sal I digested pFastBac1 resulting in the construct pFastBac-His/ORF5 (Figure 2). Subsequently pFastBac-His/ORF5 was transformed into competent DH10Bac *E. coli* cell including bacmid DNA and helper plasmid. Site-specific transposition between the mini-Tn7 site on the pFastBac-His/ORF5 and the mini attTn7 site on the bacmid DNA resulted in disruption of the LacZ gene on bacmid DNA. Recombinant colonies were selected by their LacZ - negative phenotype as white colonies and colonies expressing LacZ showed blue color in the presence of antibiotics, X-gal, and the inducer IPTG (Figure 3). About 10-12% of the transposition rate was observed. The white colonies were selected and propagated in LB medium containing antibiotics. The large recombinant bacmid DNA was isolated from the culture of white colonies using the MidiPrep kit (Promega) and analyzed by PCR with the M13 primers because restriction analysis is difficult to perform with DNA of this size. Figure 4 shows the 3.084 kb band (lane 2) of the PCR product, which is theoretically concordant with 2400 bp plus the target gene

(684 bp) whereas colonies that do not contain recombinant bacmid baculovirus contain 300 bp of bacmid alone.

1.2 ORF 5 sequence analysis of Powell PRRSV strain

There have been multiple outbreaks of "severe PRRS" in swine herds, both vaccinated and unvaccinated. The PRRSV isolated from these affected herds showed 99.2 to 99.5% sequence identity to the MLV PRRSV vaccine strain suggesting that the MLV vaccine had spread from the vaccinated herds to other non-vaccinated PRRSV-naive herds (28, 43, 44). To compare sequences, a fragment of Powell ORF 5 was amplified from recombinant bacmid DNA and directly sequenced. Ingelvac PRRS MLV vaccine-ORF5 (GenBank accession numbers; AF066183) was compared with its parent stain VR2332 (PRU87392) and with Powell which was isolated from a herd that had been vaccinated previously with an Ingelvac PRRS MLV (Figure 5). MLV-ORF5 showed 99.7% identity to VR2332-ORF5, with two nucleotide mismatches at position 37 and 451. The Powell-ORF5 showed 91.2% identity with PRRS MLV vaccine. Nucleotide sequence of PRRSV ORF 5 varies from 91-95 % homology compared to the reference strain VR2332 (45, 46). Sequence analysis showed clearly that this virus was not directly related to the vaccine strain showing a similar level of homology to other U.S. isolates.

1.3 Amplification of recombinant baculovirus and expression of rGP5 protein

To generate the recombinant baculovirus, Sf9 cells were transfected with isolated recombinant bacmid DNA using the Cellfectin and incubated in a 27°C humidified incubator and visually inspected daily for the signs of infection using an inverted phase microscope.

Baculovirus infected cells typically displayed signs of increased cell diameter, growing arrest, cell detachment, and lysis at 3 dpi. The recombinant viral solution was collected and amplified to generate the high-titer stock. In other to express the PRRSV-ORF5 gene, Sf9 cells and Hi-5 cells were infected at a MOI of 5 pfu/cell with recombinant baculovirus and incubated at 27°C until harvesting of the cultures. The cell fraction was separated from culture medium because GP5 protein is associated with the cell membrane. The cell lysates were subjected to a 12% gel for SDS-PAGE and western blot analysis using anti-His Abs. The recombinant protein was visualized in baculovirus infected Hi-5 cells after 3 dpi with peak expression reached at 4 dpi (Figure 6), whereas it was difficult to detect the presence of recombinant protein in baculovirus infected Sf9 cells in the same conditions of Hi-5 cells (data not shown) indicating that Hi-5 cells are a better host than Sf9 cells for the expression of recombinant protein. An approximately 24 kDa protein corresponding to GP5 was found. The heterologous glycosylation of this protein can result in multiple bands on western blot (lane 3, Figure. 6).

1.4 Generation of a purified rGP5 protein for vaccination

Figure 7 shows a schematic diagram of the approach used to prepare the rGP5 protein for use as a vaccine. Initially, Hi-5 cells were infected with recombinant baculovirus, which expresses GP5 protein. Hi-5 cells were harvested after 4 dpi and the recombinant protein was purified from crude lysates. The lysates were separated on a SDS-PAGE and a gel slice containing rGP5 protein based on molecular weight from 23 to 32 kDa was excised. The rGP5 protein in the gel slice was extracted by electroelution. The isolated rGP5 protein in SDS running buffer (25 mM Tris, 250 mM glycine pH 8.3, 1% SDS) was dialyzed in PBS

(50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl) and concentrated using an osmotic compound polyethylene glycol (PEG) 8000. The purified protein was then combined with alum adjuvant and used to immunize swine. Typically we were able to recover approximately 3 mg of rGP5 protein following electroelution from 10 mg of crude baculovirus-infected Hi-5 cell lysates.

2. Clinical Observations and Viremia

Next, we evaluated the efficacy of the purified rGP5 protein as a vaccine against PRRSV in a live-virus challenge experiment. The experiment consisted of four treatment groups with three swine in each group, including; control, vaccine only, virus only, and vaccine plus virus (Table 1). Immunizations were performed at 8 and 11 weeks of age and live virus was administered at week 16 of the protocol (Figure 8A). The PRRS virus used in the challenge experiments was the Powell strain, the strain from which the GP5 gene had been isolated. We monitored a number of clinical parameters and in addition we monitored the replication of PPRRV by quantitative real time RT-PCR. As shown in Figure 9, we found that infection with the Powell strain of PRRSV induced a hyperthermic response with temperatures significantly elevated for 6 days post infection. However temperatures of vaccinated pigs dropped to 38.6°C at 4 dpi and subsequently recovered to normal temperature. Infection with the Powell strain was also accompanied by a variety of clinical symptoms, including; eyelid edema, nasal secretion, inappetence, and behavior retardation (Figures. 8B and C). The rGP5 vaccine partially ablated both hyperthermia (Figure 9) and clinical signs in 2 of 3 animals in this group. One animal in this group showed an extreme hyperthermic response and signs of secondary infection and was eliminated from the study. In contrast, the vaccine did not for the most part suppress viremia (Figure 10).

3. Humoral Immune Responses

3.1 GP5 protein or virus-specific antibody responses

The appearance of anti-GP5 antibody was monitored by western blot in animals that received the vaccine, or the Powell strain of virus, or both. The purified rGP5 protein was subjected to 12% SDS-PAGE followed by immunoblot analysis using porcine sera. As shown in Figure 11, anti-GP5 antibody appeared after booster injection in vaccinated animals. The most prominent band corresponded to a size of 17-18 kDa (type A) and another band was observed of higher 24 kDa (type B). Next we examined how antibody production was affected in the vaccinated pigs by challenge by live virus (Figure 12). After challenge with the virus, GP5-specific Abs were continuously detected at 0, 7, 17, and 24 dpi and showed strong reactivity. The humoral immune response shifted to produce a predominant response to type B protein whereas reactivity with the type A protein decreased, whereas an unique antibody response to the type B protein was detected in animals receiving virus only after 17 dpi (Figure 13). In addition, an extra band between these two bands was observed in pig 1.

We also examined the ability of these Abs to recognize GP5 protein as it is expressed in Hi-5 cells and alveolar macrophages. As shown in Figure 14, Abs produced in response to either vaccination or infection recognized GP5 protein expressed in Hi-5 cells. This was not true for GP5 protein as it is expressed in alveolar macrophages. As shown in Figure 15, in western blots, we found that the Abs produced in infected animals recognized GP5 protein as it is expressed in alveolar macrophages, but that Abs produced in vaccinated animals did not. The same pattern was seen when infected macrophages were examined directly using immunofluorescence (Figure 16). Taken together, these data indicate that antibody against purified rGP5 protein does not recognize GP5 protein assembled in the virus perhaps due to

masking of the epitope.

3.2 Domain analysis of the GP5 protein

We next sought to identify the epitopes on ORF5 that are recognized by the immune response in vaccinated animals. Initially, the amino acid sequence of the GP5 protein was analyzed. The alignment of the deduced amino acid sequences for Powell and VR2332 is shown in Figure 17. There was 90.5% amino acid sequence identity. The cleavage of the signal peptide is most likely to occur between the residues 32 and 33, as previously indicated (47). The four potential glycosylation sites are located at amino acid residues 30, 34, 44, and 51. The single ectodomain cysteine at amino acid residue 48 is assumed to be involved in the disulfide-link of GP5-M heterodimer. Figure 18 gives an overview of the hydropathy profile and assumed topology of the GP5 protein of the NC Powell strain of PRRSV. The N-terminus of GP5 protein contains a predicted signal peptide (48), which is followed by a hydrophilic domain. This domain is presumed to be exposed on the outside of the virion and is therefore designated the ectodomain. The ectodomain is followed by a hydrophobic region that is presumed to span the membrane three times. The last C-terminus of the GP5 protein is thought to form the endodomain.

3.3 Identification of the epitopes recognized by ORF5 vaccinated animals

The different domains of GP5 were expressed separately in order to identify the actual epitopes recognized by vaccinated or infected animals. We separately expressed each domain of the GP5 protein, including; the signal sequence (aa 1-32), the ectodomain (aa 33-63), the transmembrane region (aa 64-134), and the endodomain (aa 135-201) (13). Each

domain was amplified by PCR and PCR products were treated with Proteinase K to increase activity of restriction enzymes by digesting off tightly attached Taq polymerase. PCR products were digested with BamHI and Xba I and visualized by agarose gel electrophoresis (Figure 19). The fragments purified from the gel were ligated into pFastBacHTb vector (Figure 20) and expressed in baculovirus express system. The expression of each domain was confirmed by western blot using anti-His Abs (Figure 21 A). Unfortunately, we found it impossible to express the ectodomain individually and therefore it was co-expressed with the signal sequence to form a fragment of 63 amino acids. Lysates expressing these fragments were then probed for reactivity with the Abs induced by vaccination with rGP5 protein or by infection with the Powell strain. Interestingly, we found that Abs from both vaccinated and infected animals targeted the C-terminal endodomain of GP5 protein (Figure 21).

4. Cell-Mediated Immune Responses

4.1 Changes in PBMC of normal pigs

Next, we sought to characterize the T cell response against rGP5 protein and the Powell virus. PBMC were collected from vaccinated animals, purified and stimulated *in vitro* (Figure 22). Then, following stimulation proliferation was measured using a lipophilic dye, PKH, whose intensity decreases with each successive round of proliferation. Alternatively, following *in vitro* stimulation, flow cytometry was used to characterize the CD phenotype of the responding T cells. As shown in Figure 23, we found that the Powell virus itself could cause proliferation in normal T cells. This effect was not seen when PBMC were treated with GP5 protein making it unlikely that the proliferation induced by Powell is a

GP5-dependent function. As shown in Figure 24, flow cytometry revealed that the responding T cells were single positive $CD4^+$ cells.

4.2 Phenotype of CD4 cells responding to virus

Interestingly the population of CD4 cells responding to virus showed the intermediate intensity of the expression of the CD4 marker by flow cytometry. Resting small lymphocytes and activated cells were independently analyzed based on their characteristic SSC and FSC profiles. Most of activated cells were $CD4^{dim}CD8^-CD25^+$ whereas resting small cells were $CD4^-CD8^-CD25^-$(Figure 25).

4.3 Changes in PBMC of pigs vaccinated with a GP5 subunit vaccine

PBMC from vaccinated animals were next examined. As shown in Figure 26, PBMC from vaccinated animals responded to rGP5 protein. Interestingly, the responding T cells displayed a different phenotype comparing with the T cells responding to the virus (Figure 27). In this case we found that the proportion of double positive $CD4^+CD8^+$ cells increased after stimulation with the purified rGP5 protein.

4.4 Changes in PBMC of pigs infected with Powell

The response of PBMC from animals infected with the Powell strain of PRRSV is shown in Figures 28 and 29. Interestingly, in these animals, we did not observe significant $CD4^+$ cell proliferation in response to the virus. Apparently, the infection *in vivo* eliminates this constitutive response. On the other hand, we found that treating these cells with rGP5 protein or with Powell virus *in vitro* did cause several changes in the composition of the T

cell population. Single positive $CD8^+$ T cells from infected animals increased in relative proportion following treatment with either rGP5 protein or inactive Powell virus. An increase in double positive $CD4^+ CD8^+$ cells, and a concomitant decrease in double negative $CD4^- CD8^-$ cells, was seen following *in vitro* treatment with inactive Powell strain of PRRSV.

4.5 Changes in PBMC of vaccinated and infected pigs

Finally, we examined the response in animals that had been vaccinated with rGP5 protein then infected with the Powell virus. Interestingly, in many ways, we found that the response of these animals was a composite of the responses when these treatments were administered separately. For example, their PBMC proliferated in response to rGP5 protein (as in vaccinated animals) but failed to proliferate in response to the Powell virus (as in the infected group) (Figure 30). We also noted an increase in the proportion of double positive $CD4^+ CD8^+$ cells, and a concomitant decrease in double negative $CD4^- CD8^-$ cells, was seen following treatment with only the Powell virus (Figure 31).

DISCUSSION

In this report, we examined immune responses in swine elicited by infection with PRRSV and following vaccination with GP5 protein. The GP5 protein is one of the major outer membrane proteins of PRRSV and is a leading target for new generation of vaccines against PRRSV. Recombinant GP5 from PRRSV has been tested previously in the Laster's lab as a vaccine for PRRSV. Hadley found it to be highly effective at preventing the clinical symptoms associated with disease (35). In these experiments the vaccine caused mild fever reduction and a slight reduction in virus titer. However our results were not as dramatic as those shown previously (35). A possible reason for the reduced effectiveness of the vaccine in these experiments is protein degradation. Our vaccine may have been partially degraded prior to administration. This is one of drawbacks of protein vaccines; proper storage and handling is essential. As an alternative, to alleviate these potential problems, GP5 could be administered as a DNA vaccine. The presentation of a protein following DNA vaccination should be very close to that seen during infection with the original pathogen. It may also be explained by genetic variation of individual pigs responding to PRRSV. Substantial individual variability in disease severity was observed on the farms that harbor a single strain of PRRSV (49). Petry DB *et al* found that the Hampshire-Duro cross pigs were more susceptible to PRRSV than Index Line pigs, indicating that underlying genetic variation existed (50).

The mechanisms by which this vaccine provides resistance to PRRSV have been analyzed previously. The antibodies in vaccinated animals recognized rGP5 but not whole virus as detected by ELISA (35). These results suggested to us that antibodies induced by

rGP5 were capable only of recognizing denatured GP5 (its form in the immunogen) and not native GP5 as it would appear in the virion. These results also raised the possibility that the antibody response was not critical for protection provided by the vaccine and that activation of T cells is critical. We have explored both of these hypotheses. We further analyzed the humoral immune response and compared antibody recognition using IFA and western blots. We found that both assays revealed antibody recognition of rGP5 when it was expressed in insect cells. On the other hand, these Abs failed to recognize rGP5 (with either assay) when it was expressed in pulmonary alveolar macrophages. We conclude, therefore, that heterodimerization of GP5 and M proteins under conditions of natural infection may mask the GP5 epitopes. Alternatively, post-translational processing in the baculovirus expression system may be different from that seen in macrophages during infection with PRRSV. Post-translational processing includes proper proteolysis, N- and O-glycosylation, acylation, amidation, carboxymethylation, phosphorylation, and prenylation. The inability to produce eukaryotic glycoproteins with complex N-linked glycans was reported as a major limitation of the baculovirus-insect cell expression system (51).

In addition, we also noted a difference in the way vaccine and virus-induced Abs recognized the rGP5 protein. In western blot analysis, vaccine-induced Abs reacted predominantly against a rGP5 protein of low molecular weight (type A). Since this protein was not detected by anti-His Abs, this protein likely represents a degradative fragment of rGP5 which has lost the His-tagged C-terminal region. In contrast, virus-induced Abs recognized only full length rGP5 (type B), which was also detected by anti-His Abs. In animals that were vaccinated by rGP5, and then challenged by the virus, this pattern changed from type A to type B recognition very quickly, suggestive of an anamnestic response against

type B. These results may indicate that the type A protein is capable of priming B cells that recognize type B Ag.

