USE OF ADJUVANTS TO INCREASE EFFICACY OF PRRSV MODIFIED LIVE VACCINES

by

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M.S., China Agricultural University, Beijing, China, 2006

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important swine diseases worldwide that leads to severe reproductive failure in sows and high mortality in young pigs. Vaccination is currently the most effective way to control this disease. The protection ability provided by vaccines however is limited due to the large diversity of field PRRSV strains. In chapter 2, we compared immune responses induced by vaccination and/or PRRSV infection by using IngelVac® Modified Live PRRSV vaccine (MLV), its parental strain VR-2332, and the heterologous KS-06 strain. Our results showed that MLV provide complete protection to homologous virus and partial protection to heterologous challenge. The protection was associated with the levels of PRRSV neutralizing antibodies at the time of challenge.

Besides developing new vaccines to combat PRRSV, adjuvants have been applied to PRRSV MLV vaccines to induce vaccination-mediated cross-protection against genetically dissimilar PRRSV strains. In chapter 3, we demonstrated that a commercial Montanide^{TM} Gel01ST adjuvant provides enhanced protection to homologous PRRSV infection by regulating the production of PRRSV-specific antibodies. In chapter 4, we tested a novel peptide nanofiber hydrogel acting as a potent adjuvant for PRRSV MLV vaccines. We found that the hydrogel adjuvant enhanced vaccine efficacy by developing of higher titers of neutralizing antibodies and stronger IFN-γ cellular immune responses.

Chinese highly pathogenic PRRSV (HP-PRRSV) variants were isolated in 2006 and they belong to genotype 2 of PRRSV. Compared with classic PRRSV, HP-PRRSV is characterized by robust proliferation ability and high morbidity/mortality with all ages of pigs. In chapter 5, we compared the difference of immune responses elicited by HV-PRRSV, a Chinese HP-PRRSV, and a US virulent strain of PRRSV NADC-20. Traditional PRRSV MLV vaccines developed in US offer no protection to HP-PRRSV. Vaccines specific to HP-PRRSV strains available in China provide protection to HP-PRRSV. In chapter 6, we demonstrated that pigs challenged with US NADC-20 strain were protected by vaccination with Chinese MLV HP-PRRSV vaccines. The availability of Chinese HP-PRRSV vaccines in North America may act to increase the preparedness of possible transmission of HP-PRRSV to North America.

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Chapter 1 - Introduction to PRRSV

1.1. History of PRRS

Porcine reproductive and respiratory syndrome (PRRS) was first characterized in 1987 in the United States of America with a clinical presentation of severe reproductive losses in late gestational sows and perinatal losses and respiratory distress of piglets [1]. The etiological agent, PRRS virus (PRRSV), was identified in Europe in 1991 and termed Lelystad virus [2]. PRRSV was subsequently isolated in the US and assigned the name VR-2332 [3]. Since then, PRRS has become an endemic disease in the global swine industry and has led to huge economic losses in the pork industry [4].

PRRSV seems to evolve by random mutation and intragenic recombination events that led to the emergence of different variants with high pathogenicity to pigs, such as the recent outbreak of Chinese highly pathogenic PRRSV in China in 2006, the high virulent 1-18-2 strain in U.S. in 2007, and east European subtype 3 PRRSV isolate Lena strain in Europe [5-7]. Classic PRRSV causes mild clinical symptoms and leads to abortion in sows and death of piglets. In contrast, these highly pathogenic strains of PRRSV lead to increased tissue atrophy and greater morbidity and mortality rate in all ages of pigs [5].

1.2. PRRS virus and strain heterogeneity

PRRSV is a small, enveloped positive-strand RNA virus, which belongs to the family *Arteriviridae* in the order of the *Nidovirales* that include members of the *Coronaviridae* and *Roniviridae* families [8]. The family *Arteriviridae* also comprises three other viruses: equine arteritis virus (EAV), simian hemorrhagic fever virus (SHFV), and lactate dehydrogenase elevating virus (LDV).

PRRSV isolates are divided into two distinct genotypes: the European (Type 1) and North American (Type 2). The two genotypes of PRRSV cause the same disease symptoms but are antigenically different. The two genotypes differ approximately 60% from each other at the genomic sequence level [9]. Within the European PRRSV genotype, three subtypes have been further delineated based on ORF5 and ORF7, namely a Pan-European subtype 1, East European subtype 2, and subtype 3 [10].

PRRSV is a roughly spherical virion with a diameter of 50-60 nm and buoyant densities of 1.13-1.17 g cm^{-3} in sucrose [11]. PRRSV is one of the most rapidly evolving viruses and its evolutionary rate of $1\text{-}3\times10^{-2}$ substitution per year is the highest among RNA viruses reported so far [12]. Field isolates of PRRSV exhibit considerable sequence heterogeneity of up to 20% [13]. Among these structural proteins, the major envelope protein GP5 is the most variable protein, with 50-100% amino acid identities among different field isolates [14], most likely because GP5 induces neutralizing antibodies and therefore exposed to selective antibody pressure. NSP2 is the most variable nonstructural protein among 14 nonstructural proteins. As the largest PRRSV protein, NSP2 is tolerant for mutations, deletions, and insertions, which further contribute to the variability of NSP2 [15]. PRRSV NSP2 is an immune-dominant protein with the ability to induce a strong humoral antibody and cellular immune response [16]. Like the variability of GP5, natural deletions and hyper-variability of NSP2 may also work as a strategy that virus compromise host immunity. All the above facts make it difficult to develop efficient vaccines with cross-protection to different field isolates.

1.3. PRRSV genome organization

The PRRSV genome is a single-stranded, positive-sense RNA, which is approximately 15 kb in length and consists of a 5'-untranslated region (UTR), nine open reading frames (ORFs), ORF1a, ORF1b, ORF2a, ORF2b, ORF3, ORF4, ORF5, ORF6, and ORF7, followed by a 3'-UTR and a poly (A) tail [11]. The ORF1a and ORF1b located in the 5'-proximal part consists of approximately 75% of the genome and encodes 14 putative non-structural proteins which are mostly involved in genome replication and subgenomic mRNA transcription. These NSPs work as viral proteases (NSP1α, NSP1β, NSP2 and NSP4), a RNA-dependent RNA polymerase (NSP9), a helicase (NSP10) and an endonuclease (NSP11) during viral replication [17]. Several

NSPs also modulate host immune response for more efficient viral replication. NSP2 contains several immunodominant B-cell epitopes that are dispensable for virus replication [18]. NSP1α, NSP1β, NSP2, NSP4 and NSP11 attenuate type I IFN response by inhibiting the activation of the interferon beta (IFN-β) promoter [19].

The 3'-proximal part of the genome encodes seven PRRSV structural proteins that are translated from a 3'-coterminal nested set of six subgenomic mRNAs. ORF2a, ORF2b, and ORFs 3-7 encode viral structural proteins GP2a, GP2b, GP3, GP4, GP5, M, and N, respectively[11]. Four of them are membrane-associated N-glycosylated proteins (GP2a, GP3, GP4 and GP5) and two are non-glycosylated membrane proteins (GP2b and M). The nucleocapsid protein (N) encapsulates the viral RNA genome. The GP5 and M proteins are the two major envelope proteins in PRRSV which exist as a disulfide-linked heterodimer in the virion [20]. The GP5 protein is also the most abundant enveloped glycoprotein containing major neutralizing epitopes [21]. GP5a, this protein is encoded by an alternative reading frame of subgenomic mRNA GP5, responsible of recognizing the cell receptor in target cells [22]. Nucleocapsid protein, existing as a dimer, is highly immunogenic and serves as the main diagnostic protein to detect antibodies to PRRSV [23]. As for the minor proteins, GP2a, GP3 and GP4 form a structural trimer important for viral tropism and entry into cells [24]. Studies on PRRSV non-structural and structural proteins provide new insights on PRRSV biology and vaccine development.

1.4. PRRSV clinical and pathological aspects

Clinical signs of PRRSV infection vary with the strains of virus. PRRSV causes piglets to develop clinical signs including dyspnea, anorexia, lethargy, cutaneous hyperemia, and decreased body weight gain [25]. The infection of PRRSV sometimes leads to reddish to blue discoloration and blotching of the skin, most often of the ears, which give PRRS the name of "Blue ear disease". Subclinical infection often occurs in finishing pigs, boars, gilts and sows, and the clinical symptoms are mild. Pregnant sows infected pigs the clinical signs including infertility, lowered farrowing rates, increased abrogation rate in late gestation, and stillborn, mummified or weak live born piglets [26]. Sows seldom develop respiratory symptoms and they can transplacentally transmit virus to their unborn piglets. HP-PRRSV infected piglets

characterized by high body temperature (>41°C), rubefaction on the skin, respiratory disorder, and high morbidity (50-100%) and mortality (20-100%) in all age of affected pigs [27]. Most of infected pigs showed obvious respiratory distress such as sneezing, coughing, dyspnea, increased eye secretion, conjunctivitis, constipation and diarrhea. As for the infected pregnant sows, the abortion rate is more than 40%, and the mortality of sows is usually 10% [27].

PRRSV produces a multi-systemic infection in pigs, but gross lesions are usually only observed in respiratory and lymphoid tissues. PRRSV-infected pigs show mottled, tan and red lung and lymph nodes are moderately to severely enlarged and tan in color. Microscopic examination reveals moderate to severe multifocal interstitial pneumonia characterized by alveolar septal infiltration by a mixed population of mononuclear cells, hypertrophy and hyperplasia of pneumocytes and marked mixed inflammatory and necrotic alveolar exudate [26]. PRRSV has a tropism for macrophages and replicates mainly in macrophages of the lymphoid tissues and lungs in the acute phase of infection and persists in tonsil and lung macrophages. However, HP-PRRSV exhibits more extensive tissue tropism than classic PRRSV [28]. Besides lymphoid tissues, immunohistochemistry examination shows that HP-PRRSV antigen can also be detected in the tissues including trachea, esophagus, liver, kidney, cerebellum, stomach, and intestine, which prove its high pathogenicity to pigs [29].

1.5. Host anti-PRRSV immunity

PRRSV infection results in weak and delayed immune responses that lead to long-lasting viremia in the blood and lymphoid tissues. PRRSV circumvents the host immune response by suppression of type I interferon (IFN) production, generation of non-neutralizing antibodies in the early stage of infection and low titers of protective neutralizing antibody (NAb), and impaired T cell-mediated immune (CMI) response [30]. The study of mechanisms of PRRSV used to escape host immunity is still undergoing, and better understanding of these mechanisms facilitates to develop more robust vaccines to control PRRS.

The innate immune response against PRRSV is very weak [19]. PRRSV does not induce IFN-α production a key element in host antiviral response, leading to a minimal production of

inflammatory cytokines and activation and recruitment of natural killer (NK) cells [31]. PRRSV infection also compromises the production of IFN-β at transcriptional and post-transcriptional level in macrophages and dendritic cells [32, 33]. PRRSV NSP1α and NSP1β were proposed to be major modulators of type I IFN cytokine expression in the early infectious process, and type I IFNs may be instrumental in effective induction of adaptive immunity and memory responses [17, 34-36]. For this reason, lack of type I interferon production is assumed to reduce the overall immune response.

PRRSV also compromises the adaptive immunity by utilizing different mechanisms. A hallmark of the swine humoral response against PRRSV is the production of non-neutralizing antibodies detected early in the infection, followed by a low neutralizing antibody titer that is detected more than 3 weeks after infection [37]. These non-neutralizing antibodies are involved in antibody-dependent enhancement (ADE) of PRRSV infection, which facilitate the entry of virus into target cells, leading to increased infectivity [38]. ADE may suppress the innate antiviral response, especially type I IFN system of the host [39]. Moreover, ADE enables the viruses to benefit from the anti-inflammatory and immunosuppressive environment created by autocrine and paracrine IL-10 production [40].

PRRSV neutralizing antibodies play a critical role in clearance of virus and are able to completely protect pigs against PRRSV re-infection [41]. As mentioned above, GP5 is the major structural protein that elicits the production of neutralizing Abs. Besides the high variability of GP5 protein among different strains of PRRSV, which leads to less cross-protective immunity, the presence of two decoy epitopes and glycan-shielding of the epitope critical for neutralization in GP5 protein were also proposed to explain the delay in NAb induction [42].