In this report we also sought to define the important antigenic regions in GP5 protein. Four different domains were separately expressed and tested for recognition by Abs from infected or vaccinated pigs. We found that the endodomain region was recognized strongly in western blots with sera collected both from infected and vaccinated pigs suggesting that C-terminal region of GP5 contains an important antigenic determinant. These results, to some extent, contradict previous epitope mapping studies with GP5 protein. Ostrowski *et al* (52) identified one immunodominant non-neutralizing epitope (aa 27-31) and three different neutralizing epitopes (aa 24, aa 29-35 and aa 37-45) in the N-terminal portion of GP5 protein using swine polyclonal sera and mouse mAbs (52-54). The antigenic area comprising amino acids 37-45 was also identified as the neutralizing epitope that was recognized by Abs used in passive transfer experiments (55). Plagemann, using serum from convalescent animals, found that the neutralizing epitope of both in the American and European strains also mapped to amino acids 37-45 of the GP5 protein (53). Together, these reports suggest an important role for the ectodomain of GP5 in Antibody responses to PRRSV. Alternatively, several previous studies have identified the endodomain as an important antigenic region. Mapping sites recognized by a panel of anti-GP5 mAbs, Dorval *et al* identified at least two domains of antigenic importance, one being located within the ectodomain (aa 27 to 41) and the other to the C-terminus (aa 180 to 197). Rodridguez also reported an immunodominant epitope in the region of aa 170-201 (56). Gonin *et al* found that the majority of convalescent pig sera reactive with the native viral protein and the *E. coli*-expressed recombinant ORF5 protein lost their reactivity following deletion of 50 aa residues from its C-terminus (57). Therefore,

we do not believe our results are likely to result from the chemical composition and structure of the Ag at the time of immunization.

The lack of ectodomain recognition may be explained by differential folding of GP5 in its native form vs. the recombinant purified form we used. The GP5 protein of PRRSV is a type I transmembrane glycoprotein that mediates both virus receptor recognition and low pH-induced membrane fusion (58). This protein can assume three different states such as the 'native' state detected at the virus surface, which is responsible for receptor binding, the activated hydrophobic state, which interacts with the target membrane as a first step in the fusion process, and the fusion-inactive conformation. These three states, which are structurally different, are in a pH-dependent equilibrium. The presence of the transmembrane domain and insertion of the protein into a lipid bilayer has been shown to be key to the correct folding of the ectodomain of Rabies virus glycoprotein (59). It is possible therefore, that the recombinant purified GP5 we used as an immunogen folds in a manner to prevent recognition of the ectodomain. Alternatively, it is possible that glycosylation of the GP5 ectodomain can block its recognition by Abs. Three putative N-linked glycosylation sites (N34, N44, and N51) are located on the GP5 ectodomain, where a major neutralization epitope also exists. Ansari *et al* (60) showed that the loss of glycan residues in the ectodomain of GP5 by mutants containing amino acid substitutions at putative N-linked glycosylation sites enhances both the sensitivity of these viruses to *in vitro* neutralization and the immunogenicity of the nearby neutralization epitope.

In these studies we also examined the T cell response to rGP5 and PRRSV. Different lymphocyte subsets have critical roles in the immune response against PRRSV and changes in their levels of expression have proven important for evaluating the immune response

against this virus (61-63). Lymphocytes from vaccinated animals proliferated strongly *in vitro* when restimulated with the GP5 vaccine protein, indicating that T cell recognition of GP5 had occurred *in vivo*. The responding cells were double positive $CD4^+$ $CD8^+$ T cells. Swine $CD4^+$ $CD8^+$ T cells have been reported to be MHC class II restricted and represent the majority of $CD4^+$ memory T cells which function as helper cells for humoral immune response (64-66). Meier *et al* also demonstrated that majority of virus-specific IFN-γ secreting cells were predominantly $CD4^+CD8^+$ T cells (15). It is likely therefore, that in our experiments, rGP5 induced production of memory T cells capable of helping both B and T cell effector responses. T cell proliferation was also detected in lymphocytes from vaccinated animals stimulated with PRRSV *in vitro* suggesting that rGP5 is capable of inducing T cells cross reactive with native PRRSV GP5. It is possible, therefore, that these T cells mediate resistance to PRRS that is conferred by this vaccine.

Interestingly, we also observed significant proliferation of naïve T cells following exposure to PRRSV *in vitro.* The phenotype of these cells proved to be single positive $CD4^{dim}CD8^-CD25^+$, indicative of an activated helper phenotype. The cause of this proliferative response is unknown. It is unlikely that this represents an antigen specific response since conventional peptide antigens normally stimulate less than 1 in 10,000 T lymphocytes (67). It also unlikely that this represents a secondary antigen specific response since these animals were raised under SPF conditions and did not display Abs to PRRSV. Our results may indicate that PRRSV can act as a mitogen. Alternatively, T cell proliferation may have been triggered through a superantigen encoded by PRRSV. Superantigens are potent activators of $CD4^+$ T cells, causing rapid and massive cell proliferation and cytokine production (68). While superantigens can stimulate a large fraction of T cells via interaction

with the T cell receptor (TCR) V beta domain, mitogens induce a direct, polyclonal stimulation of lymphocytes without a requirement for APC (69). Consequentially, differentiation between a possible mitogenic property of PRRSV and a superantigen-mediated activation may be achieved by investigating the involvement of APC in a PRRSV-induced proliferation.

A very different situation was observed in animals infected with PRRSV. In vitro, these lymphocytes did not proliferate in response to challenge with either rGP5 or PRRSV. It is likely, therefore, that infection by PRRSV suppresses or deletes the normally occurring T cells that respond to PRRSV. Porcine T cells can be suppressed by some viruses and bacteria via several mechanisms including the killing of T cells and the induction of immunosuppressive cytokines (70). In addition, Charerntantanakul *et al* reported that virulent PRRSV can suppress T cell proliferation demonstrating the significantly reduced CD25 expression in naïve porcine T cells in response to mitogen such as Concanavalin A (71). It should be noted, however, that lymphocytes from PRRSV-infected animals were not totally unresponsive. While we did not detect lymphocyte proliferation, we did detect several changes in CD phenotype. In PRRSV-infected pigs, the frequencies of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells were increased in response to virus stimulation in vitro suggesting up-regulation of surface expression of CD4 or CD8 on $CD4^-CD8^-$ T cells.

The rGP5 protein of PRRSV was generated using a baculovirus expression system. This system has several advantages, including simplicity and high levels of expression of recombinant proteins. In addition, proteins expressed in baculovirus are modified post-translationally in a manner similar to that seen in the native cell type. Traditional baculovirus expression systems use *B. mori* nuclear polyhedrosis virus (BmNPV), but we used the Bac-

to-Bac baculovirus expression system. This system utilizes a bacmid (a baculovirus shuttle vector), which has been developed for *Autographa Californica* nuclear polyhedrosis virus (AcNPV) (72). Using the bacmid system eliminates the multiple rounds of purification and amplification of viruses previously done by researchers using the BmNPV baculovirus expression system. In the Bac-to-Bac baculovirus expression system, identification of recombinant viruses is simplified because of the site-specific transposition of an expression cassette into the bacmid which eliminates multiple rounds of plaque purification.

The baculovirus expression system was used to express the entire GP5 protein as well as each domain. Expression of the His-tagged recombinant proteins was confirmed by western blot using anti-His Ab. We were successful in expressing each domain except for the region corresponding to the ectodomain. Although the sequence encoding the ectodomain of the GP5 protein was correctly inserted in the baculovirus transfer vector and in the bacmid DNA, we could not detect expression in insect cells. At present it is not clear why the ectodomain could not be expressed independently. It is possible that the ectodomain may have been secreted into the media and this possibility could be tested in future experiments, i.e., immunoprecipitation with anti-his antibody (73). Another possible explanation is due to toxicity of the ectodomain for insect cells because of its hydrophobicity (32). We alleviated this problem by cloning a composite of the ectodomain and the signal sequence and finally this product was detected on western blot.

Based on the experiments outlined herein the GP5 subunit vaccine that we studied induced strong antibody responses. The antibody recognized the rGP5 protein but not the native GP5 protein produced by NC Powell strain of PRRSV as detected by ELISA, western blot, and IFA tests, suggesting that humoral immunity is not critical for protection. In

addition, the endodomain of the GP5 protein was strongly recognized in western blot using sera collected from both infected and vaccinated pigs suggesting that the C-terminal region contains important antigenic determinants. T cells from vaccinated animals responded to rGP5 and to the NC Powell strain of PRRSV suggesting an important role in vaccine-induced resistance. Interestingly, the NC Powell strain of PRRSV caused strong proliferative response in naïve T cells from control pigs. The responding cells were CD4 single positive, suggesting the possibility of the presence of a superantigen on this highly virulent strain of PRRSV.

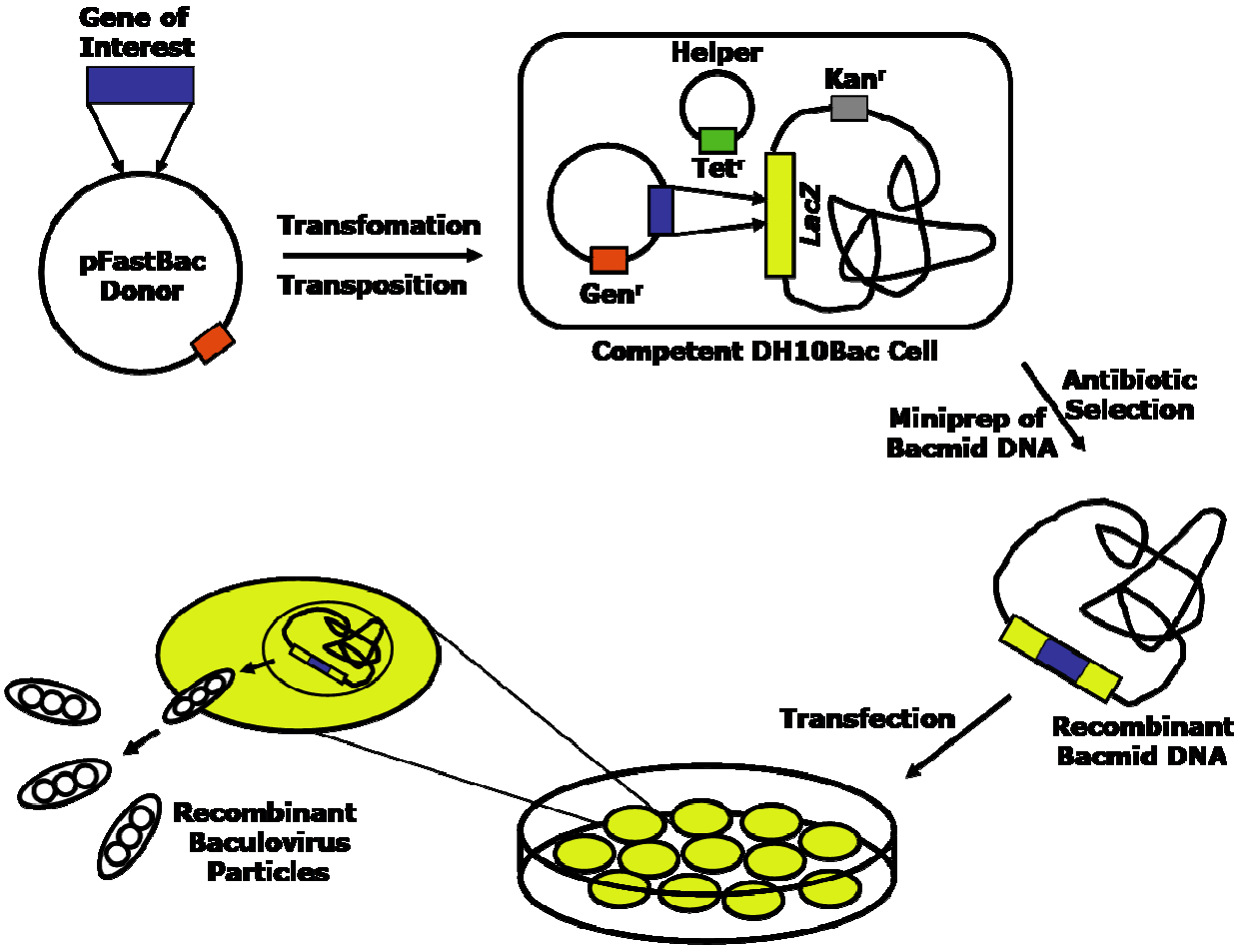


Figure 1. Protocol for generating a recombinant baculovirus by site-specific transposition in *E. coli*. The synthetic nucleotide sequence encoding full-length of ORF 5 or each domain of the GP5 protein of PRRSV is cloned into pFastBac vector. This recombinant donor plasmid is transformed into competent DH10Bac *E. coli* cell including bacmid DNA and helper plasmid. Site-specific transposition is obtained between the mini-Tn7 site on the pFastBac plasmid and the mini attTn7 site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids are identified by antibiotic selection and transposition is confirmed by blue/white screening due to disruption of the lacZ gene by transposing. The recombinant bacmid DNAs are isolated by plasmid mini-prep and then used to transfect Sf9 insect cells to generate a recombinant baculovirus. The culture media is used as the virus stock.

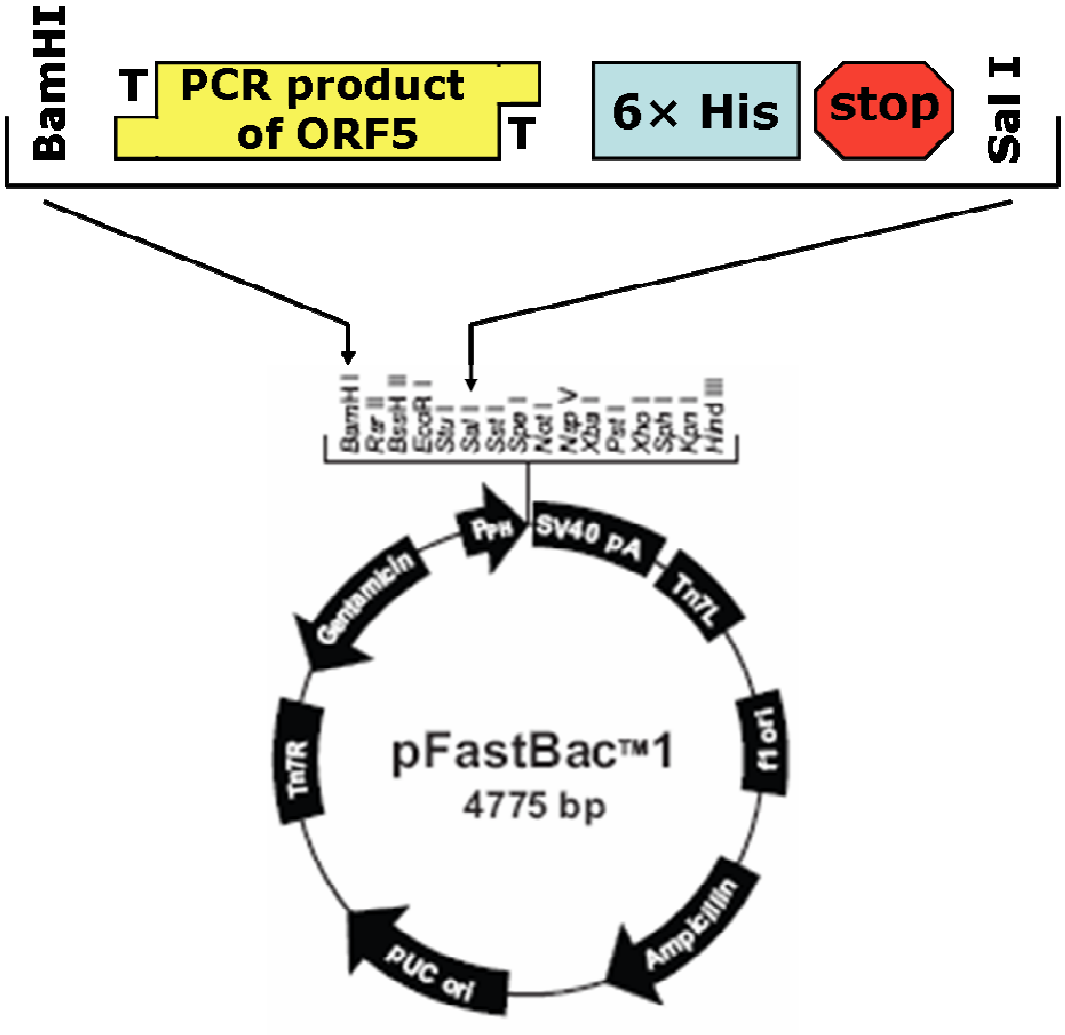


Figure 2. Diagram of baculovirus entry vector for expression of the GP5 protein of PRRSV. The recombinant pFastBac donor plasmid was constructed by ligating the insert DNA, which consists of a gene of ORF5 and a penta-His tag, into the BamHI-Sal I on the multiple cloning sites of the pFastBac vector. The insert DNA was prepared by digesting pBlueBac4.5/V5-His/ORF5 recombinant plasmid with restriction enzymes, BamHI and Sal I. (The feature of pFastBac vector was adapted from the Invitrogen website)

Figure 3. Identification of the colony containing recombinant bacmid by blue/ white screening. The colonies containing the recombinant bacmid are white due to disruption of the LacZ gene in process of transposition on the culture dish including IPTG and X-gal, while the blue colonies indicates that they do not contain the DNA coding GP5 protein in bacmid.

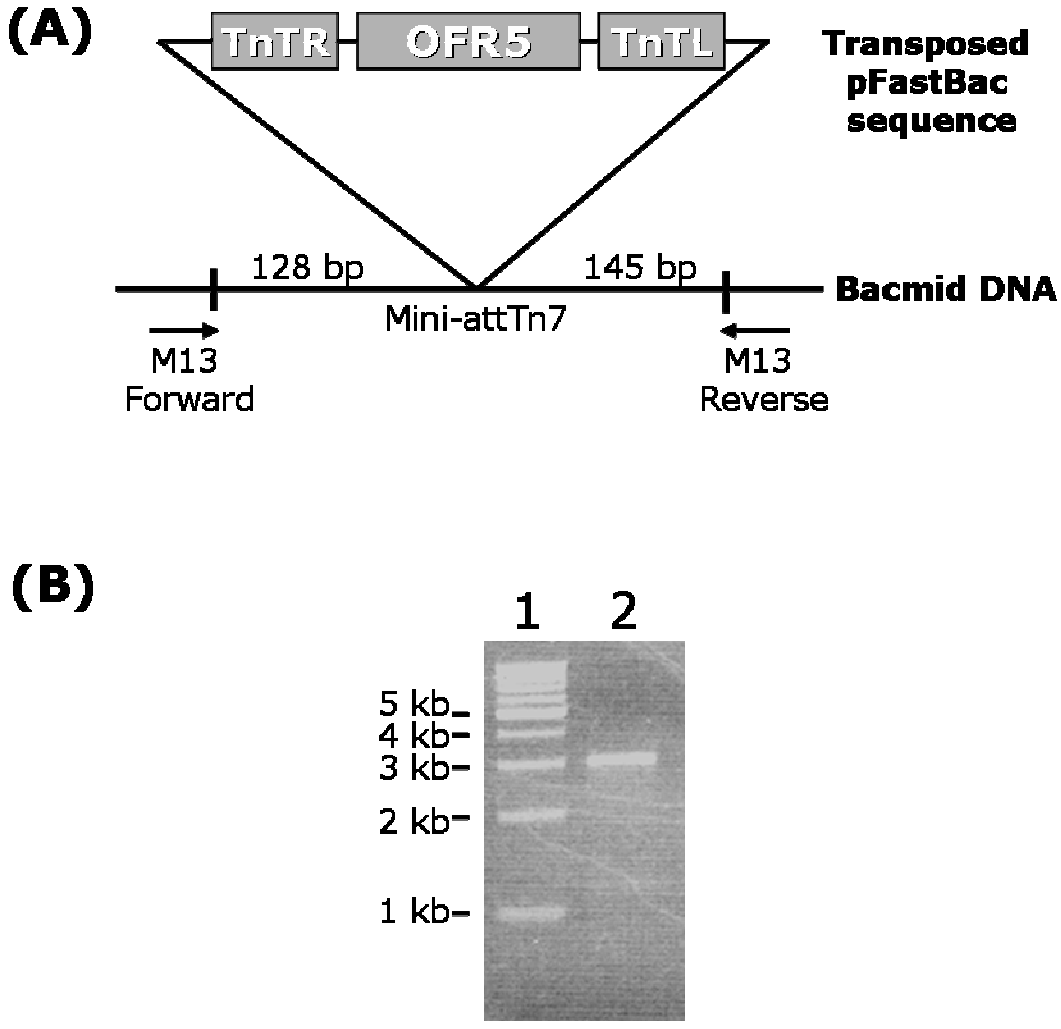


Figure 4. Verification of recombinant bacmid baculovirus. (A) Constructing diagram of recombinant bacmid baculovirus (rBac-GP5) (B) The PCR was performed on recombinant DNA using the M13 universal primers. The PCR product was electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. Lane 1, 1 kb molecular marker; lane 2, PCR product of white colony

```
                10         20         30         40         50         60         70
VR2332   ATGTTGGAGA AATGCTTGAC CGCGGGCTGT TGCTCGCGAT TGCTTTCTTT GTGGTGTATC GTGCCGTTCT
RespPRRS ---------- ---------- ---------- -------A-- ---------- ---------- ----------
Powell   ---------- ---------- ---------- ---------- ---C--T--- ---------- ----------

                80         90        100        110        120        130        140
VR2332   GTTTTGCTGT GCTCGCCAAC GCCAGCAACG ACAGCAGCTC CCATCTACAG CTGATTTACA ACTTGACGCT
RespPRRS ---------- ---------- ---------- ---------- ---------- ---------- ----------
Powell   ---------- -A---A---- -T----GG-A ---------- T---T-T--- T-------T- ----------

               150        160        170        180        190        200        210
VR2332   ATGTGAGCTG AATGGCACAG ATTGGCTAGC TAACAAATTT GATTGGGCAG TGGAGAGTTT TGTCATCTTT
RespPRRS ---------- ---------- ---------- ---------- ---------- ---------- ----------
Powell   ---------- ---------- -C-----G-- -G-------- ---------- ------C--- ----------

               220        230        240        250        260        270        280
VR2332   CCCGTTTTGA CTCACATTGT CTCCTATGGT GCCCTCACTA CCAGCCATTT CCTTGACACA GTCGCTTTAG
RespPRRS ---------- ---------- ---------- ---------- ---------- ---------- ----------
Powell   -----G---- ---------- T--------- --A-----C- ---------- ------T--- --T-G-C-G-

               290        300        310        320        330        340        350
VR2332   TCACTGTGTC TACCGCCGGG TTTGTTCACG GGCGGTATGT CCTGAGTAGC ATCTACGCGG TCTGTGCCCT
RespPRRS ---------- ---------- ---------- ---------- ---------- ---------- ----------
Powell   -T-------- C--------- ---TA----- ---------- -T-------- ---------- ----------

               360        370        380        390        400        410        420
VR2332   GGCTGCGTTG ATTTGCTTCG TCATTAGGTT TGCAAAGAAT TGCATGTCCT GGCGCTACGC GTGTACCAGA
RespPRRS ---------- ---------- ---------- ---------- ---------- ---------- ----------
Powell   ---------- ---------- -------A-- ---G-----C ---------- --------T- A---------

               430        440        450        460        470        480        490
VR2332   TATACCAACT TTCTTCTGGA CACTAAGGGC AGACTCTATC GTTGGCGGTC GCCTGTCATC ATAGAGAAAA
RespPRRS ---------- ---------- ---------- G--------- ---------- ---------- ----------
Powell   ---------- -C--C--A-- T--------- ---------- ---------- ------T--- ---------G

               500        510        520        530        540        550        560
VR2332   GGGGCAAAGT TGAGGTCGAA GGTCATCTGA TCGACCTCAA AAGAGTTGTG CTTGATGGTT CCGTGGCAAC
RespPRRS ---------- ---------- ---------- ---------- ---------- ---------- ----------
Powell   ----T--G-- ---------- --C--C--T- ---------- ---------- ---------- ----------

               570        580        590        600
VR2332   CCCTATAACC AGAGTTTCAG CGGAACAATG GGGTCGTCCT TAG
RespPRRS ---------- ---------- ---------- ---------- ---
Powell   T---T----- ---------- -T-------- ---------- --A
```

Figure 5. Nucleotide sequence alignment of the ORF5 of NC Powell, modified live vaccine (RespPRRS) and VR2332. The sequence of the VR2332 is shown on the top, and only differences are indicated in other two strains.

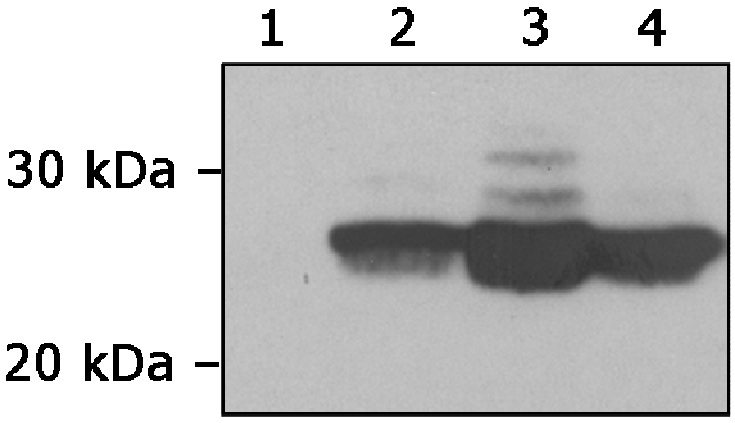


Figure 6. Western blot analysis of the recombinant GP5 expression. Hi-5 cells were infected at with rBac-GP5 at 5 MOI for 2 (lane 1), 3 (lane 2), 4 (lane 3), and 5 (lane 4) days. 20μg total Hi-5 cell lysates containing baculovirus-expressing recombinant GP5 protein were separated by 12% SDS-PAGE, blotted to nitrocellulose membranes and detected using anti-His Abs. Positions of molecular mass markers are given in kDa on the left.

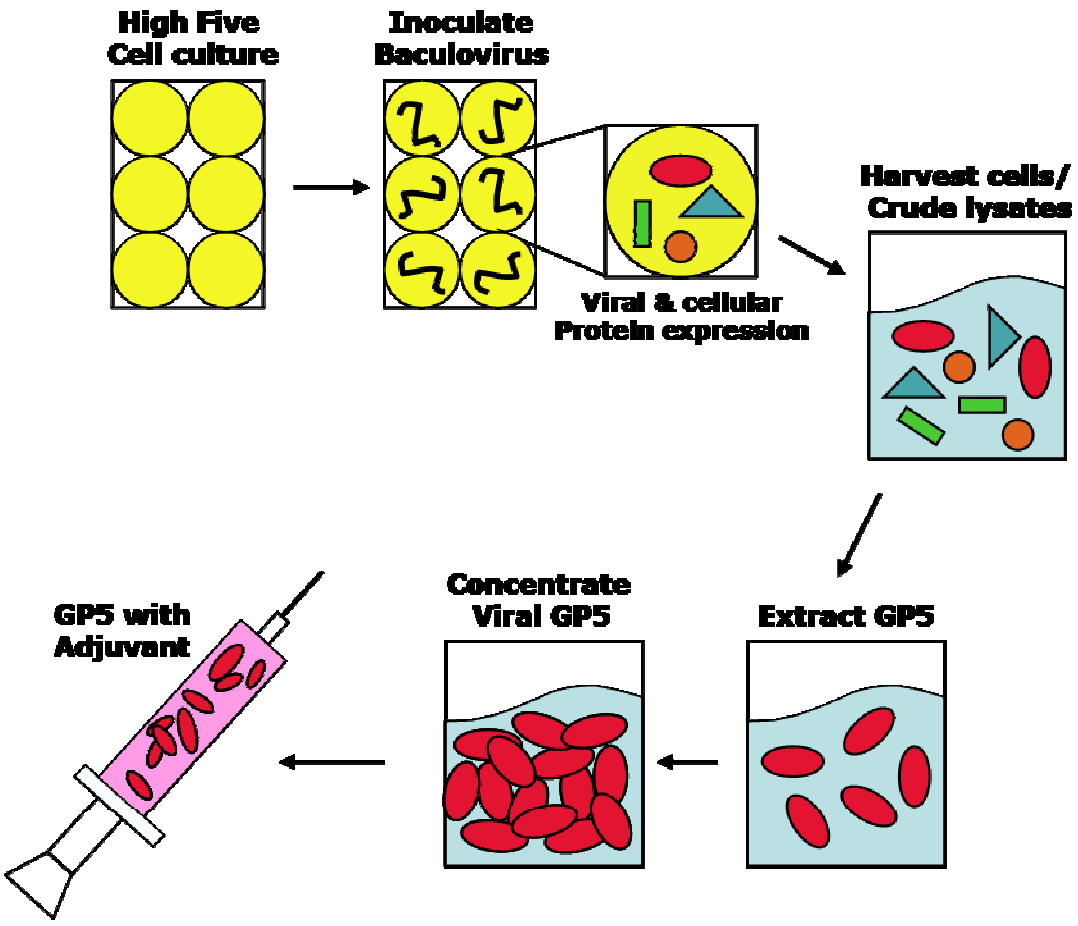


Figure 7. Schematic representation for preparing a subunit vaccine with purified rGP5 protein. The rGP5 protein was expressed in Hi-5 cells infected with the rBac-GP5. To extract rGP5 protein, the crude lysates were separated on a SDS-PAGE and the gel containing the rGP5 protein was excised based on the predicted molecular weight for the GP5 protein. The rGP5 protein was electroeluted from the sliced gel in SDS running buffer. The isolated rGP5 protein was dialyzed in PBS and concentrated using PEG 8000. The rGP5 protein prepared with an aluminum hydroxide gel adjuvant was used as a GP5 subunit vaccine.

Table 1. Description of the experimental groups. An experiment with 12 pigs was carried out. Pigs in groups B and D were vaccinated with a GP5 subunit vaccine. Pigs in groups C and D were challenged with NC Powell strain of PRRSV. Group A consisted of non-vaccinated and non-PRRSV-infected pigs as control. Groups A and B were housed in separated isolation facility from groups C and D.

Experimental Group (number of pigs)	1st Vac	2nd Vac	NC Powell challenge	Place
Group A (n=3)	-	-	-	Room I, Den I
Group B (n=3)	Yes	Yes	-	Room I, Den II
Group C (n=3)	-	-	Yes	Room II, Den I
Group D (n=3)	Yes	Yes	Yes	Room II, Den II

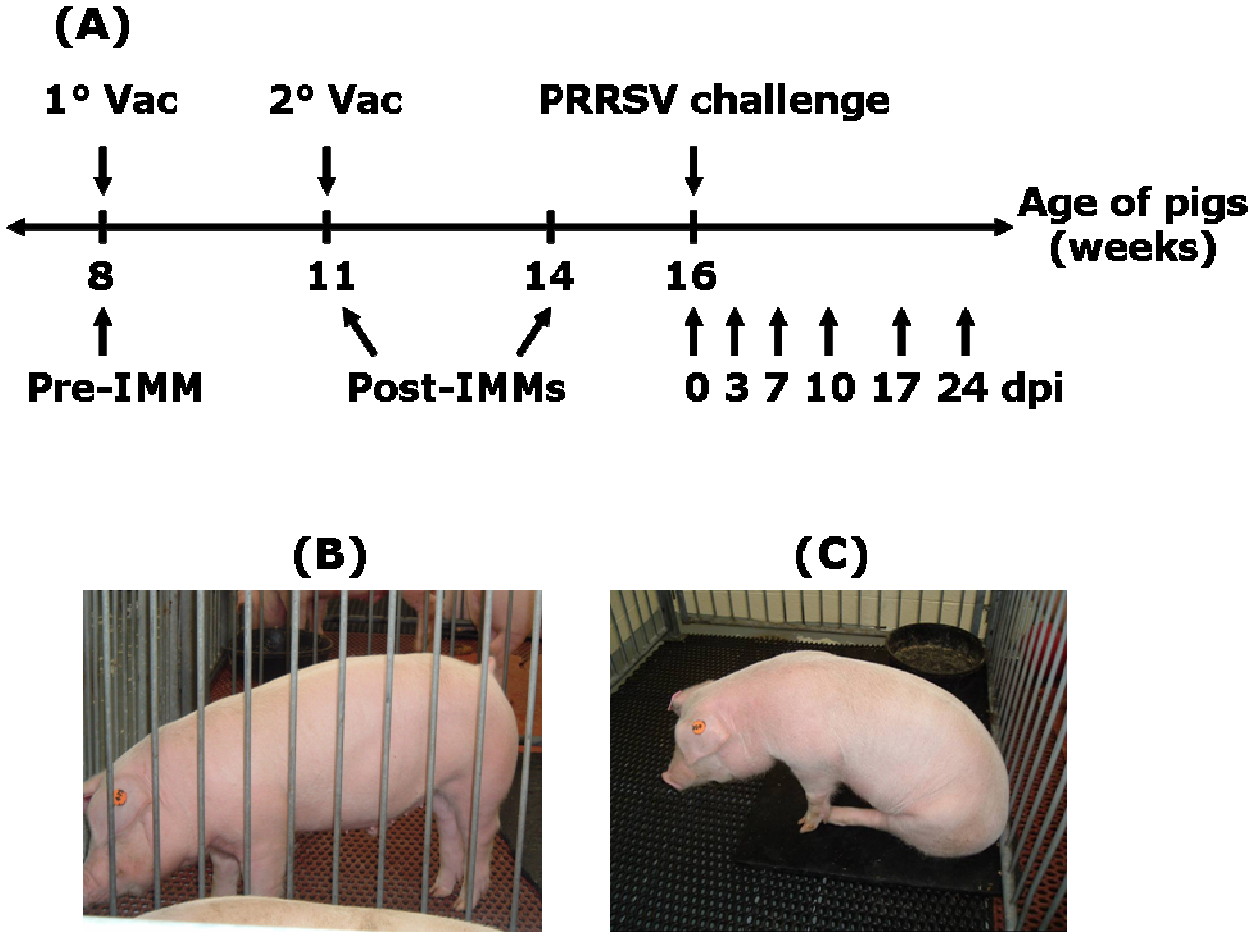


Figure 8. Description of animal experimental procedures. Pigs were vaccinated with the GP5 subunit vaccine at 8 weeks of age. Three weeks later, a booster vaccination was performed. At 16 weeks of age, pigs were challenged with the live wild type NC Powell PRRSV. Blood samples were collected at 8, 11 and 14 weeks of age and time points of 0, 3, 7, 10, 17, and 24 days post challenge. Photographs show one vaccinated (B) and one unvaccinated pig (C) at 5 days post challenge.