PRRSV infection results in a weak and delayed T cell mediated immune response that should be necessary for the elimination of the virus [43]. It has been shown that the induction of IFN-γ secreting cells, complementing neutralizing antibodies, provides partial protection against PRRSV [44,45]. However, the abundance of PRRSV-specific T cells and IFN-γ-secreting cells in infected pigs appears to be highly variable and has no apparent correlation with the viral load in lymphoid tissues [11]. Also, PRRSV participates in the generation of T regulatory cells, a cell

subpopulation producing IL-10 and TGF-β upon antigenic stimulation as a protection mechanism to reduce IFN-γ, TNF-α and IL-12 levels and suppress effector T cell activation and proliferation [46, 47]. Specific knowledge of porcine T-cell function and mechanisms of action in response to PRRSV infection is lacking due to the absence of tools and methods for selection and culture of antigen-specific T cells.

1.6. PRRSV vaccines

Vaccination is the principal means used to control and treat PRRSV infection. Attenuation of virulent pathogens by cultivation in non-native conditions and the development of adjuvants provide tools to broaden the useful range of vaccines. The current commercial PRRSV vaccines include products containing live virus derived by cell culture attenuation of virulent field isolates and inactivated preparations of attenuated PRRSV strains. Some other experimental vaccines include inactivated preparation of multiple virulent isolates enriched with viral antigens, subunit vaccines expressing selected proteins, and next generation of PRRSV vaccines based on reverse genetics [48].

Commercially available vaccines have some limitations against PRRSV infection. Killed or subunit PRRSV vaccines have been less effective in prevention of both infection and disease [49]. The outcomes of the use of inactivated vaccines to prevent PRRSV infection are not promising. The current widely used modified live vaccines based on attenuated European or North American PRRSV strains provide decent protection against challenge with homologous isolates but very limit or no protection against heterologous viruses [45]. Moreover, modified live vaccines may allow virus shedding, and could revert generating virulent phenotypes [50]. For example, acute PRRS outbreak occurred in Danish herds vaccinated with an MLV, and the outbreak was linked to reversion of the vaccine virus to a pathogenic phenotype [51]. Therefore, the design of future vaccines must take the antigenic and genetic diversity of PRRSV into consideration or PRRS will remain difficult to control.

PRRSV reverse genetics techniques provide a powerful tool to dissect the mechanism of PRRSV pathogenesis and design the next generation of PRRSV vaccines. The reverse genetics system is

defined as the generation of viruses possessing a genome derived from cloned cDNAs (infectious clones). Compared with the current PRRSV vaccines, the next generation vaccines have reduced cost and time by bypassing the need of an RNA in-vitro-transcription step. To circumvent the limited heterologous protection in the field, chimeric cDNA clones derived from different distinct PRRSV strains were created, and the reciprocal chimeric viruses have less severe pathogenicity and show protection against challenge with parental heterologous strains [52]. Generation of a marker or DIVA (differentiating infected from vaccinated animals) PRRSV vaccine based on a deletion marker (i.e., an immunogenic marker absent from the vaccine strain but present in field strains) on the viral genome by reverse genetics system will allow for differentiation and be of great value for the control and eventual elimination of PRRSV [53, 54].

1.7. PRRSV vaccine adjuvants

Besides developing new vaccines to combat PRRSV, several types of adjuvants such as mineral oils and salts, bacterial products, cytokines, peptides and liposomes have been applied to killed or live modified PRRSV vaccines to induce vaccination-mediated cross-protection against genetically dissimilar PRRSV strains [55-59]. These adjuvants can enhance the immune responses by different mechanisms such as delivering the antigen slowly to the organism (depot effect), increased antigen uptake and presentation to antigen presenting cell, recruitment and activation of macrophages and lymphocytes, and stimulation of the production of cytokines and chemokines [60, 61]. Both MLV and killed PRRSV vaccines showed improved protection ability when they were combined with different types of adjuvants by the evidence of increment on neutralizing Ab production and reduction in viremia level and clinical signs, increased levels of proinflammatory cytokines and specific cell proliferation [62, 63].

Adjuvants applied to PRRSV MLV vaccines have been reported recently. Mycobacterium tuberculosis whole cell lysate applied to PRRSV MLV vaccine as an adjuvant generated effective cross-protective immunity against PRRSV due to enhanced Th1-biased immune responses [62]. The commercial Montanide^{TM} class of flexible polymeric adjuvants combined with PRRSV MLV provided better protection to homologous viral challenge than MLV vaccine alone by regulating the production of PRRSV-specific antibodies and cytokines involved in the

development of T-regulatory cells [64]. Pigs were equally protected from challenge by vaccination with Montanide™ Gel01 adjuvanted PRRSV MLV vaccine containing only 50% of the antigen load as 100% non-adjuvanted vaccine antigen load [65]. New types of adjuvants such as thermo-sensitive hydrogel have also been applied to PRRSV MLV vaccines. Our research group was the first to report that H9e peptide hydrogel, as an adjuvant for PRRSV MLV vaccine, can enhance vaccine efficacy against two different PRRSV strains by modulating both host humoral and cellular immune responses [66]. Therefore, use of adjuvants with current PRRSV vaccines may act as a necessary supplement to help vaccines to confer full antiviral immunity against heterologous challenges and to override the immune evasion strategies of PRRSV.

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Chapter 2 - Comparison of host immune responses to homologous and heterologous type 2 PRRSV challenge in vaccinated and unvaccinated pigs[1]

Abstract: Porcine reproductive and respiratory syndrome (PRRS) is a high-consequence animal disease with current vaccines providing limited protection to infection due to the high degree of genetic variation of field PRRS virus. Therefore, understanding host immune responses elicited by different PRRSV strains will facilitate the development of more effective vaccines. Using IngelVac® Modified Live PRRSV vaccine (MLV), its parental strain VR-2332, and the heterologous KS-06-72109 strain (a Kansas isolate of PRRSV), we compared immune responses induced by vaccination and/or PRRSV infection. Our results showed that MLV can provide complete protection to homologous virus (VR-2332) and partial protection to heterologous (KS-06) challenge. The protection was associated with the levels of PRRSV neutralizing antibodies at the time of challenge, with vaccinated pigs having higher titers to VR-2332 compared with KS-06 strain. Challenge strain did not alter the cytokine expression profiles in the serum of vaccinated pigs or subpopulations of T cells. In contrast, higher frequencies of IFN-γ-secreting PBMCs were generated from pigs challenged with heterologous PRRSV in a recall response when PBMCs were restimulated with PRRSV. Thus, this study indicates that serum neutralizing antibody titers are associated with PRRSV vaccination-induced protection against homologous and heterologous challenge.

2.1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically important pandemic disease characterized by reproductive failure in sows and respiratory disease in young pigs. A

[1] Chapter 2 was reproduced from X. Li, et al, Comparison of Host Immune Responses to Homologous and Heterologous Type II Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Challenge in Vaccinated and Unvaccinated pigs. BioMed Research International (2014) 1-10.

recent study estimates that the total productivity losses in the U.S. swine industry due to PRRS is currently $664 million annually, an increase from the $560 million annual cost estimated in 2005 [1]. This indicates that not only does PRRS have a significant financial impact on the pork industry, but also current strategies for reducing the burden of PRRS virus are not adequate.

PRRS is caused by porcine reproductive and respiratory syndrome virus (PRRSV), which is a member of the genus *Arterivirus*, family *Arteriviridae*, and order *Nidovirales*. PRRSV is known to mutate rapidly in both in vitro cell culture models and in vivo in natural field infections [2]. The ability of PRRSV to mutate rapidly creates genetically extensive and antigenic diverse strains in both North American and European field isolates [3]. The high genetic mutation rate of PRRSV poses a challenge for PRRSV vaccine development [2]. Currently, both inactivated PRRSV vaccines and modified-live-virus (MLV) PRRSV vaccines are widely used to control the disease. However, inactivated vaccines as well as modified-live vaccines have been shown to be ineffective in providing protective immunity to heterologous strains of PRRSV at the herd level [4-7]. Therefore, development of a broadly protective PRRSV vaccine will be one of the most efficient solutions to control the prevalence of PRRS worldwide.

It has been shown that pigs infected with PRRSV have inadequate immune responses, such as delayed onset of neutralizing antibody as well as weak interferon (IFN)-γ responses [2,8]. Development of different types of vaccines aiming to increase host immune response and get broader protection to various field PRRSV infections has been proposed [9]. Currently, PRRSV-MLV is used to control the disease worldwide. However, the high incidence of genetic mutation during PRRSV transmission often results in vaccines based on strains of PPRSV isolated twenty years ago, such as MLV, having limited protection to new emerging viral strains. Disparity of immune responses elicited by different PRRSV strains was reported previously [10]. However, the role of humoral and cellular immune responses was not clearly elucidated in these reports with regard to the protection of virus challenge with different PRRSV strains. Therefore, dissecting the mechanisms of immune responses that are predictive of protection against heterologous PRRSV challenge will be valuable for the development of more efficacious vaccines. In this study, we investigated the differential profiles of host immune responses in naive or vaccinated pigs challenged with homologous and heterologous PRRSV strains.

2.2. Materials and Methods

2.2.1. Cells and virus

MARC-145 cells were maintained in Modified Eagle's medium (MEM) supplemented with 7% fetal bovine serum (FBS) containing 100 U penicillin/ml and 100 µg streptomycin/ml at 37°C with 5% CO2. Virus stocks were prepared and titered in MARC-145 cells and stored in aliquots at -80°C until use. For virus infection and titration, MEM supplemented with 2% FBS was used. PRRS modified live virus vaccine (Ingelvac® PRRS MLV) was purchased from Boehringer Ingelheim Vetmedica Inc. PRRSV strains VR-2332, KS-06-72109 (KS-06) and NVSL97-7895 have been described previously [11, 12].

2.2.2. Pigs, vaccination and challenge

Twenty conventional Large White-Duroc crossbred weaned specific-pathogen free piglets (3 weeks of age) were divided into four groups and were kept in separate rooms within the Large Animal Research Center (LARC) facility, Kansas State University. These piglets were confirmed sera-negative for antibodies to PRRSV by ELISA and PRPSV-free in the blood by RT-PCR. Pigs were allowed to acclimate for an additional week before initiation of the experiment. During our study, all animals received food and water *ad libitum*. The first two groups were immunized intramuscularly on day post-vaccination (DPV) 0 with vaccine (PRRS-MLV, 1 x 10^6 $TCID_{50}$/ pig). The other two groups were used as control groups before challenge and remained unvaccinated (Fig. 2.1A). After four weeks the pigs were challenged with 2 x 10^5 $TCID_{50}$/pig of VR-2332 or KS-06 PRRSV. Necropsy was performed at 14 days post challenge (DPC). Pigs were monitored for rectal temperature for the first 9 days after challenge and body weight once a week for the duration of this experiment.

2.2.3. Collection of blood samples for analysis

Blood was collected on DPV 0, 7, 14, 21, 28 and DPC 7 and 14. Serum was separated from clotted blood and preserved at -20°C. Serum was used for evaluation of viral titers, serum neutralizing antibody titers, PRRSV-specific ELISA antibody titers (Herdchek Porcine Reproductive and Respiratory Syndrome Antibody test Kit, IDEXX Laboratories), and cytokine

expression as described previously [12]. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by Ficoll-Hypaque gradient centrifugation using Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO). PBMCs were used for ELISpot assay and flow cytometry analysis as described previously [12].

2.2.4. Gross lung lesion analysis

Pigs were humanely euthanized on DPC 14 as approved by the Kansas State University Institutional Animal Use and Biosafety Committee. The lungs were macroscopically and microscopically evaluated as previously described [13]. Briefly, the dorsal and ventral surfaces of each lung lobe were given a score representing the approximate proportion that was consolidated. Individual lobe scores were used to determine an overall lung score representing the percentage of the total lung macroscopically pneumonic. Sections of each of the 4 lobes of the right lung were fixed in 10% buffered neutral formalin, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H & E). Scoring of microscopic lung pathology was done in a blinded fashion by two veterinary pathologists in the Kansas State Veterinary Diagnostic Laboratory. Grading was on a 4 point scale as previously described [13].

2.2.5. Analysis of PRRSV circulating in the blood

Total RNA was extracted from pig serum and one-step SyBR Green real-time PCR (Bio-Rad) was performed to evaluate the PRRSV ORF7 expression level as previously described [14]. For quantification, total RNA of a known $TCID_{50}$ of virus was 10-fold serially diluted and was used to generate a standard curve. The virus quantities of unknown samples were determined by linear extrapolation of the Ct value plotted against the standard curve.

2.2.6. Virus neutralizing antibody titer

Virus neutralizing antibody titers were assayed as previously described [12, 14]. Briefly, serum samples were heat inactivated (56°C, 30 min) and serially diluted before the titration. The serial dilutions of serum samples were mixed with equal volume of PRRSV strains: VR-2332, KS-06, or NVSL97-7895 containing 200 $TCID_{50}$ of the virus. After incubation at 37°C for 1 h, the mixtures were transferred to MARC-145 monolayers in 96-well plates and incubated for an additional 72 h at 37°C in a humidified atmosphere containing 5% CO2. Cells were then examined for cytopathic effects (CPE). CPE was used to determine the end-point titers that were

calculated as the reciprocal of the highest serum dilution required to neutralize 200 $TCID_{50}$ of PRRSV in 90% of the wells.

2.2.7. Analysis of PRRSV circulating in the blood

Half million PBMCs were plated in enriched RPMI in a 96-well multiscreen plate (Millipore, Billerica, MA) pre-coated overnight with capture IFN-γ mAB (BD Pharmingen, San Diego, CA). PBMCs were re-stimulated with three different strains of PRRSV (VR-2332, KS-06 or NVSL97-7895) at 0.1 MOI for 24 h at 37°C. IFN-γ-secreting cells were detected by biotinylated anti-pig IFN-γ detection antibody and visualized using the immunospot image analyzer (Cellular Technology, Cleveland, OH). The number of PRRSV-specific IFN-γ-secreting cells was calculated by "total IFN-γ-secreting cells after PRRSV re-stimulation" minus "total IFN-γ-secreting cells after MARC-145 cell lysis re-stimulation". Data were presented as the mean numbers of antigen-specific IFN-γ-secreting cells per 10^6 PBMCs from duplicate wells of each sample.

2.2.8. Flow cytometry analysis

Flow cytometry analysis was performed to determine different lymphocyte populations based on the cell surface marker phenotype: T-helper cells ($CD3^+CD4^+CD8^-$), cytotoxic T lymphocytes ($CD3^+CD4^-CD8+$), Th/memory cells ($CD3^+CD4^+CD8^+$), and γδ T cells ($CD8^+TcR1N4^+$). Mouse anti-pig TcR1N4 antibody was purchased from VMRD (Pullman, WA), and the rest of the antibodies used in this study were purchased from BD Biosciences. Immuno-stained cells were acquired using a FACS Caliber (BD Biosciences) flow cytometer as described previously [12, 14]. Briefly, PBMC was treated with 2% pig serum to block Fc receptors. Cells were then stained with an appropriate Ab which was either directly conjugated to a specific fluorochrome or with a purified Ab to pig specific immune cell surface marker (TcR1N4). For cells stained with a purified Ab, labeled cells were treated with anti-species isotype specific secondary Ab conjugated with fluorochrome. Finally, cells were fixed with 1% paraformaldehyde before flow cytometer reading. Percentages of each lymphocyte population were analyzed by 100,000 unique events using FlowJo software (Tree Star, Inc., OR, USA).

2.2.9. Analysis of cytokine responses

Pig sera were collected at DPC 7 to evaluate IL-4, IL-8, IL-10, IFN-γ, TNF-α (Life Technologies, Carlsbad, CA) and IFN-α (Abcam, Cambridge, MA) secretion profiles by ELISA. Procedures were performed as per the manufacturer's instructions. For a given sample, the OD450 was then transformed to concentration by applying a linear regression formula calculated from the results of the standards provided in each kit.

2.2.10. Statistical analysis

All data were expressed as the mean value of five pigs ± SEM. The differences in the level of body temperature, body weight, lung pathology score, humoral response, cytokine production and viremia among each group were determined by the one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test using SigmaPlot 11 software (Systat Software Inc., San Jose, CA). The difference in the percentage of different T cell subpopulations was determined by the paired t test using SigmaPlot 11 software.

2.3. Results

2.3.1. Vaccination with PRRSV-MLV induced complete protection to homologous PRRSV challenge and partial protection to heterologous challenge

To compare host immune responses to challenge by different PRRSV isolates, pigs were either vaccinated with PRRSV-MLV or a mock vaccine (PBS) on day 0 and then challenged with homologous VR-2332 or heterologous KS-06 PRRSV on day 28 (Fig.2.1A). Clinically, the mean body temperature of unvaccinated pigs challenged with the KS-06 strain of PRRSV was higher compared with that in the other three groups at DPC 4 (Fig. 2.1B). The body weight of all pigs was tracked throughout the study and weights of all groups were similar during the vaccination phase. Interestingly, pigs vaccinated with MLV and challenged with VR-2332 had a slightly higher weight gain than that of the other groups on DPC 14 (data not shown). Unvaccinated pigs that were challenged with either VR-2332 or the KS-06 strain had higher lung lesion scores on DPC 14 compared with that in vaccinated pigs (Fig.2.1C). Vaccinated pigs challenged with VR-2332 showed full protection against PRRSV with average lung scores being normal and no lung damage observed during pathological analysis. Additionally, vaccinated pigs challenged with the

KS-06 strain had moderate protection as shown by decreased lung scores compared with that in unvaccinated-KS-06 challenged pigs (Fig. 2.1C).

In addition, complete protection in vaccinated pigs against homologous challenge was confirmed with the absence of PRRS viral RNA in the serum on DPC 7. As shown in Figure 2.1D, pigs vaccinated with MLV had efficiently cleared the VR-2332 challenge virus from the blood to undetectable levels and vaccinated pigs challenged with the KS-06 strain had less circulating PRRSV in the blood than that in unvaccinated-KS-06 challenged pigs, but the difference was not statistically significant. By DPC 14, the levels of PRRSV virus circulating in the blood were reduced significantly in all vaccinated groups. Therefore, our results suggest that PRRSV MLV can protect pigs from homologous challenge and provide moderate protection against heterologous PRRSV challenge.

2.3.2 Serum neutralizing antibody titer is associated with PRRSV vaccination-induced protection against homologous and heterologous challenge

It has been suggested that vaccine induced PRRSV-specific antibody production is important for inducing protection against subsequent challenges [2,15,16]. To verify that, we analyzed PRRSV-specific ELISA antibodies in homologous- and heterologous-challenged pigs using commercial IDEXX ELISA kit. Serum samples were collected at various time points and used to determine the PRRSV-specific antibody levels. As shown in Figure 2.2A, vaccinated pigs produced PRRSV-specific antibodies starting from DPV 14. Interestingly, the antibody titers in vaccinated pigs were not further enhanced by PRRSV challenge. Additionally, it was found that unvaccinated pigs challenged with the KS-06 isolate showed a faster onset and higher ELISA antibody titers than unvaccinated pigs challenged with VR-2332 (Fig. 2.2A).

PRRSV-specific neutralizing antibodies play a critical role in anti-PRRS immunity. A previous study showed that passive transfer of neutralizing antibodies with a titer of 8 to recipient piglets protected them from challenge-induced viremia, while transfer of serum titers of 32 produced sterilizing immunity [15], suggesting that neutralizing antibody titers over 8 can protect pigs from PRRSV. The ability to induce neutralizing antibodies by PRRSV isolates is isolate-specific

and influenced by both mutation of PRRSV epitopes and virus virulence. Therefore, we analyzed the PRRS virus neutralizing antibody (VN) titers in the serum of different treatment groups. As shown in Figure 2.2B, MLV vaccinated pigs began to develop VN titers to VR-2332 at DPV 28 and the titers were significantly higher at the end of the study as compared with that in unvaccinated pigs. It is worth noting that high titer of VN antibodies against the KS-06 stain were detected only in pigs vaccinated with MLV but not in unvaccinated pigs after both groups of pigs were challenged with the KS-06 strain (Fig. 2.2C). To assay for broad neutralizing activity, another PRRSV strain, NVSL97-7895, was used to measure the VN titer of all serum samples. As shown in Figure 2.2D, VN antibodies against NVSL97-7895 were developed only in vaccinated pigs, and the serum VN titers in vaccinated pigs challenged with the KS-06 strain were higher than that in vaccinated pigs challenged with the homologous VR-2332. This indicates that prime-boost (vaccination-challenge) with different strains of PRRSV may generate antibodies with a broader neutralizing spectrum.

2.3.3. PRRSV-dependent cytokine expression patterns are PRRSV challenge strain specific

Compared with MLV vaccinated pigs challenged with the KS-06 strain, unvaccinated pigs displayed significantly higher IFN-α level in the serum (Fig. 2.3A). In contrast, the difference in IFN-α production was not detected between vaccinated and unvaccinated pigs after they were challenged with VR-2332. Interestingly, vaccinated pigs produced significant higher levels of IL-8 compared with unvaccinated pigs after they were challenged with VR-2332 (Fig. 2.3A). TNF-α expression levels were low in all pigs and there was no significant difference among treatment groups. Furthermore, serum IL-10 levels were significantly higher in unvaccinated pigs after KS-06 PRRSV challenge than that in vaccinated pigs (Fig. 2.3B). In contrast, vaccinated pigs displayed a higher level of serum IL-4 after VR-2332 challenge compared with unvaccinated pigs (Fig. 2.3B). There was no significant difference in serum levels of IFN-γ among all treatment groups.

Vaccination with PRRS-MLV has been shown to induce the production of IFN-γ-secreting cells as a mechanism of protecting pigs against PRRSV viremia [17]. Therefore, the frequency of

IFN-γ-secreting cells in PBMCs was evaluated on DPC 14 in a recall response in which PBMCs were re-stimulated with VR-2332, KS-06 or NVSL97-7895 PRRSV. As shown in Figure 2.3C, when re-stimulated with VR-2332, PBMCs from vaccinated pigs challenged with the KS-06 strain developed more IFN-γ-secreting cells than that from the other three groups. When re-stimulated with KS-06 or NVSL97-7895, PBMCs from KS-06 challenged pigs produced significantly higher amount of IFN-γ-secreting cells than that from pigs challenged with VR-2332. Finally, the ratios of IFN-γ-secreting cells in PBMCs re-stimulated with KS-06 PRRSV in all treatment groups were significantly lower than that in PBMCs re-stimulated with VR-2332 or NVSL97-7895.

2.3.4. T lymphocyte sub-populations vary between unvaccinated and vaccinated groups and are independent of PRRSV challenge strain

T lymphocyte sub-populations are reported to vary in pigs after challenge with different PRRSV strains [18]. In this study, we evaluated the changes in frequency of various lymphocyte populations before and after PRRSV challenge in all experimental groups. On DPV 28, the frequencies of T-helper cells (Fig. 2.4A), cytotoxic T lymphocytes (CTLs; Fig. 2.4B), and γδ T cells (Fig. 2.4D) in PBMCs were similar in vaccinated and unvaccinated pigs, while the frequencies of Th/memory cells in unvaccinated pigs were lower compared with that in vaccinated pigs (Fig. 2.4C). On DPC 14, the frequencies of T-helper, Th/memory, and γδ T cells in PBMCs from vaccinated pigs were higher than that from unvaccinated pigs. It is worth noting that the frequencies of various T cell populations in PBMCs from vaccinated or unvaccinated pigs were not affected by the difference in challenge strains (VR-2332 vs. KS-06), suggesting that PRRSV challenge strain does not affect T cell subpopulations.

2.4. Discussion

As one of the most prevalent diseases in swine, PRRS has caused vast economic losses to the pig industry worldwide. Adding to its devastation, the rapid evolution rate of PRRS virus worldwide generates countless genetically distinct field isolates, many of which have increased pathogenic ability [2,10,18]. Recent outbreaks of PRRSV in China were characterized by high morbidity/mortality and commercially available PRRSV vaccines offered no protection [19] [20]. This demonstrates that current commercial vaccines offer limited or no protection to newly

emerging PPRSV field strains. Therefore, studies on the difference of immune responses to homologous and heterologous challenge lay an important foundation for the development of effective vaccines and eradiation strategies. The present study evaluated the differences of immune responses between vaccinated and unvaccinated pigs when challenged with homologous or heterologous PRRSV. We have demonstrated that serum neutralizing antibody titers are associated with PRRSV vaccination-induced protection against homologous and heterologous challenge.

PRRSV neutralizing activity is associated with antibodies directed against both nonstructural and structural proteins including NSP2, GP2, GP4, and GP5. A recent review suggests that the variability within GP5 may explain the deficiency in cross-protection of current vaccines against heterologous strains of PRRSV [5]. VR-2332 (homologous) and KS-06 strain (heterologous), the PRRS viruses used for challenge experiments in this study, share 99.7% or 90.2% similarity with the PRRSV-MLV vaccine strain based on GP5 amino acid sequence, respectively. From gross lung pathology and viremia results, homologous VR-2332 PRRSV infection was fully prevented after vaccination with PRRSV-MLV as evidenced by lack of virus in sera on DPC 7 and normal gross lung pathology scores (Fig.2.1C, D). Viremia and gross lung pathology scores in the vaccinated pigs challenged with the KS-06 strain were decreased compared with that in the unvaccinated pigs, which indicate MLV vaccination can lead to partial protection to heterologous PRRSV. These results allow us to compare the immune responses from pigs with complete, partial, and no (unvaccinated) protection against PRRSV challenge.