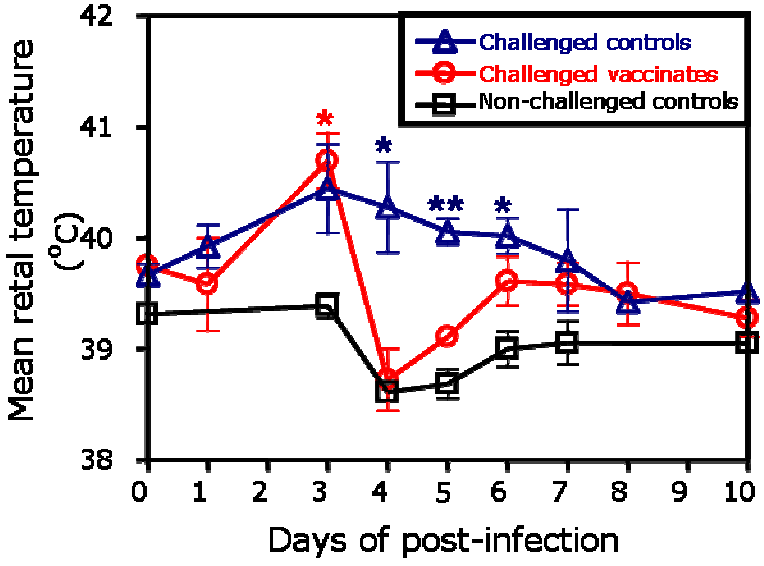


Figure 9. Mean rectal temperature of pigs after challenge with wild type NC Powell PRRSV. Temperatures of naïve non-challenged control pigs (n=3), challenged vaccinated pigs with rGP5 protein (n=2), and challenged control pigs (n=3) were observed for 10 dpi. The asterisk ($p<0.05$) and double asterisk ($p<0.005$) indicate that groups are significantly different from the control group.

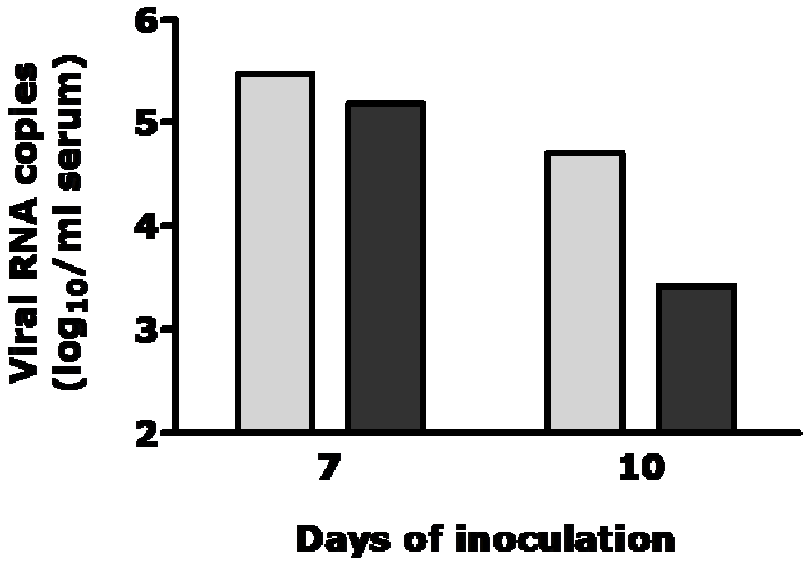


Figure 10. Mean PRRSV concentration in serum by real time RT-PCR. PRRSV RNA copy numbers in serum of challenged control pig (gray bar) and challenged vaccinated pig (black bar) were measured at 0, 3, 7, 10, 17, and 24 days post challenge with the wild type NC Powell PRRSV by real time RT-PCR. No viral RNA was detected at 0, 3, 17, and 24 days post challenge.

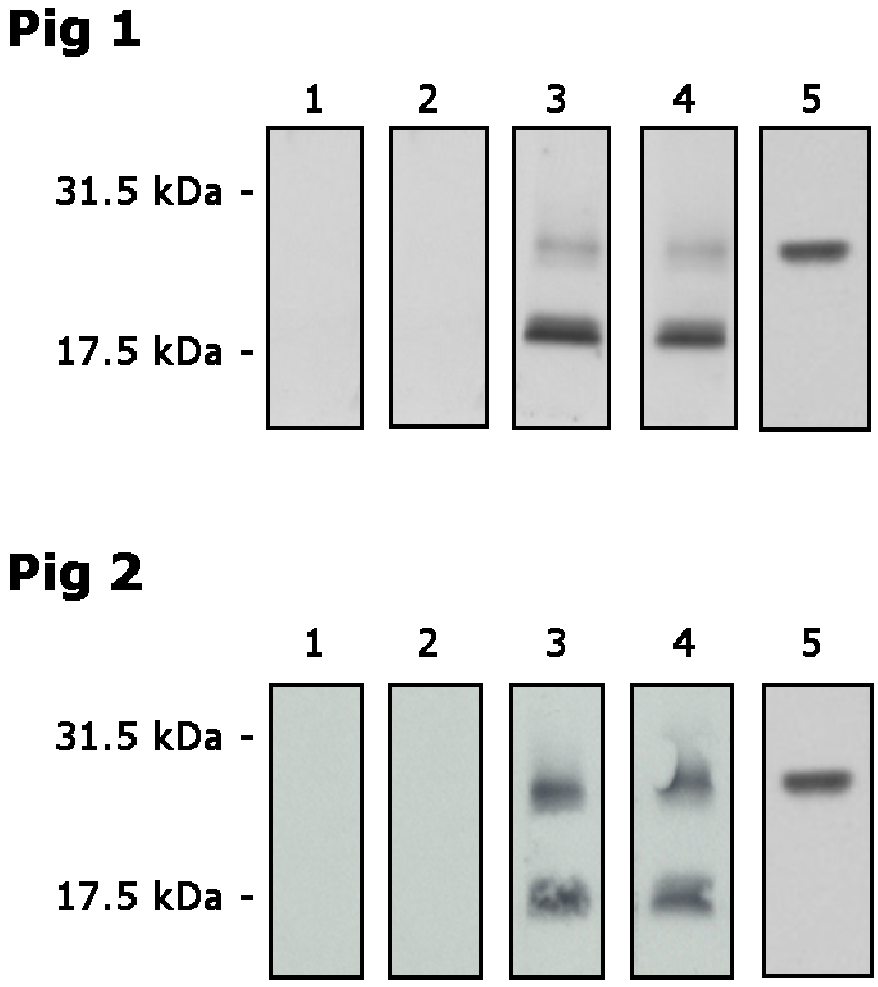


Figure 11. Western blot analysis of rGP5-specific antibody in the vaccinated pigs. 250 ng purified rGP5 protein was separated by 12% SDS-PAGE, blotted to nitrocellulose membranes and detected using sera at 1:500 dilution and anti-swine IgG at 1:10,000 dilution. The used sera were collected from the rGP5 vaccinated pigs at 8 weeks (lane 1; Pre-IMM), 11 weeks (lane 2; Post-IMM), 14 weeks (lane 3; Post-IMM), and 16 weeks (lane 4; 0 dpi) of old. Lane 5 indicates the location of the His-tagged purified rGP5 protein using anti-His Abs. Positions of molecular mass markers are given in kDa on the left.

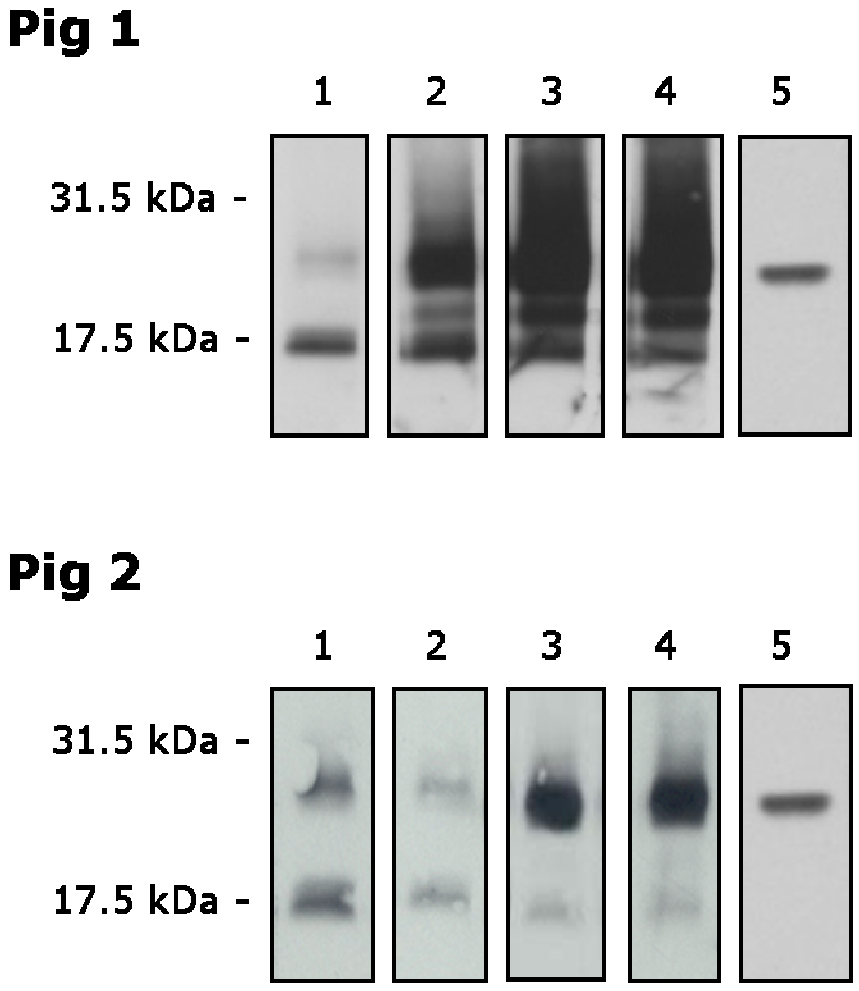


Figure 12. Western blot analysis of rGP5-specific antibody in the vaccinated pigs after challenge with wild type PRRSV. 250ng purified rGP5 protein was separated by 12% SDS-PAGE, blotted to nitrocellulose membranes and detected using sera at 1:500 dilution and anti-swine IgG at 1:10,000. The used sera were collected from pigs at 0 (lane 1), 7 (lane 2), 17 (lane 3), and 24 (lane 4) days post challenge with the wild type NC Powell PRRSV. Lane 5 indicates the location of the His-tagged purified rGP5 protein using anti-His Abs. Positions of molecular mass markers are given in kDa on the left.

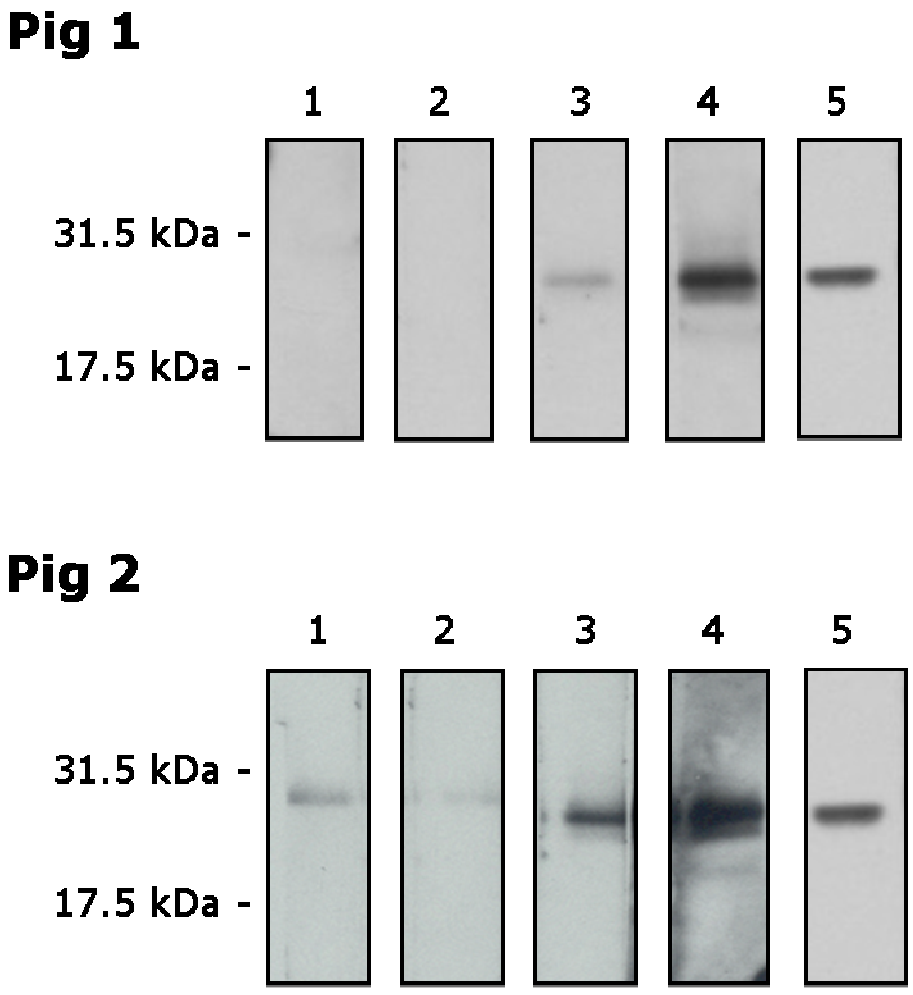


Figure 13. Western blot analysis of rGP5-specific antibody in pigs infected with wild-type PRRSV. 250ng of purified rGP5 protein was separated by 12% SDS-PAGE, blotted to nitrocellulose membranes and detected using sera at 1:500 dilution and anti-swine IgG at 1:10,000. The used sera were collected from pigs at 0 (lane 1), 7 (lane 2), 17 (lane 3), and 24 (lane 4) days post challenge with the wild type NC Powell PRRSV. Lane 5 indicates the location of the His-tagged purified rGP5 protein using anti-His Abs. Positions of molecular mass markers are given in kDa on the left.