By DPV 14, antibodies specific for N proteins of PRRSV, as measured by the IDEXX ELISA kit, were detected in vaccinated pigs and increased throughout the experimental period. PRRSV-specific antibodies were similar between vaccinated groups throughout the study, suggesting that anti-N protein antibodies are not predictive of PRRSV protection. Interestingly, we did observe that KS-06 PRRSV challenge induced a faster anti-PRRSV antibody response as compared with the vaccine strain, suggesting that more virulent strains could induce a stronger antibody response.

In contrast to anti-N protein antibodies, virus neutralizing antibodies (VNs) have been shown to correlate with protection to PRRSV [15,19]. We found that VNs to different PRRSV strains did not start to emerge until DPV 28 in the vaccinated pigs. At the time of PRRSV challenge (DPV 28), vaccinated pigs developed higher VN titers to VR-2332 (Fig. 2.2B) than to KS-06 strain (Fig. 2.2C), suggesting an association between PRRSV strain-specific VN titer and level of protection to PRRSV. Vaccinated pigs did not develop VNs to KS-06 after vaccination, but developed significantly higher VN titers to KS-06 as compared with the other three groups two weeks after challenge, which suggests that the KS-06 specific VN could be induced by KS-06 challenge (Fig.2.2C). Also, vaccinated and KS-06 challenged pigs developed a higher level of VN antibodies to the heterologous NVSL97-7895 PRRSV strain (Fig.2.2D). This result supports the notion that two vaccinations with different PRRSV strains can generate higher neutralizing Abs and broader cross-protection against various PRRSV field strains. Similar observation has been reported in influenza virus vaccination strategy studies [21].

It was reported that PRRSV can inhibit the expression of IFN-α [22]. However, we found that the level of IFN-α was increased in unvaccinated pigs challenged with KS-06 virus. Similar to previous reports, serum level of IFN-α is not associated with the PRRS virus clearance in pigs after viral challenges [18]. The serum level of inflammatory cytokine IL-8 in vaccinated pigs challenged with homologous VR-2332 virus was the highest among all treatment groups (Fig.2.3A). Our results are consistent with previous studies which have shown that low level of serum IL-8 is seen in persistent PRRSV infection, and elevated IL-8 levels in serum is correlated with the clearance of PRRS virus [23]. However, it remains to be determined how elevated IL-8 may contribute to the clearance of PRRS virus in vaccinated pigs and whether the level of serum IL-8 can be used to predict vaccination-induced protection in pigs.

The expression of IL-4 was significantly higher in vaccinated pigs as compared with that in unvaccinated pigs after KS-06 challenge. This and our previous study [12] and results from others [24] suggest that increased IL-4 expression may play a positive role in vaccination-mediated clearance of heterologous PRRS virus. However, IL-4 level in the serum may not have a direct role in protecting pigs from PRRSV infection since pigs challenged with homologous PRRSV (VR-2332) did not show increased IL-4 production. Thus, whether or not IL-4 plays an

important role in the development of vaccination-induced protection against PRRSV has yet to be explored in future studies.

PRRSV infection has been shown to induce a strong immunosuppressive response characterized by promoting the secretion of IL-10 to antagonize the protective Th1 immune response [25]. In our study, we found that IL-10 production in the serum was increased in unvaccinated pigs, but not in vaccinated pigs, when they were challenged with the KS-06 strain (Fig.2.3B). In contrast, both unvaccinated and vaccinated pigs challenged with VR-2332 had similar levels of serum IL-10. The level of serum IL-10 in PRRS infection has been reported to be virus strain-dependent, which may be related to the virulence of each viral isolate [25]. Thus, the difference in IL-10 production between the two challenged groups may be due to the fact that the KS-06 isolate is more virulent than the VR-2332 isolate.

IFN-γ is a key cytokine that is associated with host cell-mediated immunity (CMI) response, which is secreted by natural killer cells and several different T cell subpopulations. Report has shown that the expression level of IFN-γ after PRRSV infection was variable and showed no correlation to virus load [26]. In our study, we did not observe any changes to serum levels of IFN-γ among the four treatment groups (Fig.2.3B). In a recall response, IFN-γ-secreting cells from memory lymphocytes was calculated after removal of the control background data of cells which was stimulated with MARC145 cell lysis. MLV vaccination generated higher frequency of IFN-γ-secreting cells. However, PBMCs isolated from vaccinated and KS-06 challenged pigs generated more IFN-γ-secreting cells when re-stimulated with homologous or heterologous PRRSV as compared with that from unvaccinated pigs (Fig.2.3C). We found that the lowest number of IFN-γ-secreting cells was from PBMCs re-stimulated with the KS-06 strain, as compared with another heterologous strain NVSL97-7895 or VR-2332 stimulation. This may be due to the fact that the KS-06 isolate is more virulent than the other two strains and can cause a stronger immunosuppression during infection [18]. Our results suggest that increased IFN-γ expression does not correlate with protection against PRRSV as evidenced by lower levels of IFN-γ in fully protected vaccinated pigs challenged with VR-2332 compared with partially protected vaccinated pigs challenged with KS-06 strain. Therefore, the role of IFN-γ in the protection to PRRSV infection needs to be further explored.

A high frequency of γδ T cells in pigs is considered to be related to the activation status of the innate immune system, and $CD4^+CD8^+$ double positive T cells possess memory, T-helper and cytolytic properties [27, 28]. Although significant increases in the frequency of T helper, Th/memory and γδ T cells in PBMCs were observed in vaccinated pigs compared with that in unvaccinated pigs, and this may suggest a protective role of these cells against PRRSV infection, this parameter cannot predict the level of protection since changes in T cell subpopulations are similar between fully and partially protected groups of pigs.

Author Disclosure Statement

The authors declare that they have no competing financial interests.

2.5. References

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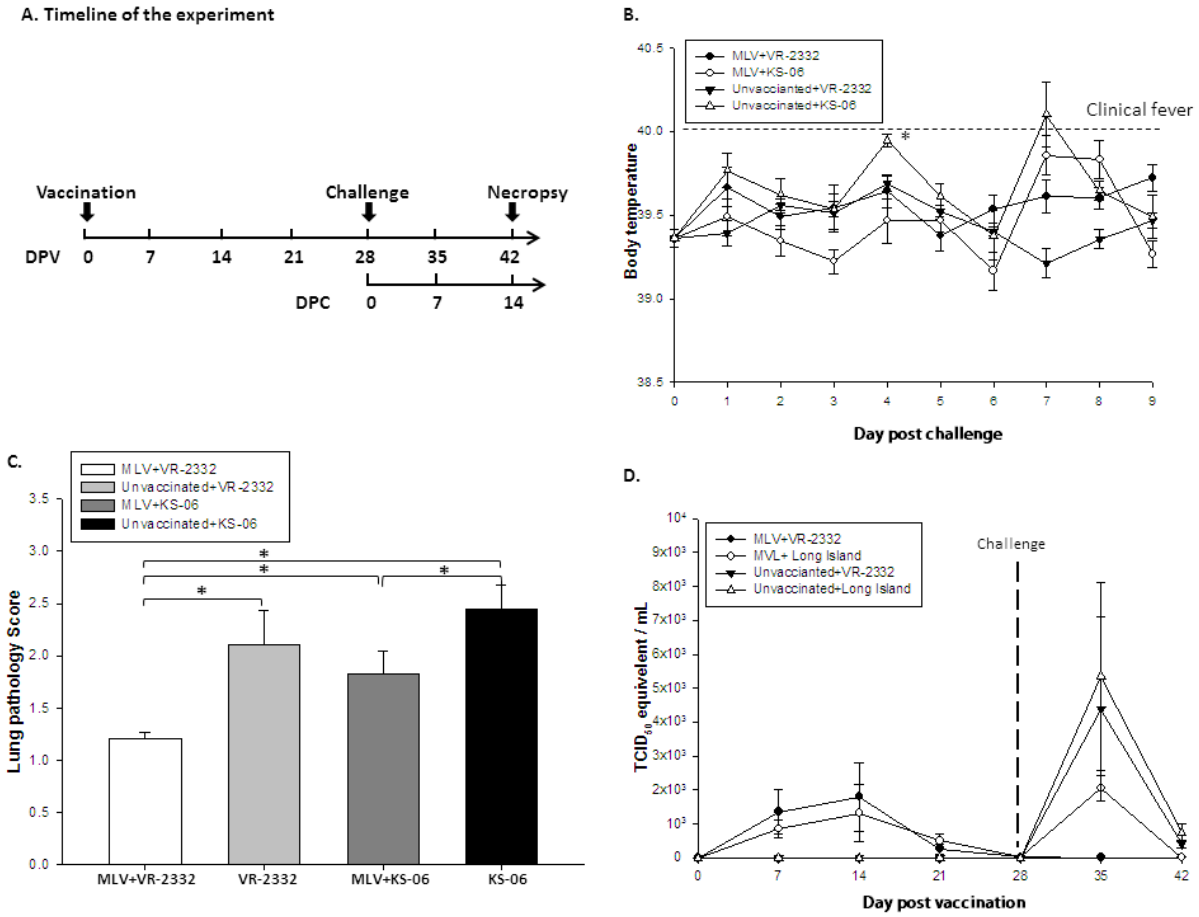

Figure 2.1. Vaccination with PRRSV-MLV induced complete protection to homologous PRRSV challenge and partial protection to heterologous challenge. (A) Experimental timeline. (B) Rectal temperature of pigs was monitored daily after PRRSV challenge. (C) Gross lung lesion scores present in all lung lobes on DPC 14 were scored using a 4 point scale. (D) PRRSV viral RNA in the serum was determined by qPCR, a standard curve was used for calculation of $TCID_{50}$ of viral RNA. Each bar represents the average of samples from five pigs ± SEM. *$p<0.05$.

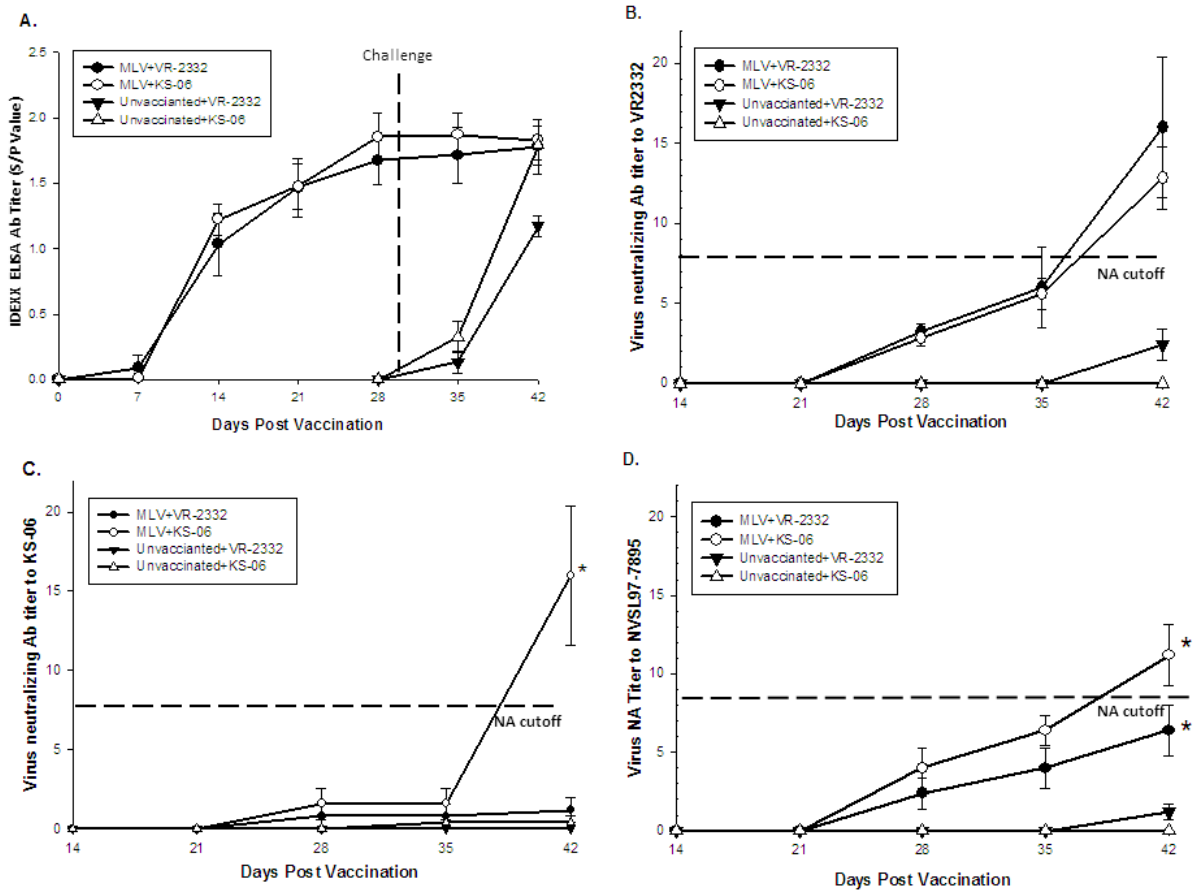

Figure 2.2. Serum neutralizing antibody titer is associated with PRRSV vaccination-induced protection against homologous and heterologous challenge. (A) PRRSV-specific antibodies were detected in the serum using IDEXX ELISA kit. The threshold for positive sera was set at a sample to positive (s/p) ratio of 0.4 according to the manufacturer's instructions. (B-D) Serum samples were titrated on MARC-145 cells and the levels of anti-PRRSV neutralizing Abs were determined as the reciprocal of the highest dilution that could inhibit CPE. Data were shown as mean ± SEM for 5 pigs per group. *p <0.05.

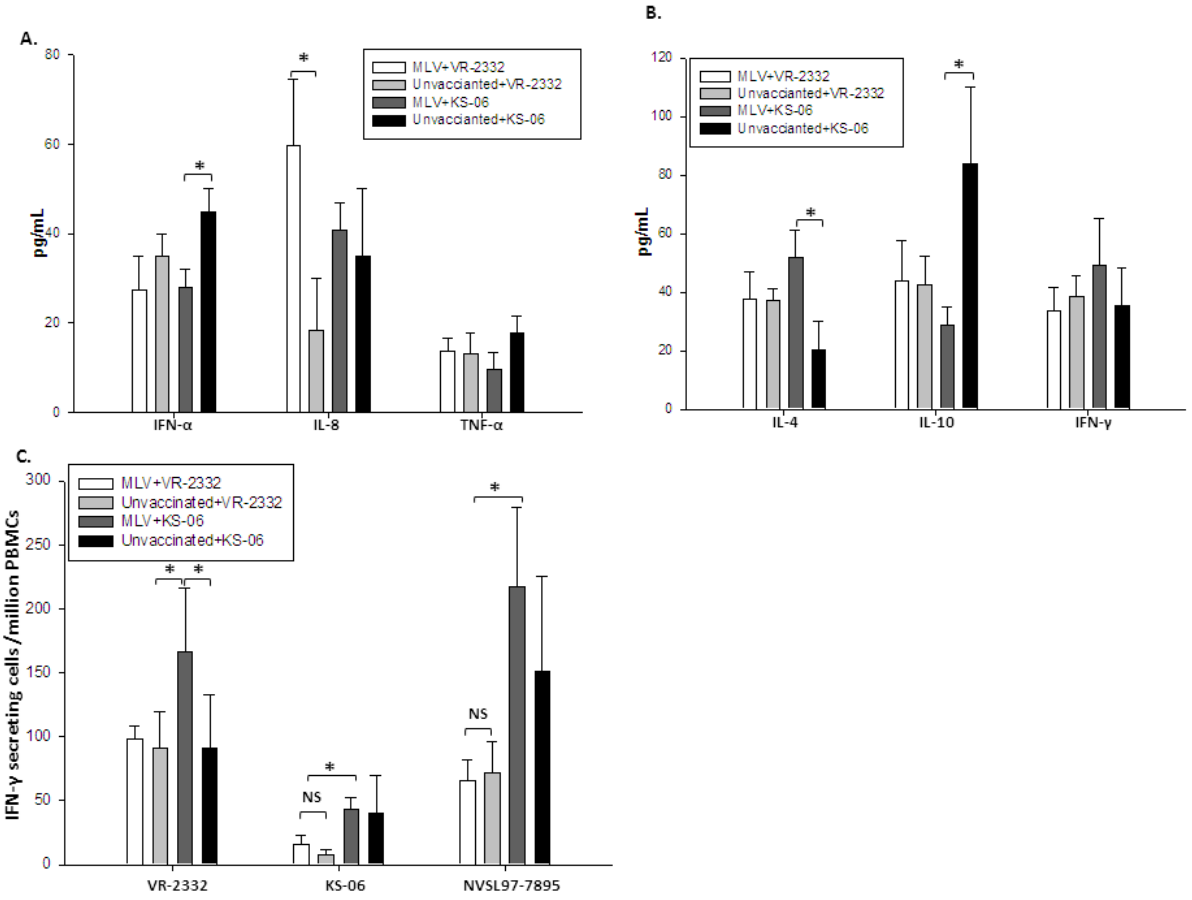

Figure 2.3. PRRSV-dependent cytokine expression patterns are PRRSV challenge strain specific. (A) Innate and (B) adaptive cytokine expression profiles in the sera of pigs at DPC 7 were tested by ELISA. (C) PBMCs collected at DPC 14 were re-stimulated with VR-2332, KS-06 or NVSL97-7895 strains of PRRSV. IFN-γ-secreting cells were then analyzed by ELISpot assay. Data were shown as mean ± SEM for 5 pigs per group. *p <0.05. NS= Not significant.

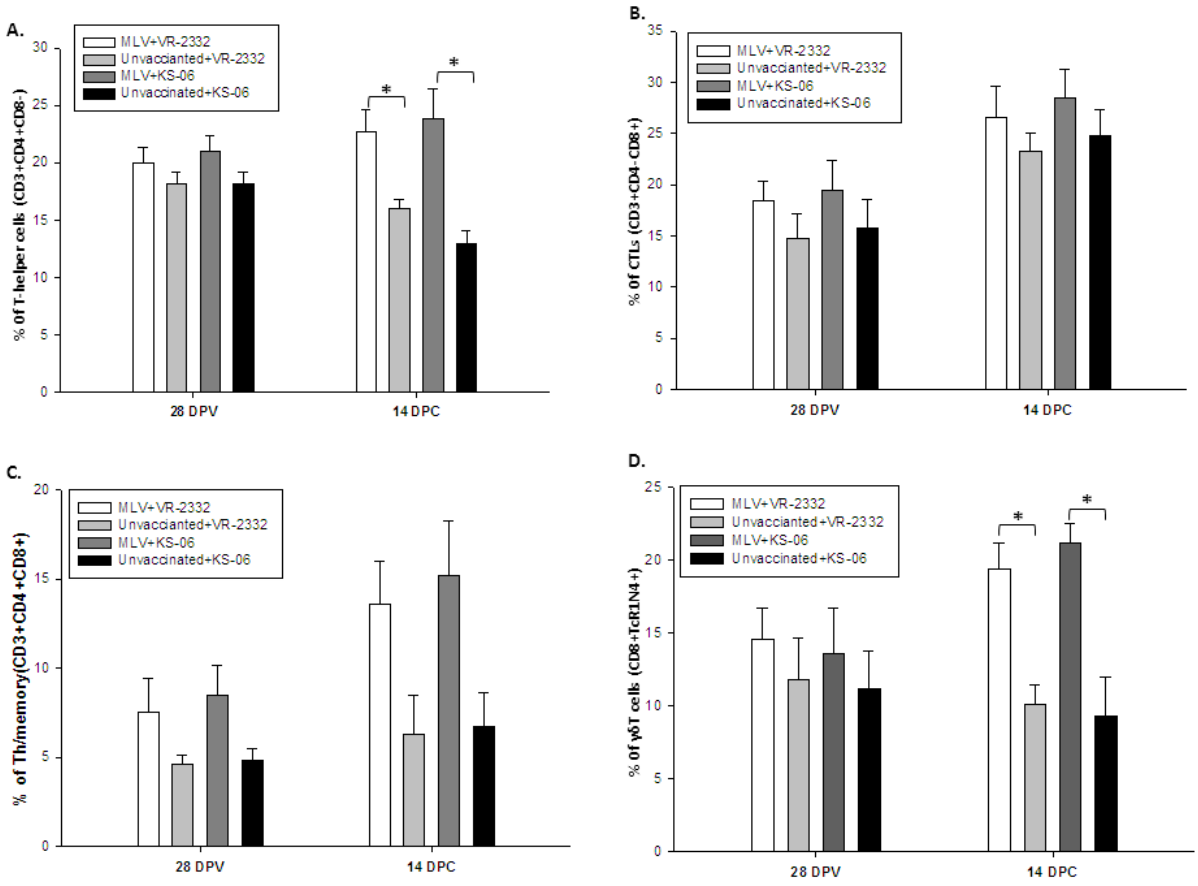

Figure 2.4. T lymphocyte sub-populations vary between unvaccinated and vaccinated groups and are independent of PRRSV challenge strain. PBMCs were isolated from pigs at necropsy (DPC 14) and T cell subsets were determined by flow cytometry analysis according to their phenotypes. Shown are the percentages of (A) T-helper cells that were $CD3^+/CD4^+/CD8^-$, (B) Cytotoxic T lymphocytes that were $CD^+CD4^-CD8^+$, (C) Th/memory cells that were $CD3^+CD4^+CD8^+$, and (D) γδ T cells that were $CD8^+TcR1N4^+$. Data were shown as mean ± SEM for 5 pigs per group. *p <0.05.

Chapter 3 - Montanide™ Gel 01 ST adjuvant enhances PRRS modified live vaccine efficacy by regulating porcine humoral and cellular immune responses[2]

Porcine reproductive and respiratory syndrome (PRRS) is a devastating disease caused by the PRRS virus. The Montanide™ class of flexible polymeric adjuvants has recently been shown to enhance protective immunity against PRRSV infection in piglets when used in combination with PRRS modified live vaccines (MLV). In this study, we explored the efficacy and imunological mechanisms of protection of Montanide™ Gel 01 ST (Gel01) adjuvanted modified live PRRS vaccine in pigs challenged with two genetically distinct strains of PRRSV. Gel01-MLV reduced lymph node pathology scores in pigs challenged with VR-2332 (parental strain of MLV vaccine) but not that in pigs challenged with MN184A (heterologous strain), when compared with that in pigs vaccinated with un-adjuvanted MLV. Pigs vaccinated with Gel01-MLV had higher levels of PRRS-specific antibodies, as measured by IDEXX ELISA and virus neutralizing antibodies, after vaccination and VR-2332 challenge. In addition, pigs vaccinated with Gel01-MLV had decreased levels of IFN-γ, IL-10, and T-regulatory lymphocytes in the blood as compared with that in pigs vaccinated with MLV alone. Interestingly, we found that addition of Gel 01 ST did not change the profile of other T lymphocyte populations after PRRSV challenge. These results demonstrate that the MLV adjuvanted with Gel01 provides enhanced protection against homologous PRRSV infection, possibly by regulating the production of PRRSV-specific antibodies and cytokines involved in the development of T-regulatory cells. Thus, Gel 01 ST is a promising adjuvant that can be formulated with PRRSV MLV vaccines to reduce disease severity and tissue damage caused by PRRSV infection in pigs.

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3.1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is currently one of most devastating swine diseases worldwide, causing immense economic losses in the swine industry [1]. It was estimated that the US pork industry alone has annual losses of $664 million due to the prevalence of PRRS [2]. The causative agent, PRRS virus (PRRSV), belongs to the family *Arteriviridae*, order *Nidovirales*, and causes reproductive failure in sows including still births, mummification, week-born piglets and high pre-weaning mortality [3]. Currently, commercially available PRRS modified live vaccines (MLVs) are widely being used in the US to control PRRSV infection [4]. However, the efficacy of these MLVs is debated due to limited protection against antigenically diverse heterologous PRRS virus isolates [5].