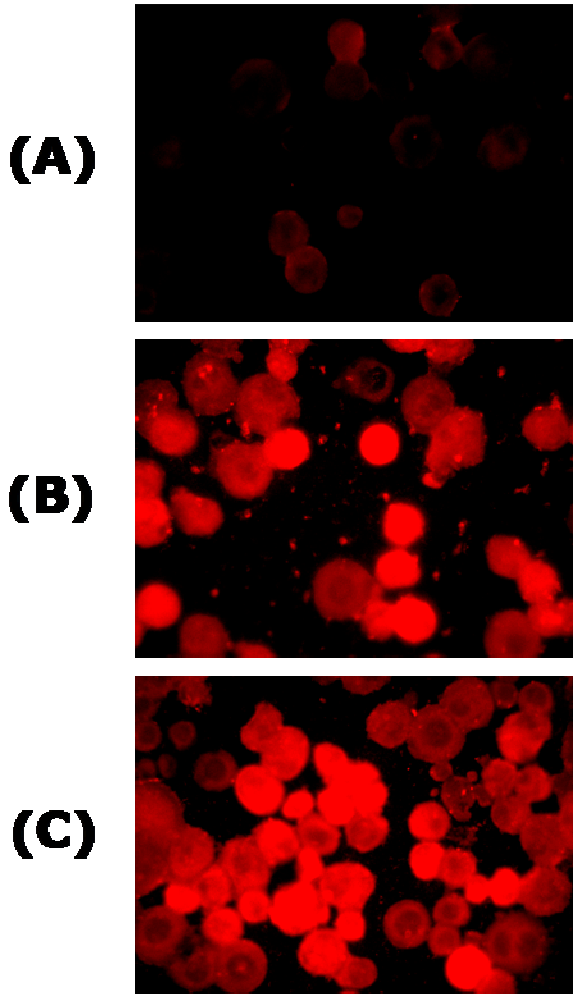


Figure 14. Immunofluorescent test of rGP5-specific antibody. Immunofluorescent cell staining was conducted in rGP5 protein expressing Hi-5 cells using sera collected from a uninoculated control pig (A), from a vaccinated pig at 3 weeks post booster vaccination (B), and a challenged pig at 24 days post infection (C). Hi-5 cells were inoculated with recombinant baculovirus at 5 MOI for 4 days. 2×10^4 cells were plated on the 8 well chamber slides, fixed with 1:1 solution of acetone and methanol, and incubated with the sera at 1:500 dilution followed by rhodamine-conjugated anti-swine IgG at 1:2,000 dilution. The fluorescence was visualized by a fluorescent microscope at 40× magnification.

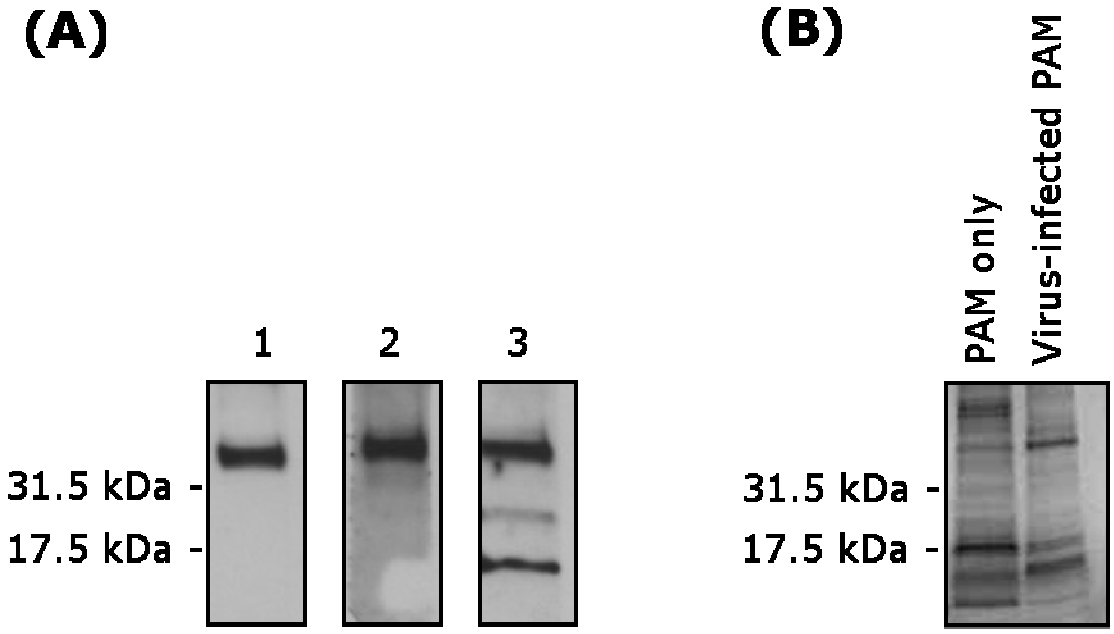


Figure 15. PRRSV-specific antibody. Panel A is a western blot analysis of PAM lysates from pigs infected with the NC Powell strain of PRRSV at 0.1 $TCID_{50}$/cell. 20 ug total cell lysates were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes, and detected using sera at 1:500 dilution and anti-swine IgG at 1:10,000. The used sera were collected from an uninoculated control pig (lane 1), from a vaccinated pig at 3 weeks post booster vaccination (lane 2), and a challenged pig at 24 days post infection (lane 3). Panel B is a silver staining of uninfected and virus-infected PAM lysates. Positions of molecular mass markers are given in kDa on the left.

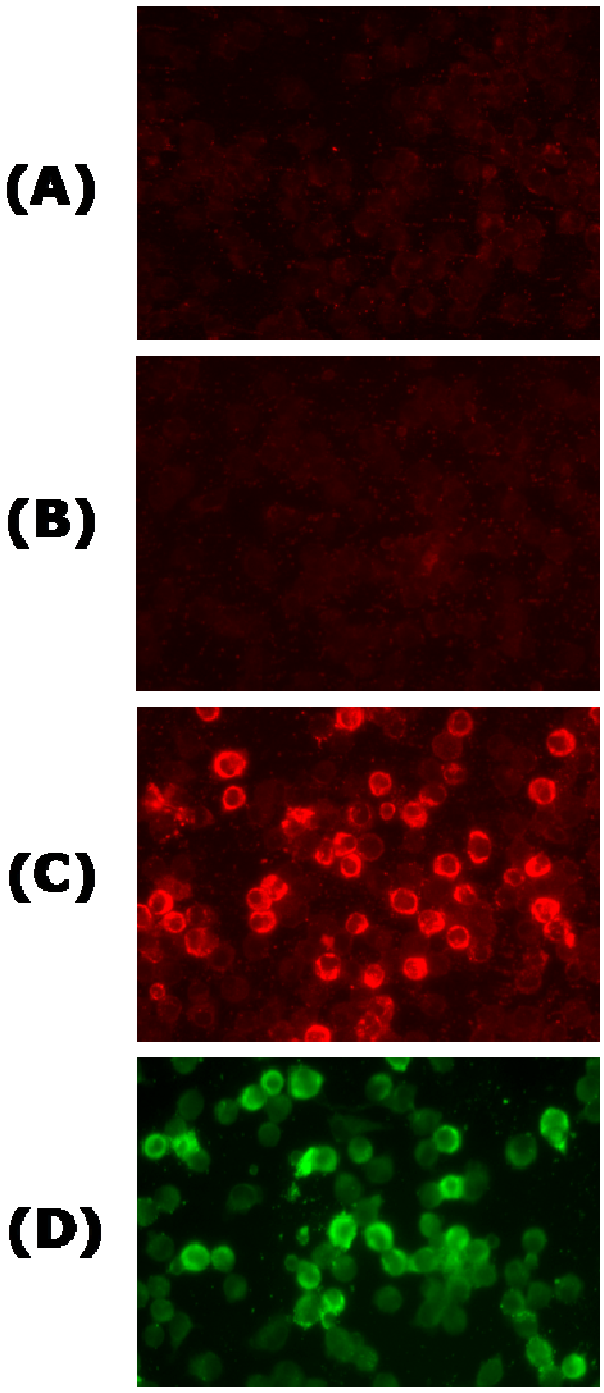

(A)
(B)
(C)
(D)

Figure 16. Immunofluorescent test of PRRSV-specific antibody. Immunofluorescent cell staining was conducted in a monolayer of PAM infected with the NC Powell strain of PRRSV using the sera collected from an uninoculated control pig (A), from a vaccinated pig at 3 weeks post booster vaccination (B), and a challenged pig at 24 days post infection (C). 2×10^5 cells were plated on the 8 well chamber slides and incubated with the NC Powell strain of PRRSV at 0.1 $TCID_{50}$/cell for 2days. The virus infected cells were fixed with 1:1 solution of acetone and methanol and incubated with the sera at 1:500 dilution followed by rhodamine-conjugated anti-swine IgG at 1:2,000 dilution. As positive control, PRRSV-infected cells were stained with FITC-conjugated SDOW-17, a mAb against the N protein of PRRSV (D). The fluorescence was visualized by a fluorescent microscope at 40× magnification.

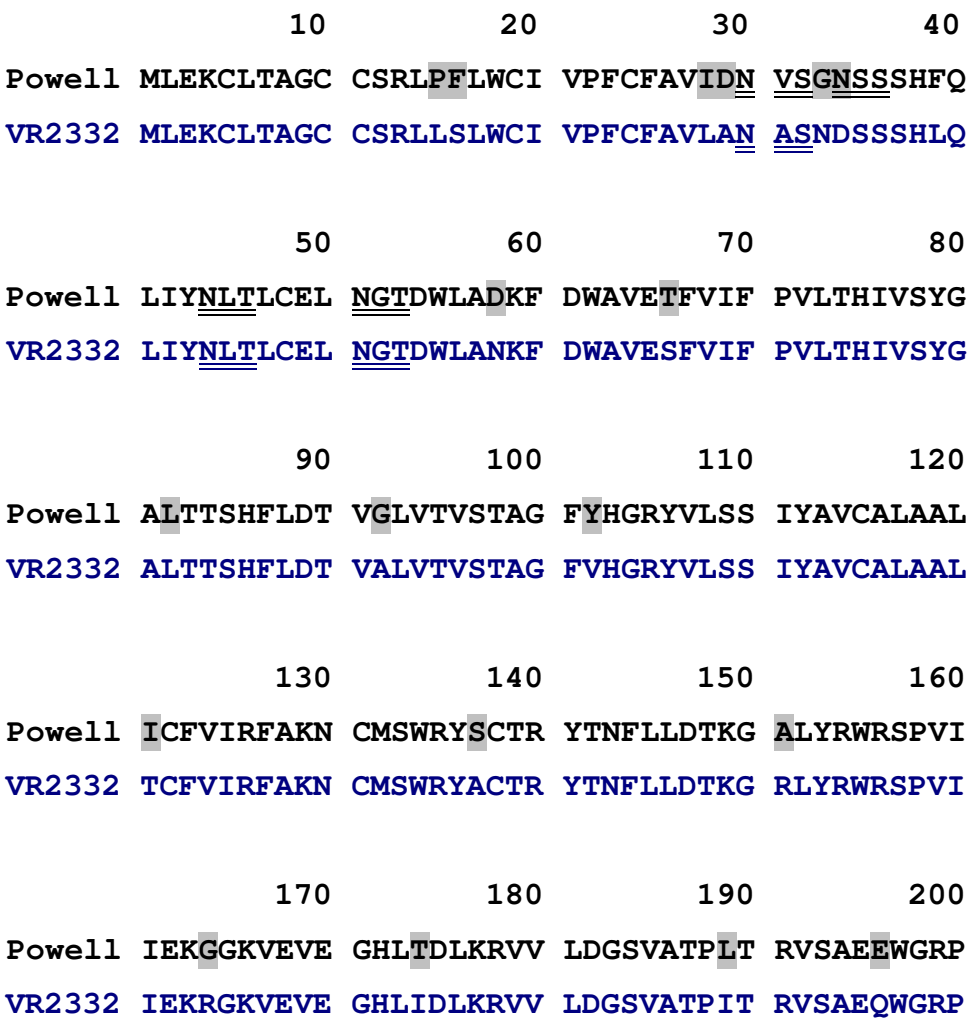


Figure 17. Alignment of the deduced amino acid sequence of the GP5 protein from NC Powell and VR2332, PRRSV strains. Different sequences from VR2332 are indicated in gray. Potential N-linked glycosylation sites (NXT or NXS) are double underlined.

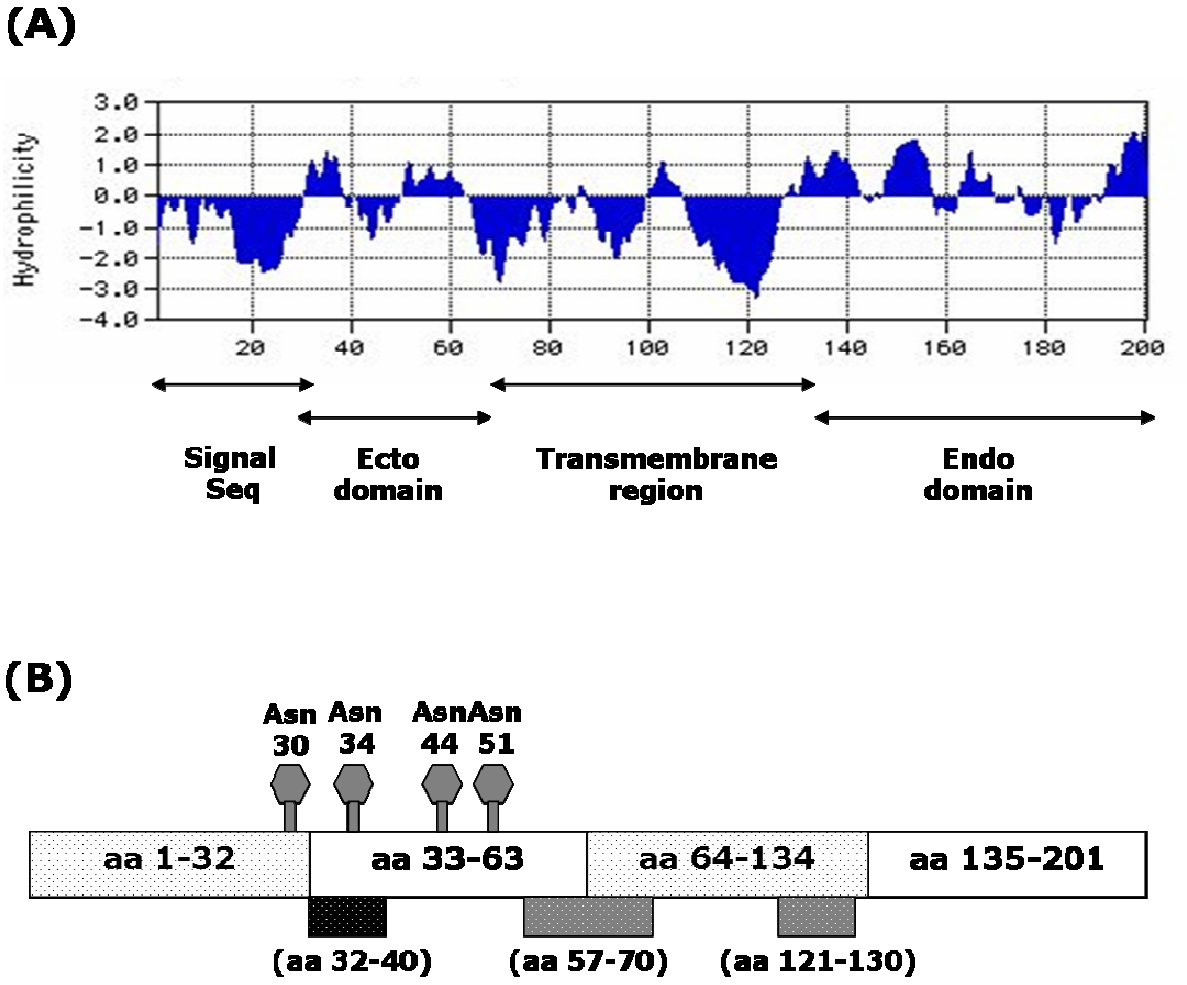
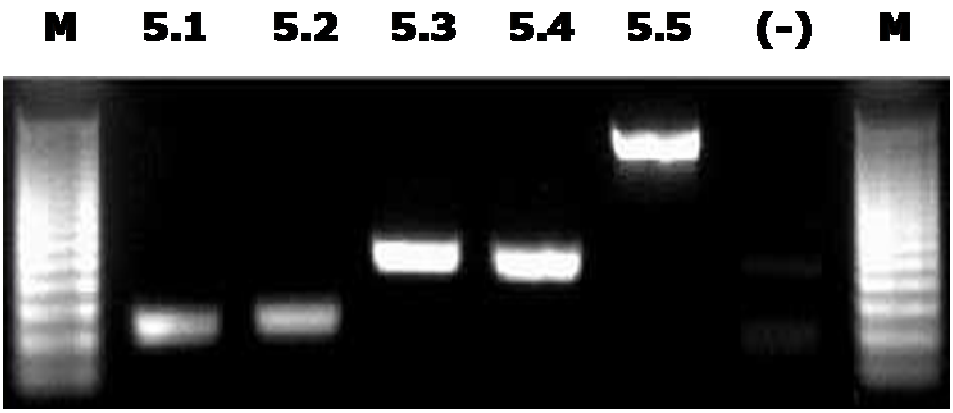
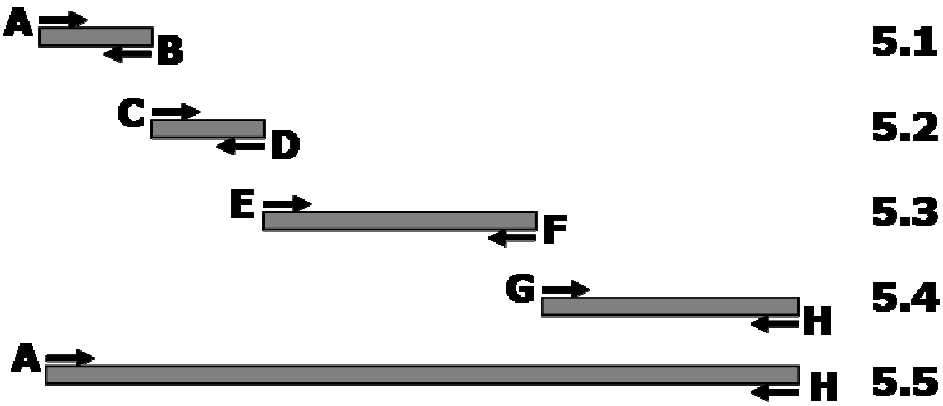


Figure 18. Schematic depiction of the GP5 protein with topology and genetic variability. The hydropathy profile of the deduced amino acid sequence of the GP5 protein from Powell, PRRSV strain is shown in (A). The amino acid residue numbers are shown on the horizontal axis, the average hydropathy are shown on the vertical axis. Hydrophilic regions appear above the middle line, hydrophobic regions appear below the middle line. The arrows identify the 4 putative domain regions; 1-32 (signal sequence), 33-63 (ectodomain), 64-134 (transmembrane region), and 135-201 (endodomain). The locations of genetically variable regions of GP5 protein among PRRSV isolates and glycosylation sites of the GP5 protein of Powell are indicated in (B). Glycosylation sites (grey) as well as hyper-variable region (black box) and variable regions (grey boxes) are depicted.