Current formulations of MLV do not contain adjuvants since the multiplication and infectious properties of attenuated live PRRS virus have been shown to induce sufficient protection to PRRSV infection [6]. However, due to the high degree of genetic variation of PRRSV, new strains are quickly emerging that current vaccine formulations may not be able to protect against. Recently, several studies showed that addition of adjuvants, such as *Mycobacterium tuberculosis* whole cell lysate, to PRRS modified live vaccine (PRRS-MLV) can induce enhanced cross-protective immunity to PRRSV [7]. However, the addition of these experimental adjuvants to commercially available vaccines still requires large-scale trials and certification by USDA before they can be brought to market. By contrast, the Montanide™ class of adjuvants is a well-established brand of vaccine adjuvants, which are already approved in Europe and included in several registered commercial veterinary vaccines for food animals including cattle, poultry, and fish. These Montanide™ adjuvants have been shown to enhance disease protection when combined with diverse types of antigens [8]. Recently, one research group used Montanide™ Gel 01, a polymer based adjuvant, to adjuvant PRRS attenuated live vaccine and found the addition of Gel 01 enhanced protection from PRRS in vaccinated animals, even in formulations containing half the dose of the modified live PPRSV [9]. However, they did not evaluate the cross-protection potential of the Gel01 adjuvant or mechanism of increased protection. Therefore, using Gel 01 ST as an adjuvant, we evaluated PRRS MLV-induced humoral and cellular immune responses to homologous and heterologous PRRSV challenges and explored

whether Gel01-adjuvanted PRRS MLV can provide broader cross-protection to field strains of PRRSV.

3.2. Materials and Methods

3.2.1. Cells, virus and adjuvant preparation

Marc-145 cells were maintained in Modified Eagle's medium (MEM) supplemented with 7% fetal bovine serum (FBS) containing 100U penicillin/ml and 100ug streptomycin/ml at 37°C with 5% CO2. Virus stocks were prepared and titrated in Marc-145 cells and stored in aliquots in -80°C until use. For virus infection and titration, MEM supplemented with 2% FBS was used. Modified live virus vaccine (PRRS-MLV) was purchased from Boehringer Ingelheim Vetmedica Inc. PRRSV VR-2332, the parental strains of MLV, was purchased from ATCC. PRRSV MN184a was a kind gift from Dr. Kay Faabberg in United States Department of Agriculture. Montanide^{TM} Gel 01 ST (Gel01) polymeric adjuvant was a kind gift from Dr. Robert Parker (SEPPIC Inc.). A final 10% of Gel 01 was added into diluted PRRS modified live vaccine and mixed by manual shaking.

3.2.2. Pigs, vaccination and challenge

Thirty-five conventional Large White-Duroc crossbred weaned specific-pathogen free piglets at 3 weeks of age were housed at the Large Animal Research Center (LARC) facility, within Kansas State University. These piglets were confirmed sera-negative for antibodies to PRRSV by ELISA and PRPSV-free in the blood by RT-PCR. Pigs were allowed to acclimate for an additional week before initiation of the experiment. Pigs were immunized intramuscularly on day post-vaccination (DPV) 0 with MEM (placebo) or vaccine (PRRS-MLV, $1x10^6$ $TCID_{50}$/ pig) formulated with or without 10% Gel 01 adjuvant. After four weeks, the pigs were challenged with either homologous PRRSV VR-2332 (1×10^6 $TCID_{50}$) or heterologous PRRSV MN184a (5×10^5 $TCID_{50}$) Pigs were monitored for body weight once a week and clinical signs of infection, including rectal temperature, for 7 days post challenge (DPC).

3.2.3. Collection of blood samples for analysis

Blood was collected on DPV 0, 7, 14, 21, 28, DPC 7 and 14. Serum was separated from clotted blood and preserved at -20°C until used in assays. Serum was used for evaluation of viremia, viral titer, serum neutralizing antibody titers, and ELISA antibody titer (HerdCheck Porcine Reproductive and Respiratory Syndrome Antibody test Kit, IDEXX Laboratories) to PRRSV as previously described. Peripheral blood mononuclear cells (PBMCs) were isolated from a heparinized blood sample by Ficoll-Hypaque gradient centrifugation using Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO). PBMCs were used for ELISpot assay, flow cytometry and real-time PCR analysis.

3.2.4. Gross lung and lymph node lesion analysis

Pigs were humanely euthanized on DPC 14 as approved by Kansas State University Institutional Animal Use and Biosafety Committees. To evaluate lung and lymph node histopathology, slices of lung tissue from each lobe and lymph nodes were formalin-fixed, paraffin embedded, sectioned and stained with hematoxylin and eosin (H&E). Scoring of macroscopic and microscopic lung/lymph node pathology was done in a blinded fashion by two veterinary pathologists in the Kansas State Veterinary Diagnostic Laboratory (KSVDL).

3.2.5. Virus neutralizing antibody titration

Serum samples were heat inactivated (56°C, 30min) and serially diluted before the titration. The serial dilutions of serum were mixed with equal volumes of PRRSV VR-2332 or MN184a, respectively, containing 200 $TCID_{50}$ viruses. After incubation at 37°C for 1h, the mixtures were transferred to Marc-145 monolayers in 96-well plates. After incubation for 72h at 37°C in a humidified atmosphere containing 5% CO2, cells were examined for cytopathic effects (CPE). CPE was used to determine the end-point titers that were calculated as the reciprocal of the highest serum dilution to neutralize 200 $TCID_{50}$ of PRRSV in 50% of the wells.

3.2.6. ELISpot assay

Briefly, 5 x 10^5 PBMCs were plated in enriched RPMI in a 96-well multiscreen plate (Millipore, Billerica, MA) pre-coated overnight with capture IFN-γ mAB (BD pharMingen, San Diego, CA). PBMCs were re-stimulated with three different strains of PRRSV at 0.1 MOI for 24h at 37°C. IFN-γ-secreting cells were detected by biotinylated anti-pig IFN-γ detection antibody and visualized using the immunospot image analyzer (Cellular Technology,Cleveland, OH). Data were presented as the mean number of antigen-specific IFN-γ-secreting cells per 10^6 PBMCs from duplicate wells of each sample.

3.2.7. Flow cytometry analysis

Flow cytometry analysis was performed to determine different lymphocyte populations based on the cell surface marker phenotype: T-helper cells ($CD3^+CD4^+CD8^-$); cytotoxic T lymphocyte ($CD3^+CD4^-CD8^+$); Th/memory cells ($CD3^+CD4^+CD8^+$), T-regulatory cells ($CD4^+FoxP3^+CD25^+$) and γδ T cells ($CD8^+$ $TcR1N4^+$). Mouse anti-pig TcR1N4 antibody was purchased from VMRD (Pullman, WA), and the rest antibodies used in this study were purchased from BD Biosciences. Immuno-stained cells were acquired using a FACS Caliber (BD Biosciences) flow cytometer. Frequencies of individual lymphocyte were analyzed by 100,000 events using FlowJo software (Tree Star, Inc., OR, USA).

3.2.8. Analysis of serum PRRS virus titer

Total RNA was extracted from 100ul serum using TRIzol® Reagent. One-step SyBR Green real-time PCR (Bio-Rad) was performed to evaluate PRRSV ORF7 expression level as previously described [10]. For quantification, total RNA of known $TCID_{50}$ of virus were 10-fold serially diluted and were used to generate standard curve. Virus quantity of unknown samples was determined by linear extrapolation of the Ct value plotted against the standard curve.

3.2.9. Analysis of IL-10 cytokine response

Pig sera collected at necropsy and culture supernatants harvested after in vitro restimulation of one million of PBMC, TBLN, and lung MNC were analyzed by ELISA kit (Invitrogen, CA) for secretion of IL-10 cytokine.

3.2.10. Statistical analysis

All data were expressed as the mean value of five pigs ± SEM. The differences among each group were determined by the paired t test (Prism5.0, GraphPad Software, SanDiego, CA). Differences were considered statistically significant when $P<0.05$.

3.3. Results

3.3.1. Addition of Montanide™ Gel 01 ST adjuvant to MLV provided enhanced protection against homologous VR-2332 challenge but not heterologous MN184a PRRSV challenge in pigs

Currently, the most effective vaccines for PRRS, including MLV, are cell-culture attenuated strains of PRRSV. However, the high incidence of genetic mutation during PRRSV transmission often results in vaccines based on strains of PRRSV isolated twenty years ago, such as MLV, having limited protection to new emerging viral strains. Therefore, there is a growing need to develop new vaccines or significantly improve the ones currently available. Previous studies have shown that the Montanide™ line of adjuvants may be able to improve the protection potential of commercially available PRRSV MLV to emerging field isolates of PRRSV [9]. To determine the cross-protection potential of Gel 01, pigs were mock vaccinated or vaccinated with modified live PRRSV vaccine formulated with or without Gel 01 adjuvant. Twenty eight days after vaccination, pigs were challenged with homologous VR-2332 (isolated in 1992) or heterologous MN184a (isolated in 2002) strains of PRRSV. Gel01 was tested to be safe when combined with MLV in our vaccination protocol. We did not observe injection site reactions in any group (data not shown) and pigs vaccinated with MLV adjuvanted with Gel01 had equivalent net body weight gain compared with control challenged pigs (Fig.3.1A). Clinically, unvaccinated pigs developed typical PRRSV symptoms including slight fever and lethargy after

challenge. The mean body temperature of unvaccinated pigs challenged with VR-2332 or MN184a was 0.3 or 1.0 ^{o}C higher, respectively, compared with vaccinated pigs with no differences between MLV and Gel01-MLV groups (data not shown).

At necropsy, 14 days post challenge (DPC), Gel01-MLV vaccinated pigs had slightly lower lung lesion scores, although not statistically significant, compared with MLV vaccinated pigs challenged with VR-2332 (Fig.3.1B). However, lymph node pathology scores were significantly lower in Gel01-MLV pigs than MLV vaccinated pigs with homologous VR-2332 challenge (Fig.3.1C). Interestingly, Gel01 adjuvant addition was unable to reduce MN184a-induced lung lesion and lymph node pathology scores. Protection from disease in vaccinated pigs with or without Gel01 was also associated with a significantly reduced PRRS virus titer at DPC14 (Fig.3.1D). Circulating VR-2332 PRRSV was cleared in the blood by DPC14 in both MLV and Gel01-MLV vaccinated groups, and a reduced MN184a PRRSV titer in the blood was observed in both vaccinated groups. However, there was no difference between Gel01-MLV and MLV vaccinated groups for the level of viremia (Fig.3.1D). Taken together, our results suggest that addition of Gel01 to MLV can enhance protection of homologous but not heterologous PRRSV infection in pigs.

3.3.2. Pigs vaccinated with Gel01 adjuvanted MLV have enhanced PRRSV-specific antibodies and virus neutralizing antibodies after homologous PRRSV challenge

Since pigs vaccinated with Gel01-adjuvanted MLV demonstrated enhanced protection against homologous PRRSV challenge, we next wanted to explore the immunological mechanisms of improved vaccination efficacy. The presence of vaccine-induced PRRSV-specific antibodies has been shown to correlate with the protection against disease [11]. Therefore, serum samples were analyzed for PRRSV specific ELISA antibodies and neutralizing antibodies before and after PRRSV challenge. Pigs vaccinated with Gel01-MLV developed significantly higher IDEXX ELISA antibody titers (indicated by value of S/P) on 21 DPV than the MLV vaccinated or unvaccinated pigs (Fig.3.2A and 3.2B). After challenge, the ELISA antibody titers were only significantly higher in Gel01-MLV vaccinated pigs than that in MLV vaccinated pigs when challenged with homologous VR-2332 (Fig.3.2A).

The presence of VR-2332 and MN184a PRRSV strain-specific neutralizing antibodies were also assayed in the serum of all groups of pig at 14 DPC. Neutralizing antibody titers to VR-2332 were higher in Gel01-MLV pigs than that in MLV vaccinated pigs when challenged with VR-2332 (Fig.3.2C). But there was no difference in neutralizing antibody titers to MN184a between these two vaccinated groups when pigs were challenged with VR-2332 or MN184a. Therefore, our results suggest that Gel01 adjuvant may be facilitating the production of PRRSV-specific antibodies, including neutralizing antibodies, leading to enhanced protection against VR-2332 challenge.

3.3.3. Pigs vaccinated with Gel01 adjuvanted MLV have decreased PRRSV-specific IFNγ and IL-10 cytokines after PRRVS challenge.

Our results thus far show that the addition of Gel01 adjuvant to the MLV PRRSV vaccine acts to increase the humoral immune response in pigs challenged with homologous PRRSV. In addition to antibody responses, cytokines expression profiles and cell-based immune responses are involved in the resolution of PRRSV infections [12]. In order to determine whether cellular immune responses were also enhanced by Gel01 adjuvant, PBMCs were isolated from blood samples in each group at 14 DPC. We found that the Gel01-MLV group of pigs challenged with VR-2332, as compared with MLV group, developed a lower frequency of IFN-γ-secreting cells when re-stimulated with the homologous virus (Fig.3.3A). Additionally, the secretion of immunosuppressive cytokine IL-10 by PBMCs was also reduced in the Gel01-MLV vaccinated pigs, but not in MLV pigs, challenged with VR-2332 or MN184a (Fig.3.3B). To further confirm the decreased IL-10 cytokine expression induced by the addition of the Gel01 adjuvant, serum IL-10 and IL-10 secreted by lung MNCs were also analyzed. As shown in figure 3.3C and D, reduced IL-10 cytokine secretion was also found in the serum, but not in the supernatant of lung MNC, of the Gel01-MLV vaccinated pigs challenged with homologous VR-2332 but not heterologous MN184a virus. Therefore, these results suggest that Gel01 adjuvant may increase MLV-mediated protection against homologous PRRSV infection using a mechanism that involves decreased production of circulating IFN-γ and IL-10.