A 5′–CGGGATCC**ATG**TTGGAGAAATGC–3′

B 5′–GCTCTAGA**CTA**GCTGACGTTGTCGATCAC–3′

C 5′–CGGGATCC**ATG**GGCAACAGCAGCTC–3′

D 5′–GCTCTAGA**CTA**AGCCCAATCAAATTTGTCA–3′

E 5′–CGGGATCC**ATG**GTGGAGACTTTTGTC–3′

F 5′–GCTCTAGA**CTA**CCAGGACATGCAGT–3′

G 5′–CGGGATCC**ATG**CGCTACTCATGTACC–3′

H 5′–GCTCTAGA**CTA**AGGACGACCCCAT–3′

Figure 19. PCR productions of each domain of GP5 protein. The fragments were constructed using PCR on the pBlueBac-His/ORF5 clone. Each PCR product was subjected to electrophoresis and stained with ethidium bromide. The 5.1, 5.2, 5.3, 5.4, and 5.5 indicate PCR products belong to signal sequence, ectodomain, transmembrane, endodomain, and whole ORF5, respectively. M indicates 0.5 kb molecular weight marker and (-) represents a negative control. Primers used in PCR are indicated by single letters and primer sequences are shown below. Underline indicates BamHI or Xba I restriction site. Boldface indicates translation initiation or stop codon.

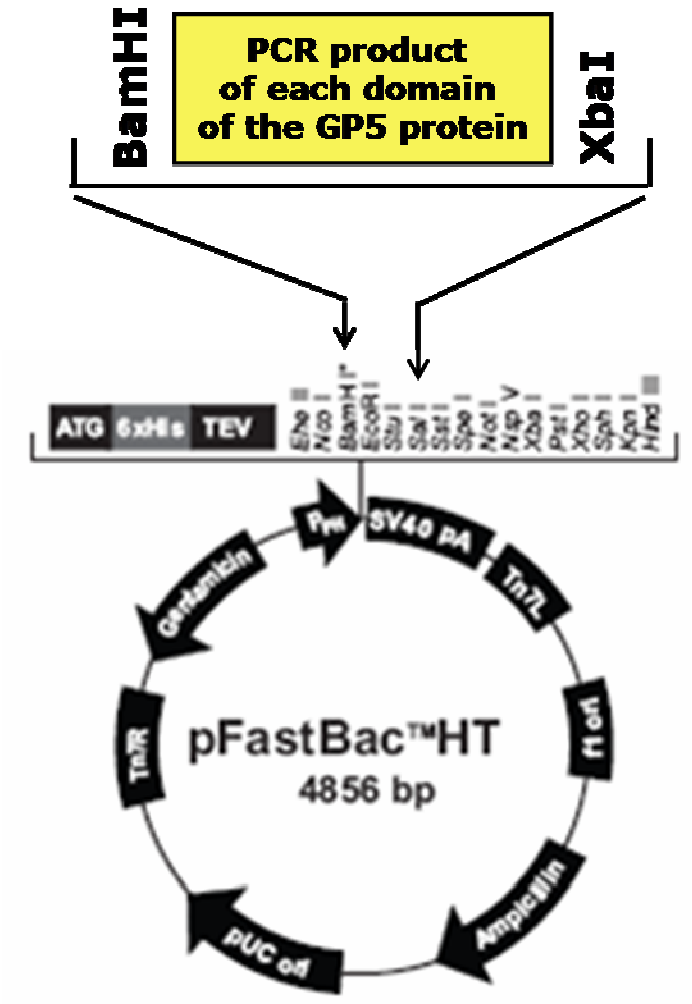
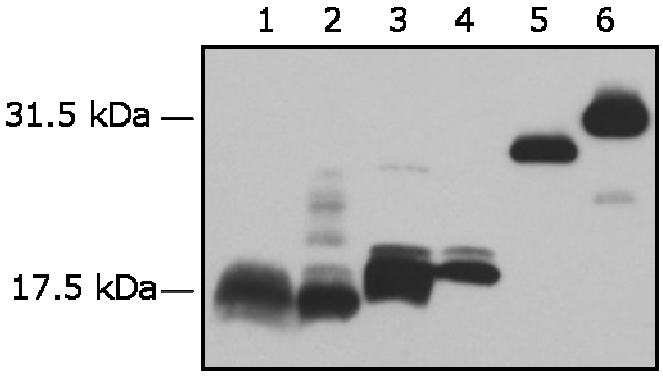
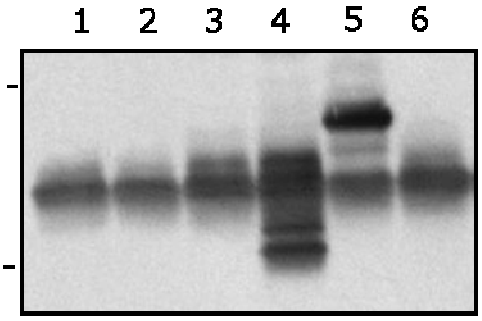


Figure 20. Diagram of baculovirus entry vector for expressing each domain of the GP5 protein. The recombinant pFastBac HTb donor plasmid was constructed by ligating the insert DNA into the BamHI-Sal I on the multiple cloning sites of the pFastBac HTb vector. The insert DNA was prepared by digesting the PCR products encoding each domain of the GP5 protein with restriction enzymes, Bam HI and Xba I. (The diagram of pFastBac HT vector was adapted from the Invitrogen website)

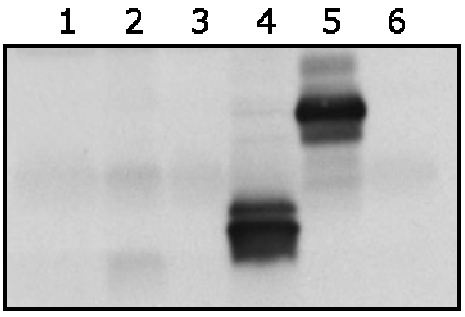


(A) Anti-His

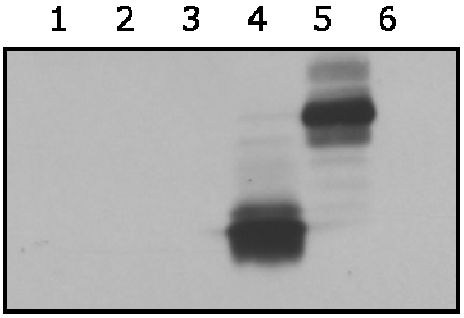
(B) Control



(C) Post-Vac



(D) Post-Vac/INF



(E) Post-INF

Figure 21. Epitope mapping of the GP5 protein. Western blot analysis was conducted with the domains of GP5 protein expressed in baculovirus infected Hi-5 cells. Cell lysates were separated by 16% SDS-PAGE, transferred to PVDF membranes, and immunostained with a panel of sera and anti-His Abs. Individually expressed domains of GP5 protein present in each lane (lane 1: aa 1-32 of GP5; lane 2: aa 1-63 of GP5; lane 3: aa 64-134 of GP5; lane 4: aa 135-201 of GP5; lanes 5: aa 1-201, whole GP5; lanes 6: CAT protein as a positive control). Panel A is a western blot developed with anti-His Abs to confirm the expression of each domain of GP5 protein. Panel B, C, D, and E show western blots developed with the porcine sera obtained from control pig (B), 3 weeks post second-vaccinated pig (C), 24 days post virus-infected pig after vaccination (D) and 24 days post virus-infected pig (E).

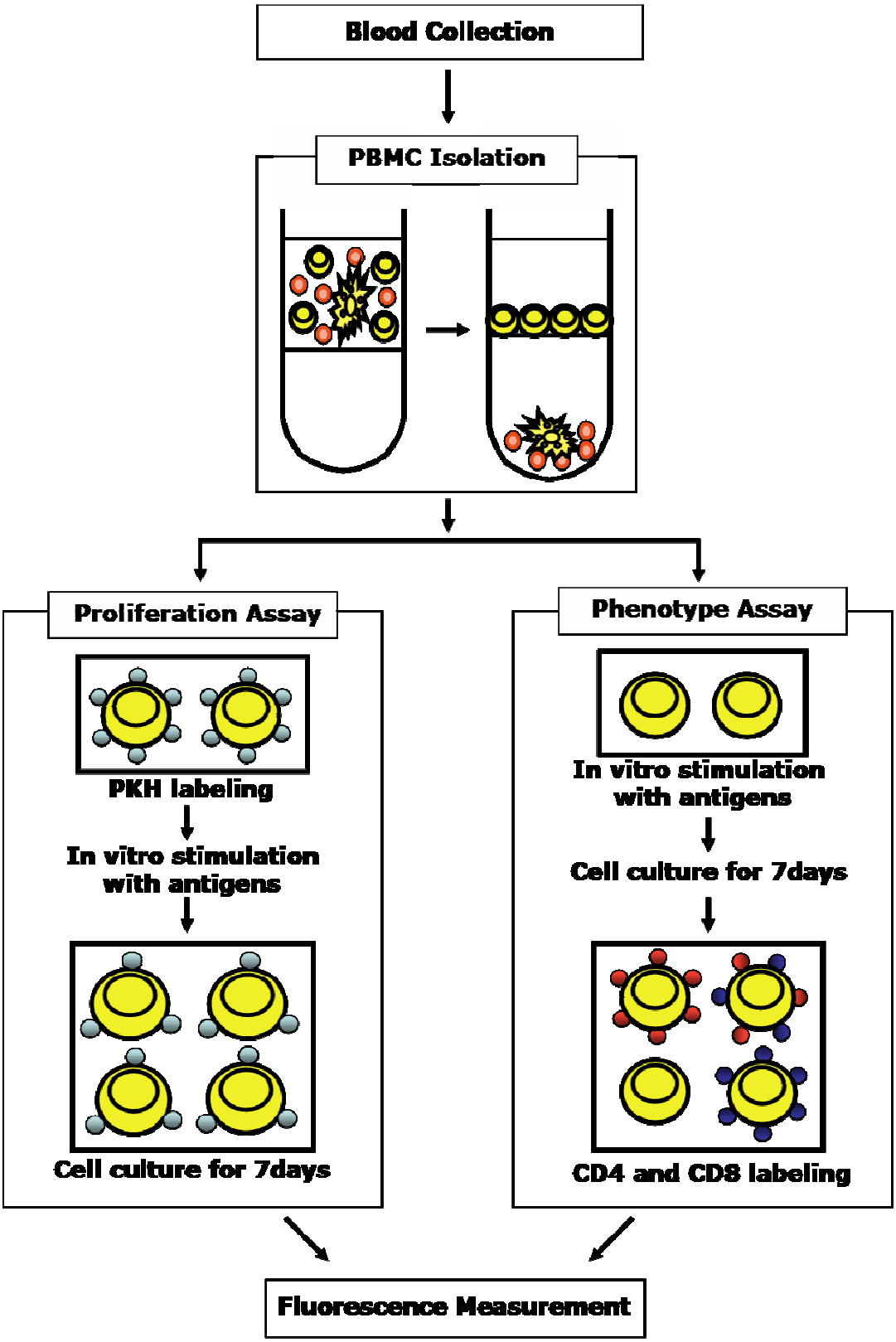

Blood Collection
PBMC Isolation
Proliferation Assay
PKH labeling
In vitro stimulation with antigens
Cell culture for 7days
Phenotype Assay
In vitro stimulation with antigens
Cell culture for 7days
CD4 and CD8 labeling
Fluorescence Measurement

Figure 22. Schematic representation for analyzing T cell responses using PBMC. Blood samples were collected from pigs and PBMC were isolated from whole blood by Ficoll-Hypaque centrifugation. The band of mononuclear cells consisting of lymphocytes was recovered at the interface. PBMCs were cultured in presence of *in vitro* stimulators. Cells were labeled with PKH for proliferation assay or with fluorochrome labeled anti-CD4 and anti-CD8 Abs for surface marker phenotype. Individual cells were analyzed using a flow cytometer.

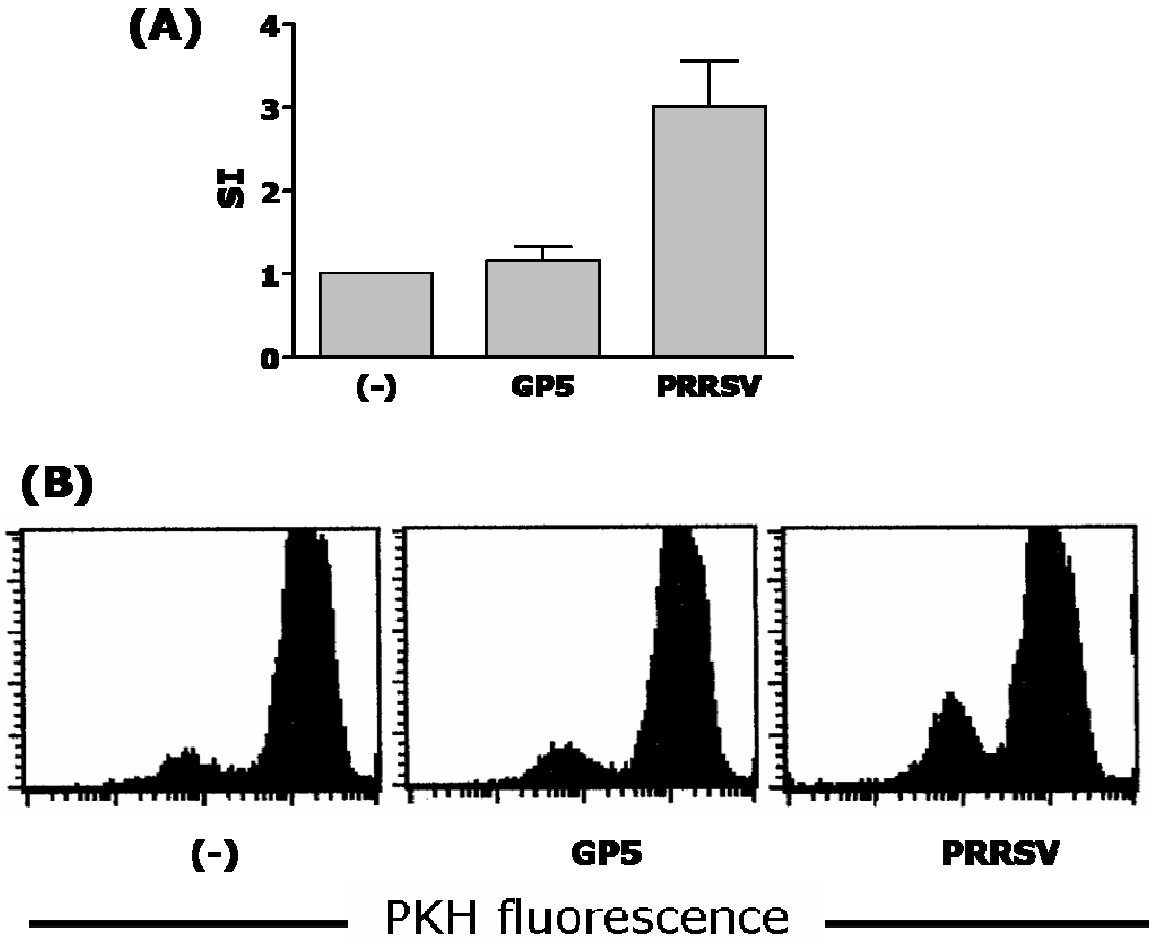


Figure 23. Proliferative response of lymphocyte in peripheral blood of normal pigs. 10^6 PBMCs/ml from the normal pigs at 14 weeks of age were labeled with 4×10^{-6} M PKH67 green fluorescent dye and then cultured in presence of *in vitro* stimulators (rGP5; 5 µg/ml or UV-inactivated NC Powell strain of PRRSV; 10 µl/ml of 10^3 $TCID_{50}$/ml) for 7 days. Antigen-stimulated cells were analyzed by evaluating the level of PKH staining. The results are displayed by Stimulation Index (SI) (A) and histograms of PKH fluorescent intensity (B). SI is defined as the ratio of the value of PKH^{dim} cells with the antigen to the value of PKH^{dim} cells with medium only (-). Bars represent mean value of the two animals ± standard deviation. The graph shown in (B) is representative of the two experiments.