3.3.4. Pigs vaccinated with Gel01 adjuvanted MLV had reduced T-regulatory cell populations after PRRSV challenge

Finally, since the immune response and cytokine expression patterns are modulated by different T cell sub-populations, the phenotype and frequency of various lymphoid immune cells in pigs were also analyzed by flow cytometry. The frequency of different immune cells at 14 DPC are shown in table 3.1. Interestingly, we found a significant decrease of the T-regulatory cell population in TBLNs and lung MNCs in Gel01-MLV vaccinated pigs compared with the MLV vaccinated pigs after both VR-2332 and MN184a challenge (Fig.3.4A and B). We did not observe any significant differences among total T cell population, T-helper cells, cytotoxic T cells, Th/memory cells, or γδ T cells (Table3.1). Taken together, our results suggest that, when combined with PRRSV MLV, Gel01 adjuvant can enhance the protection against homologous PRRSV infection by regulating the development of T regulatory cells.

3.4. Discussion

Modified live vaccines are widely used in veterinary medicine, as well as in human medicine, to control many infectious diseases in a wide variety of hosts [13]. Currently, almost all commercially available PRRSV vaccines are modified live vaccines based on cell culture-attenuated strains of PRRS virus. In general, MLV provides decent protection against homologous virus infection; however the antigenic disparity of rapidly emerging field isolates leads to partial protection against heterologous viruses [1]. Furthermore, newly emerging isolates are more virulent then parental strains and more prevalent in swine farms across the world, leading to devastating economic losses [2]. Thus, there is a growing need to improve the current PRRS vaccination practices in swine farms.

The efficacy of current PRRS modified live vaccines could be enhanced with the addition of adjuvants. In fact, a recent study demonstrated that the addition of adjuvant to MLV led to broadened cross-protection to PRRSV field isolates and reduced lung and lymph organ damage [7], suggesting that adjuvant addition to MLV would be an effective way to reduce PRRS disease. However, *Mycobacterium tuberculosis* whole cell lysate was used as the adjuvant in that

study and it will be too expensive for food animal vaccine markets. On the other hand, a more cost-effective commercially available Gel01 adjuvant is proven to be easy to use and stable in a variety of veterinary vaccines. The addition of Gel01 adjuvant to PRRS MLV has been reported previously [9]. It was reported that Gel01 adjuvant can improve the efficacy of PRRS MLV even with half of the antigen load. Gel01-adjuvanted PRRS MLV can reduce viremia after challenge and generate equivalent ELISA antibody titers as MLV alone. A better protection was shown by the reduced duration of hyperthermia and lung pathological score with administration of Gel01-adjuvanted PRRS MLV after viral challenge.

Our study shows similar results in that Gel-MLV was able to better protect pigs challenged with VR-2332 than pigs vaccinated with MLV alone. However, when pigs were challenged with a heterologous strain of PRRSV (MN184a), addition of Gel01 adjuvants did not enhance protection (Figure 3.1). The amino acid similarity of structural proteins between MLV and VR-2332 is more than 99.2%, yet MN184a share only 89.4%, which may have contributed to lack of cross-protection after MN184a challenge. Therefore, Gel01 may not be an ideal adjuvant for all strains of PRRSV, but rather is strain-specific in the ability to enhance the protective properties of MLV.

In our study, pigs vaccinated with Gel0-MLV developed higher titers of PRRSV-specific antibodies after vaccination and VR-2332 challenge, as measured by IDEXX ELISA (Fig.3.2A and B). However, these ELISA antibodies are non-neutralizing antibodies that are mainly directed towards the nucleocapsid (N) protein, and did not provide animals with any protection against PRRSV infection [14]. In contrast, PRRSV structural proteins are reported to induce protective neutralizing antibody (NA) and PRRSV-specific cellular immune response after PPRSV infection [15]. To further evaluate the immunological mechanisms of Gel01-mediated adjuvanticity, we found that pigs vaccinated with Gel01-adjuvanted MLV, as opposed to MLV alone, generated higher NA titer when challenged with VR-2332 (Fig.3.2C). Interestingly, pigs vaccinated with Gel01-MLV showed higher NA titer to both VR-2332 and MN184a than that in pigs vaccinated with MLV alone after they were challenged with PRRSV MN184a . Similar results have also been observed on other recently isolated field strains of PRRSV. These results are consistent with the speculation that the prime (vaccination-MLV) and boost with

heterologous PRRSV strain (challenge-MN184a) are able to generate higher NA titers, a concept which has already been shown to occur in influenza virus vaccination [16].

There are several immunomodulatory cytokines that are believed to be responsible for the clearance of PRRS virus. Specifically, vaccine-mediated up-regulation of pro-inflammatory cytokine IFN-γ has been suggested to be important in the combat against PRRSV infection [17]. Interestingly, in our study, pigs receiving Gel01 adjuvanted MLV were better protected against homologous PRRSV infected than MLV vaccinated pigs; however, Gel01-MLV pigs had decreased IFN-γ (Figure 3.3A). These results suggest that IFN-γ maybe be playing a negative role in protecting pigs from disease and agents that can reduce IFN-γ levels in vaccinated pigs and may lead to better protection. Additionally, pigs vaccinated with the MLV alone had increased IL-10 production as compared with unvaccinated animals and the addition of Gel acted to decrease IL-10 to levels to at or below unvaccinated animals (Figure 3.3B and C). During PRRSV infection, a significant correlation has previously been observed between the inability to effectively protect against disease and the increased expression of cytokine IL10. This could be in part due to IL10-mediated reduction of IFN-α, IFN-γ, IL-12 and TNF-α expression, cytokines involved in dampening the cellular immune response [5]. Therefore, our results suggest that Gel01 adjuvant may act to enhance the protective properties of MLV by decreasing IL-10 production.

The expression of IL-10 is mainly regulated by T-regulatory cells, which consist of a small subpopulation of T lymphocytes [18]. Consistent with IL-10 expression, we found that the frequency of T regulatory cells in Gel01-adjuvanted vaccinated pigs was dramatically reduced in the TBLNs and lung MNCs (Figure 3.4). Therefore the reduced T-regulatory cell population could have contributed to the decreased expression of IL-10 in pigs after vaccination with Gel01-MLV and challenge with PRRSV.

In summary, our results show that addition of Gel01 adjuvant to PRRSV modified live vaccine can confer increased protection to homologous but not heterologous PRRSV challenge, presumably through higher titers of ELISA and neutralizing antibodies and reduced IFN-γ and

IL-10 cytokine production. Therefore, the commercially available Gel01 adjuvant may be a useful tool in improving the efficacy of live PRRS vaccines.

Author Disclosure Statement

The authors declare that they have no competing financial interests.

3.5. References

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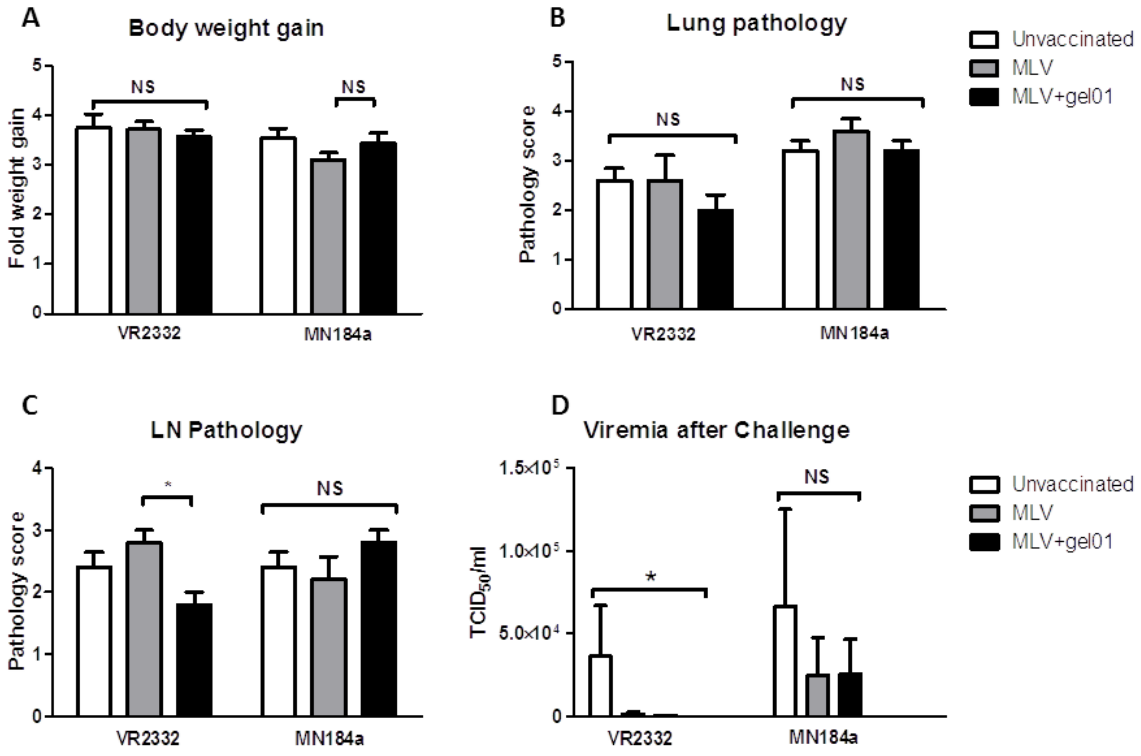

Figure 3.1. Addition of Montanide^{TM} Gel 01 ST adjuvant to MLV provided enhanced protection against homologous VR-2332 challenge but not heterologous MN184a PRRSV challenge in pigs (a) The body weight of pigs was monitored weekly for 6 weeks starting on the day of vaccination (DPV 0) and concluding 14 days post PRRSV challenge (14 DPC). Fold body weight gain of each individual pig was calculated by normalizing the weight of the pig on DPV 0 to 1. (b) Lung tissue harvested on 14 DPC was sectioned, stained with H&E, examined, and given an estimated score of 0 to 4 based on the severity interstitial pneumonia. (c) Lymph node sections harvested on 14 DPC were examined and given a score from 1 to 3 according to the amount of hyperplasia. (d) PRRS-specific viral RNA in serum was detected by real-time PCR on 14 DPC. Data are shown as mean ± SEM for five pigs per group. An asterisk denotes statistically significant ($P<0.05$)