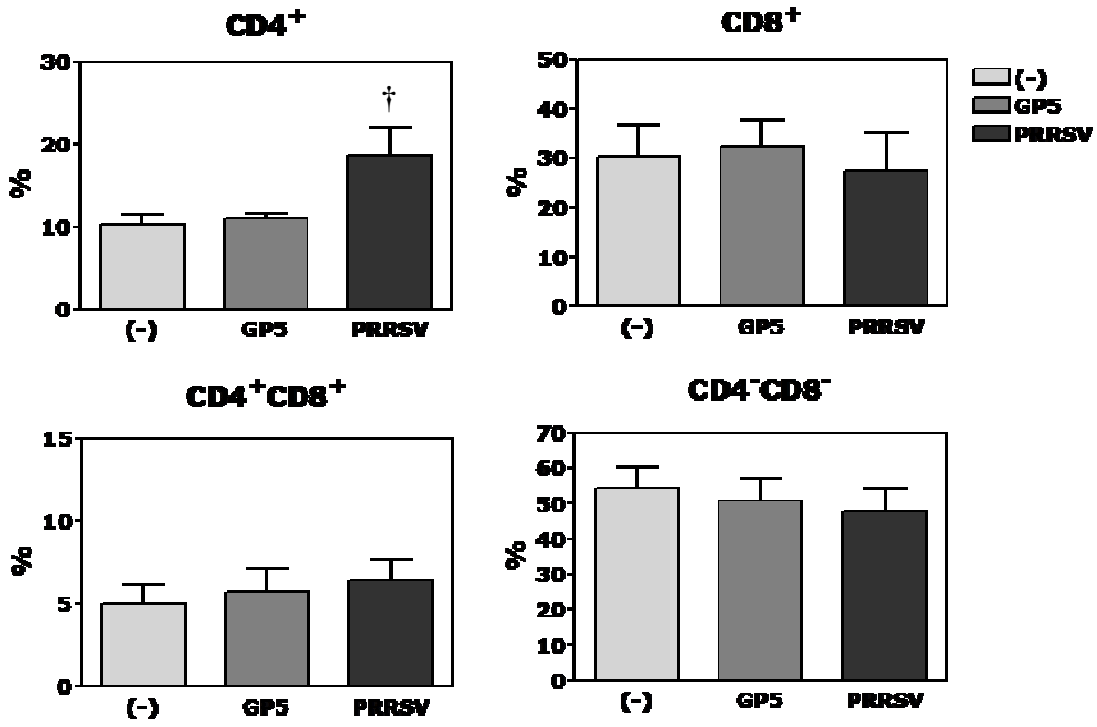


Figure 24. Percentage of lymphocyte subpopulations in peripheral blood of normal pigs. 10^6 PBMCs/ml from the three normal pigs at 14 weeks of age were cultured in presence of *in vitro* stimulators (rGP5; 5 µg/ml or UV-inactivated NC Powell strain of PRRSV; 10 µl/ml of 10^3 $TCID_{50}$/ml) for 7 days. Antigen stimulating cells were analyzed by evaluating cell surface CD4 and CD8 phenotypes using two-color cytometry. Bars represent mean value ± standard deviation. † ($p = 0.0741$)

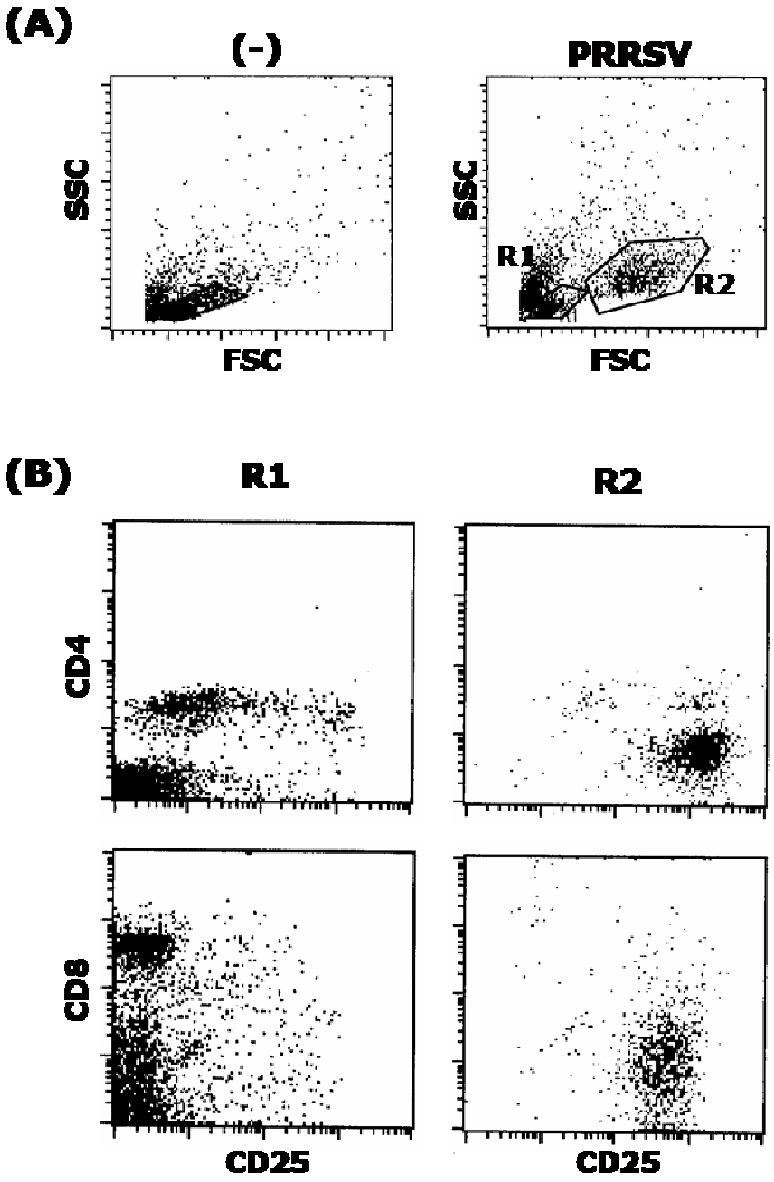


Figure 25. Two-color flow cytometric analysis of CD4, CD8, and CD25 expression on naïve PBMCs responding to NC Powell PRRSV. PBMC from naive pigs was cultured in presence of UV-inactivated NC Powell strain of PRRSV at 10 µl/ml of 10^3 $TCID_{50}$/ml for 7 days. Antigen stimulating cells were analyzed by evaluating cell surface CD4, CD8, and CD25 phenotypes using two-color cytometry. Regions corresponding to resting (R1) and activated cells (R2) were defined based on their characteristic SSC and FSC profiles as indicated in the dot-plot on the (A). Samples of cells responding to PRRSV are CD4 $^{dim}CD8^{-}CD25^{+}$ (B).

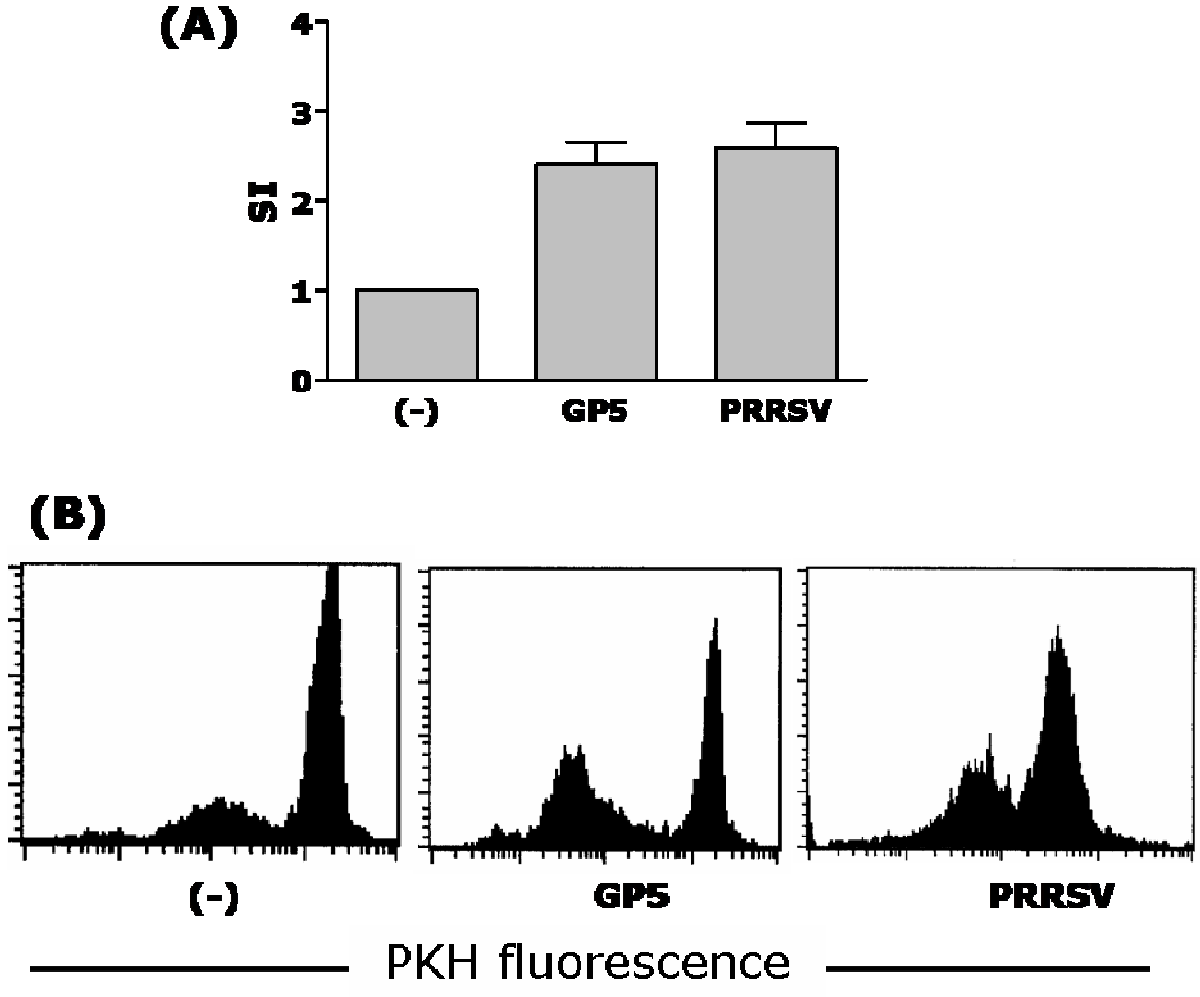


Figure 26. Proliferative response of lymphocyte in peripheral blood of the rGP5 - vaccinated pigs. 10^6 PBMCs/ml from the rGP5 vaccinated pigs at 3 weeks post booster vaccination were labeled with 4×10^{-6} M PKH67 green fluorescent dye and then cultured in presence of *in vitro* stimulators (rGP5; 5 µg/ml or UV-inactivated NC Powell strain of PRRSV; 10 µl/ml of 10^3 $TCID_{50}$/ml) for 7 days. Antigen-stimulated cells were analyzed by evaluating the level of PKH staining. The results are displayed by Stimulation Index (SI) (A) and histograms of PKH fluorescent intensity (B). SI is defined as the ratio of the value of PKH^{dim} cells with the antigen to the value of PKH^{dim} cells with medium only (-). Bars represent mean value of the two animals ± standard deviation. The graph shown in (B) is representative of the two experiments.

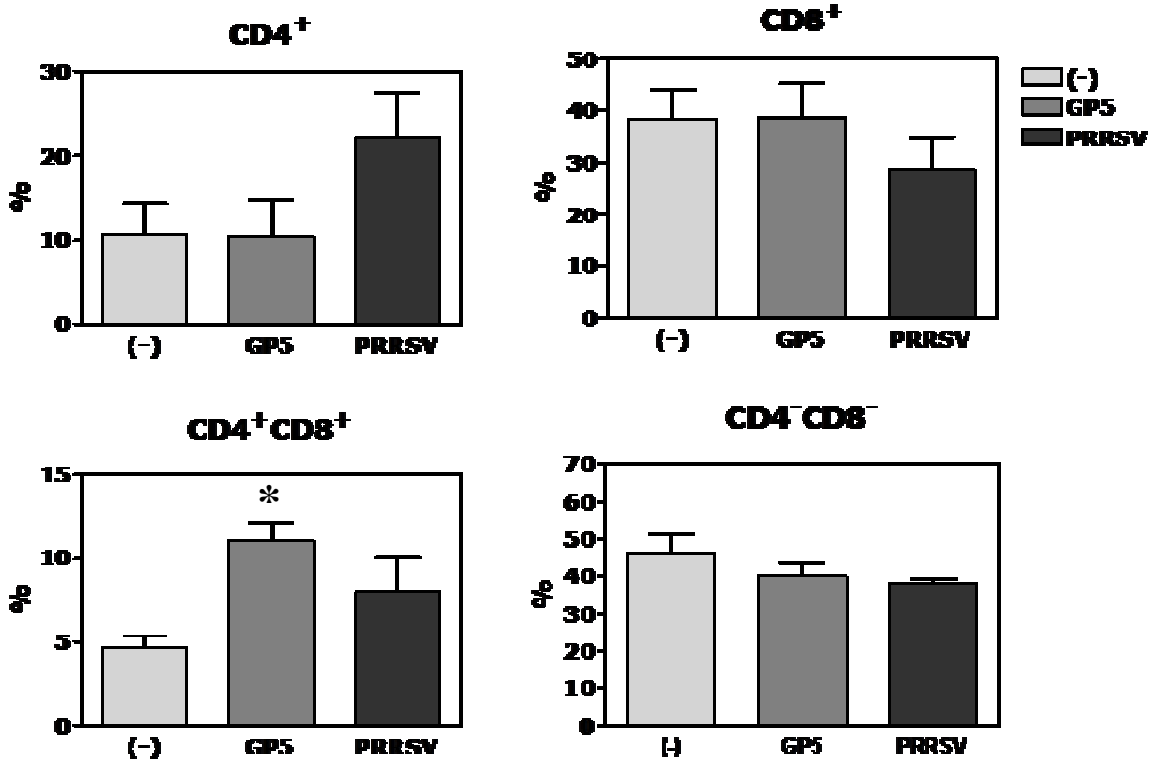


Figure 27. Percentage of lymphocyte subpopulations in peripheral blood of the rGP5 - vaccinated pigs. 10^6 PBMCs/ml from the three vaccinated pigs at three weeks post booster vaccination were cultured in presence of *in vitro* stimulators (rGP5; 5 µg/ml or UV-inactivated NC Powell strain of PRRSV; 10 µl/ml of 10^3 $TCID_{50}$/ml) for 7 days. Antigen stimulating cells were analyzed by evaluating cell surface CD4 and CD8 phenotypes using two-color cytometry. Bar represents mean value ± standard deviation. * ($p<0.05$) Statically significant as compared with un-stimulated (-) control.

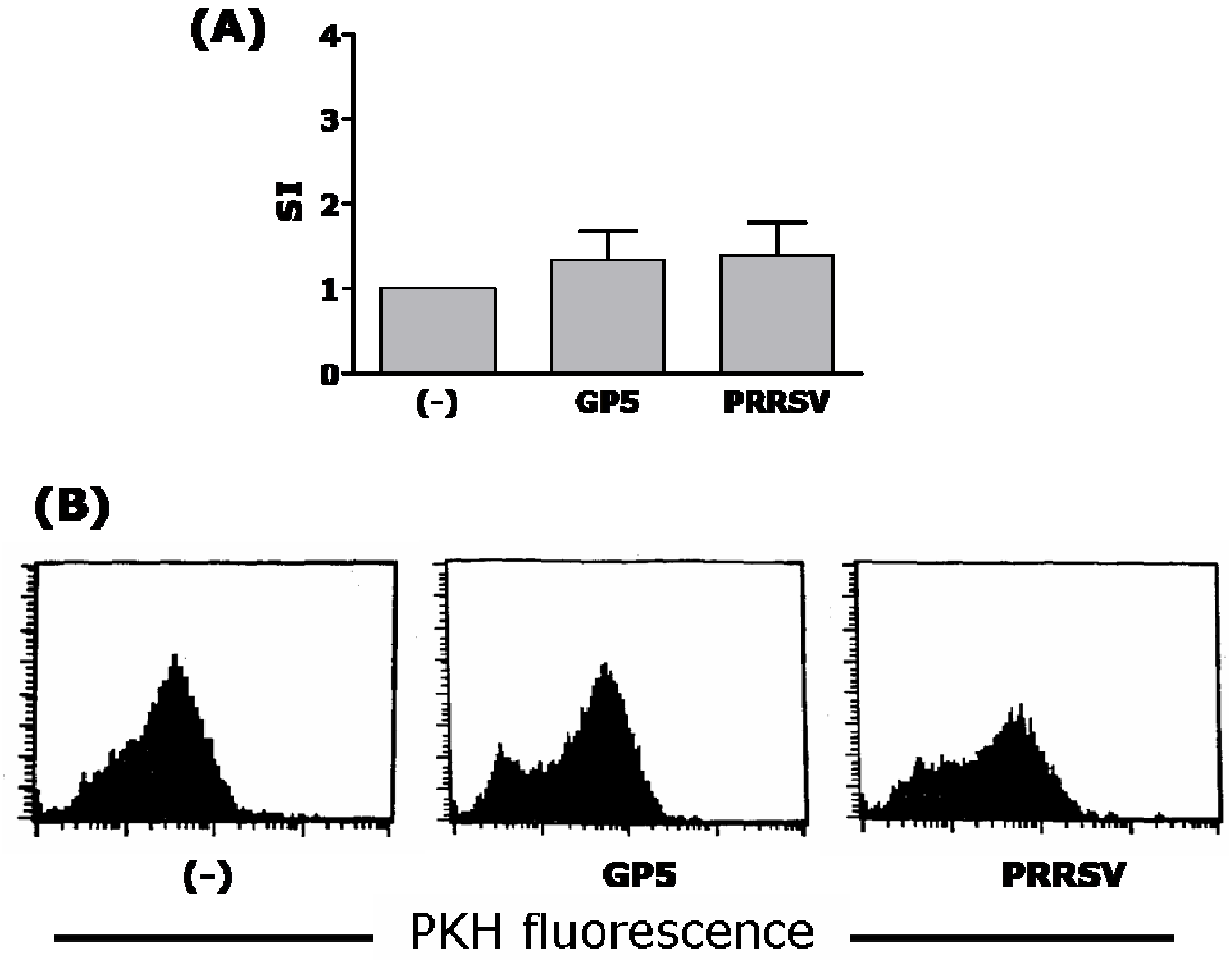


Figure 28. Proliferative response of lymphocyte in peripheral blood of NC Powell PRRSV-infected pigs. 10^6 PBMCs/ml from the NC Powell PRRSV-infected pigs were labeled with 4×10^{-6} M PKH67 green fluorescent dye and then cultured in presence of *in vitro* stimulators (rGP5; 5 µg/ml or UV-inactivated NC Powell strain of PRRSV; 10 µl/ml of 10^3 $TCID_{50}$/ml) for 7 days. Antigen-stimulated cells were analyzed by evaluating the level of PKH staining. The results are displayed by Stimulation Index (SI) (A) and histograms of PKH fluorescent intensity (B). SI is defined as the ratio of the value of PKH^{dim} cells with the antigen to the value of PKH^{dim} cells with medium only (-). Bars represent mean value of the two animals ± standard deviation. The graph shown in (B) is representative of the two experiments.

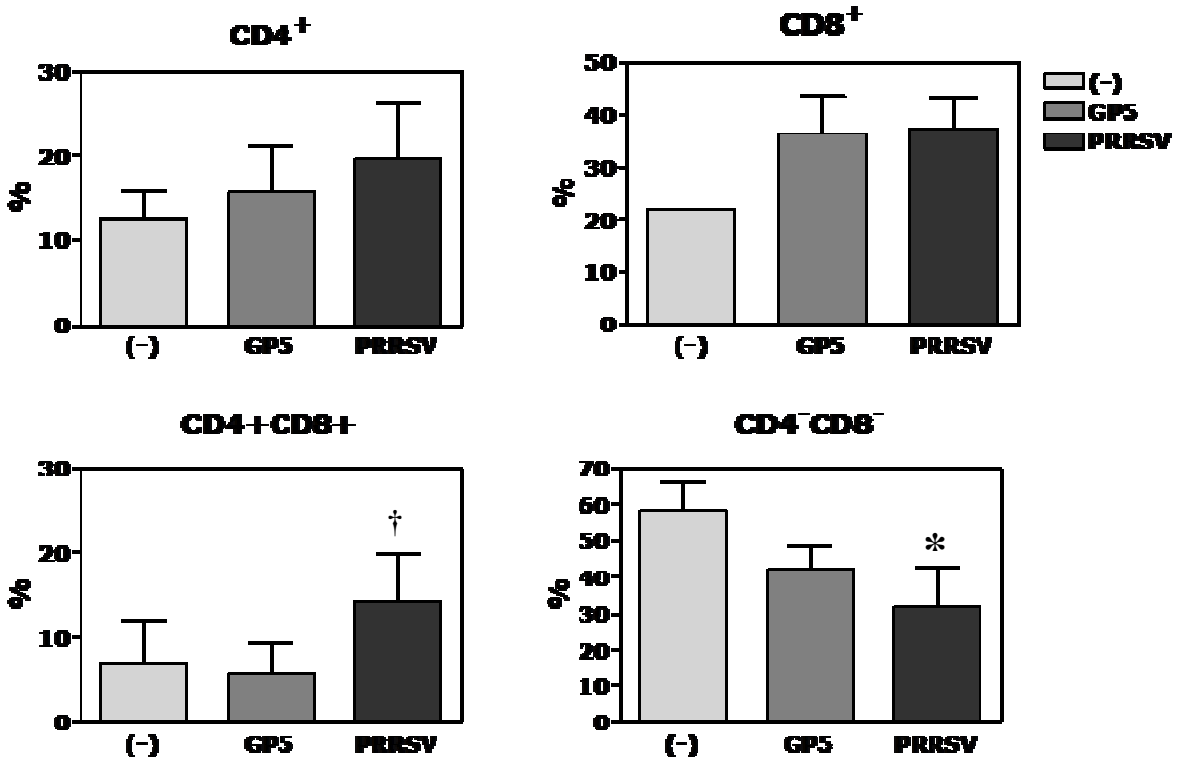


Figure 29. Percentage of lymphocyte subpopulations in peripheral blood of NC Powell PRRSV-infected pigs. 10^6 PBMCs/ml from three NC Powell PRRSV-infected pigs cultured in presence of *in vitro* stimulators (rGP5; 5 µg/ml or UV-inactivated NC Powell strain of PRRSV; 10 µl/ml of 10^3 $TCID_{50}$/ml) for 7 days. Antigen stimulating cells were analyzed by evaluating cell surface CD4 and CD8 phenotypes using two-color cytometry. Bar represents mean value ± standard deviation. * ($p<0.05$) Statically significant as compared with un-stimulated (-) control. † ($p = 0.0533$)

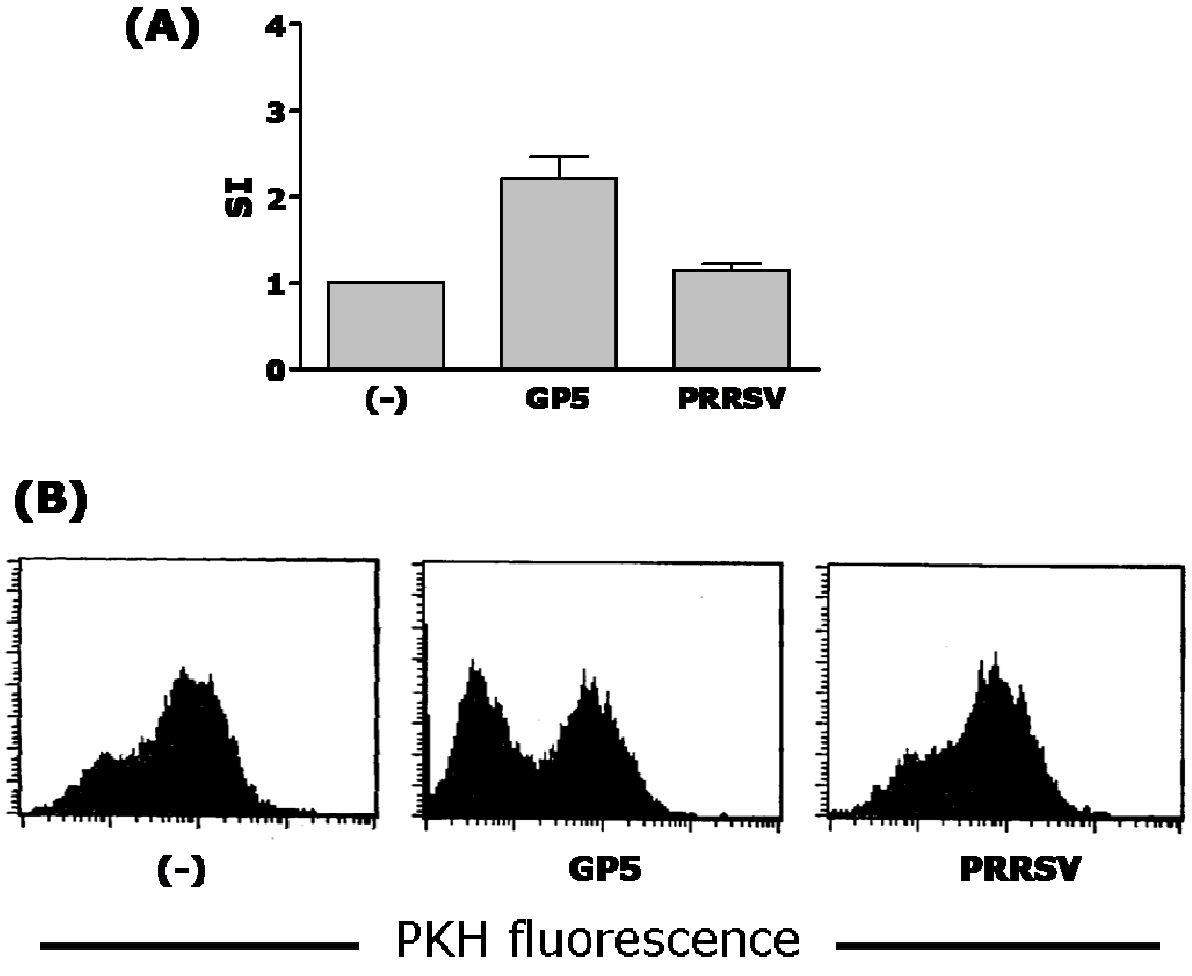


Figure 30. Proliferative response of lymphocyte in peripheral blood of the rGP5-vaccinated and NC Powell PRRSV-challenged pigs. 10^6 PBMCs/ml from the two rGP5-vaccinated and subsequently NC Powell PRRSV-challenged pigs were labeled with 4×10^{-6} M PKH67 green fluorescent dye and then cultured in presence of *in vitro* stimulators (rGP5; 5 μg/ml or UV-inactivated NC Powell strain of PRRSV; 10 μl/ml of 10^3 $TCID_{50}$/ml) for 7 days. Antigen-stimulated cells were analyzed by evaluating the level of PKH staining. The results are displayed by Stimulation Index (SI) (A) and histograms of PKH fluorescent intensity (B). SI is defined as the ratio of the value of PKH^{dim} cells with the antigen to the value of PKH^{dim} cells with medium only (-). Bars represent mean value of the two animals ± standard deviation. The graph shown in (B) is representative of the two experiments.

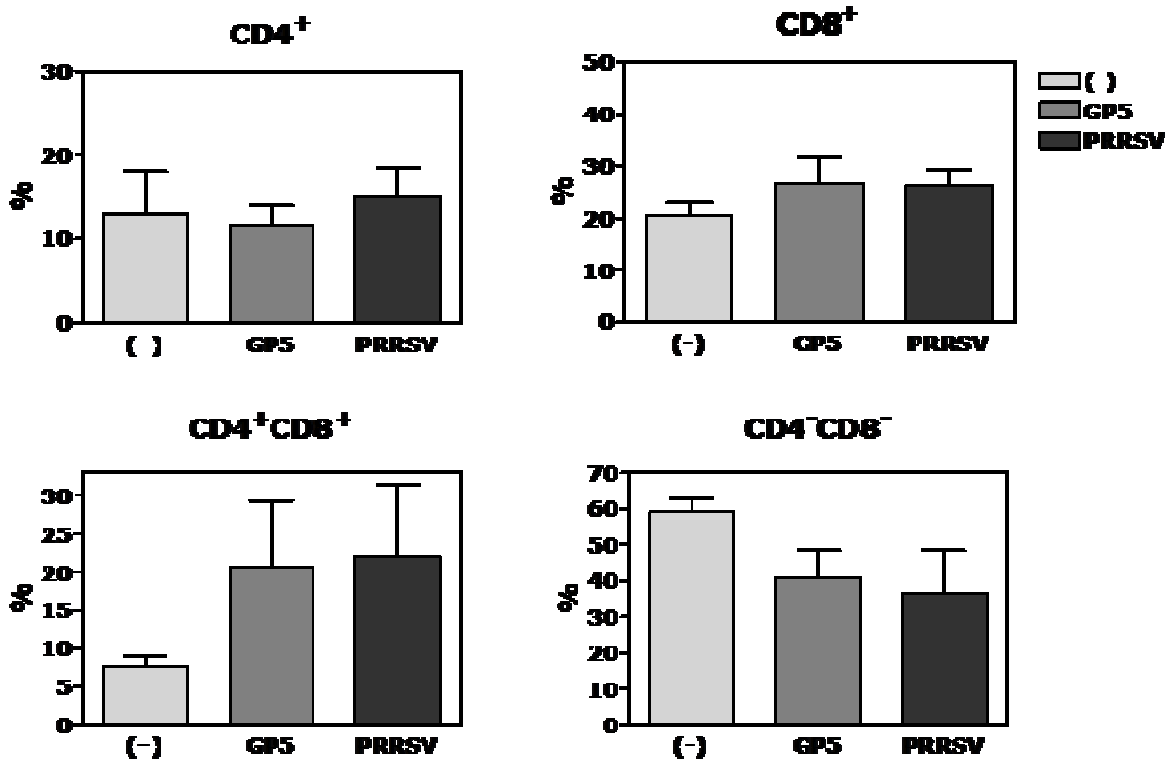


Figure 31. Percentage of lymphocyte subpopulations in peripheral blood of the rGP5 - vaccinated and subsequently NC Powell PRRSV-challenged pigs. 10^6 PBMCs/ml from the two rGP5-vaccinated and subsequently NC Powell PRRSV-challenged pigs were cultured in presence of *in vitro* stimulators (rGP5; 5 µg/ml or UV-inactivated NC Powell strain of PRRSV; 10 µl/ml of 10^3 $TCID_{50}$/ml) for 7 days. Antigen stimulating cells were analyzed by evaluating cell surface CD4 and CD8 phenotypes using two-color cytometry. Bar represents mean value ± standard deviation.

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