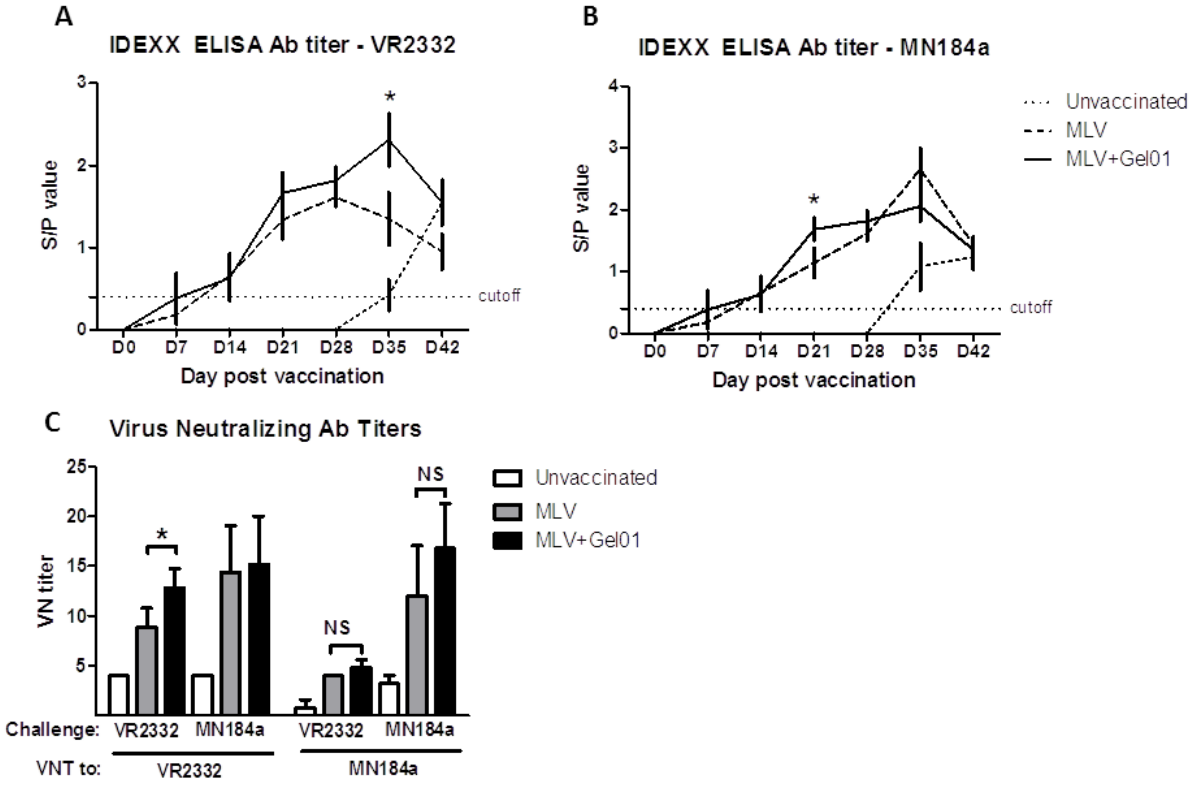

Figure 3.2. Pigs vaccinated with Gel 01 ST adjuvanted MLV have enhanced PRRSV-specific antibodies and virus neutralizing antibodies after homologous PRRSV challenge (a,b) PRRSV-specific IDEXX ELISA S/P ratio in each group after vaccination and challenge. The ELISA threshold for positive sera was set at a sample to positive (s/p) ratio of 0.4 according manufacturer's instructions. (c) Individual serum samples collected on 14 DPC were titrated in Marc145 cells. Anti-PRRSV neutralizing Ab titers were determined as the highest serum dilution that could inhibit CPE. Data are shown as mean ± SEM for five pigs per group. An asterisk denotes statistically significant ($P<0.05$).

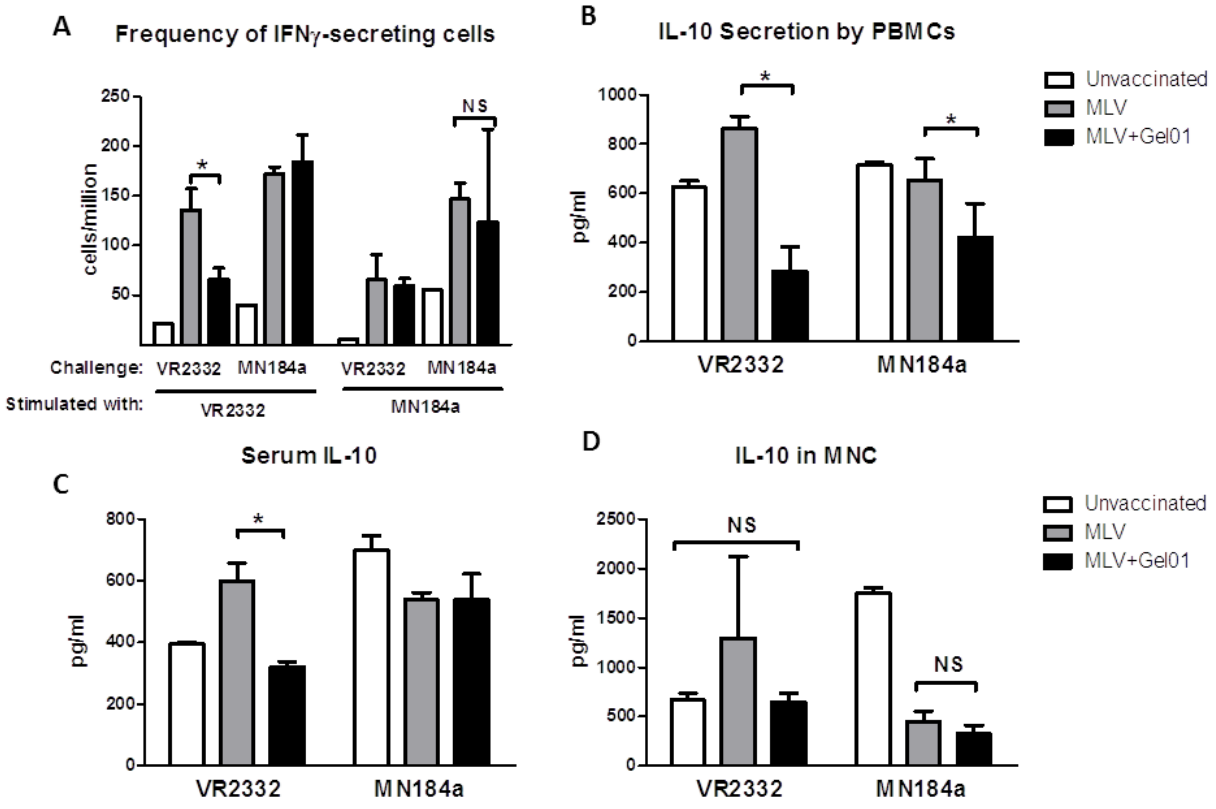

Figure 3.3. Pigs vaccinated with Gel 01 ST adjuvanted MLV have decreased PRRSV-specific IFN-γ and IL-10 cytokines after PRRVS challenge. (a) PBMCs collected from pigs at 14 DPC were re-stimulated with VR-2332 or MN184a for 24 hrs. IFN-γ-secreting cells were then analyzed by the ELISpot assay. (b-d) Blood samples were collected from pigs at 14 DPC. Serum and PBMC supernatants were then subjected to ELISA analysis for IL-10 secretion. Lung MNCs were also collected at necropsy (14 DPC), re-stimulated with PRRSV, and subjected to IL-10 detection by ELISA. Data are shown as mean ± SEM for five pigs per group. An asterisk denotes statistically significant ($P<0.05$).

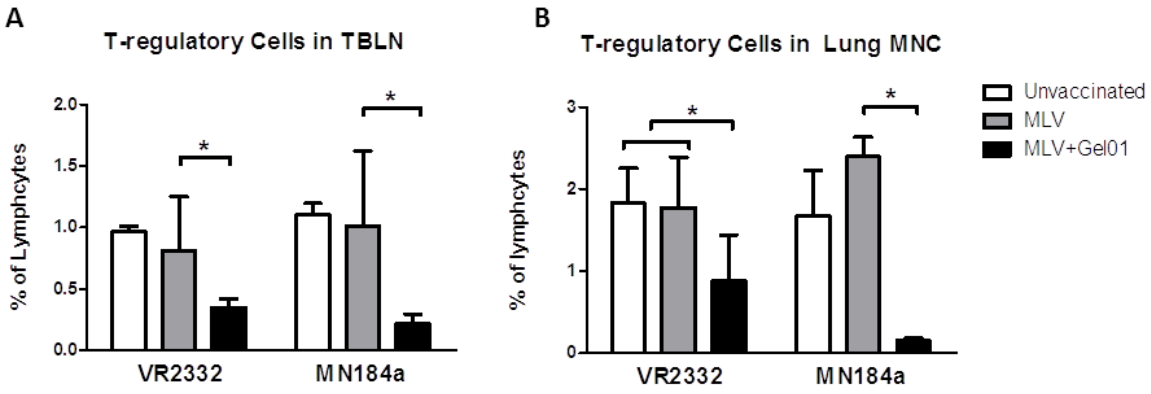

Figure 3.4. Pigs vaccinated with Gel 01 ST adjuvanted MLV had reduced T-regulatory cell populations after PRRSV challenge. (a,b) TBLN cells and lung MNCs were isolated from pigs at necropsy (14 DPC). Total cell population was gated as $CD4^+$ cells, T-regulatory cells were further gated on $CD25^+$ and $FoxP3^+$ expression. Each bar is an average percent of T-regulatory cells from five pigs± SEM. An asterisk indicates a statistically significant difference ($P<0.05$).

Table 3.1. Frequency of T cell subpopulations in pigs after challenge with PRRSV

Immune cells	Mock	Unvaccinated		PRRSV-MLV		PRRSV-MLV+Gel01	
A.Blood	(-)	VR2332	MN184a	VR2332	MN184a	VR2332	MN184a
T cell	57.2±2.4	45.3±3.6	42.1±1.7	53.7±5.5	50.4±4.9	57.8±5.3	58.9±6.5
Th cell	10.3±2.1	21.9±3.0	19.8±4.3	18.7±9.1	17.8±9.4	19.7±1.9	16.5±1.1
Activated Th	4.3±3.0	5.0±1.9	5.7±2.4	6.5±3.4	3.9±1.9	5.1±1.6	5.1±2.1
CTL	30.8±4.2	30.2±8.9	28.4±6.6	28.0±4.8	29.8±6.1	33.2±4.7	37.1±4.9
NK cell	10.1±3.2	4.3±2.0	3.2±1.6	11.4±3.1	27.3±12.7	16.8±1.7	20.8±1.2
γδ T cell	12.2±2.9	10.8±3.3	9.7±4.6	11.7±1.7	12.7±2.8	11.8±1.8	11.7±1.3
T-reg cell	0.15±0.02	0.25±0.03*	0.41±0.12	0.28±0.04*	0.22±0.16	0.14±0.03*	0.26±0.04
Th/memory	9.2±1.1	7.5±2.6	9.1±3.1	16.7±1.7	16.0±2.8	14.6±3.1	10.9±2.7
B.TBLN	(-)	VR2332	MN184a	VR2332	MN184a	VR2332	MN184a
T cell	54.3±6.2	58.3±4.7	59.1±5.5	59.0±3.0	63.7±12.0	59.0±9.2	64.2±3.5
Th cell	26.4±3.1	33.5±3.5	37.1±7.9	31.3±1.9	33.9±7.1	30.4±3.4	39.5±4.0
Activated Th	5.7±1.2	5.0±1.5	4.7±2.3	6.7±1.3	8.2±2.3	4.0±1.1	4.9±0.4
CTL	4.07±1.2	3.6±2.1	10.2±3.7	11.9±2.8	12.5±6.2	13.5±4.4	17.2±5.6
NK cell	10.3±2.3	7.3±1.5	5.6±2.4	10.5±5.9	12.0±4.3	9.7±4.5	11.1±3.1
γδ T cell	17.1±4.0	10.6±3.5	13.1±4.4	17.3±6.8	17.1±6.3	14.9±9.5	16.8±6.9
T-reg cell	2.0±0.8	1.1±0.4*	1.0±0.2	1.0±0.4*	0.8±0.4	0.4±0.2*	0.5±0.2
Th/memory	4.1±1.7	5.3±2.4	3.6±1.9	5.4±2.7	6.2±2.8	4.0±1.7	6.1±2.3
C. Lung	(-)	VR2332	MN184a	VR2332	MN184a	VR2332	MN184a
T cell	61.5±5.0	85.9±9.7	81.4±7.2	73.2±4.7	77.9±6.9	81.9±5.6	78.3±8.3
Th cell	7.2±2.2	9.6±1.5	9.7±2.4	10.8±2.8	12.8±3.1	11.6±2.7	13.7±1.8
Activated Th	3.6±1.0	4.1±0.9	5.4±1.3	3.9±0.5	3.1±0.6	6.0±0.8	5.4±1.4
CTL	21.2±4.1	37.9±9.1	38.2±6.7	40.2±9.1	41.3±9.9	38.7±7.4	35.6±5.3
NK cell	2.8±0.7	1.4±0.2	2.3±0.9	1.3±0.3	1.1±0.6	2.2±0.6	2.8±0.4
γδ T cell	20.0±2.6	28.9±7.1	37.0±10.2	31.5±13.1	34.9±7.9	32.3±4.5	31.4±6.0
T-reg cell	0.5±0.2	1.1±0.1*	2.3±0.7	2.4±0.3*	3.8±1.1	0.2±0.1*	0.9±0.2
Th/memory	1.3±0.3	1.2±0.4	1.3±0.4	1.6±0.4	2.1±0.5	2.4±0.4	3.3±0.8

Table 3.1. PBMCs were isolated from blood collected from pigs at necropsy (14 DPC), and T cell subsets were counted by flow cytometry according to their phenotypes. Each number is expressed as the average percent of total PBMCs from five pigs ± SEM. An asterisk indicates a statistically significant difference between unvaccinated or MLV vaccinated and Gel01-MLV pigs.

Chapter 4 - Peptide nanofiber hydrogel adjuvanted live virus vaccine enhances cross-protective immunity to porcine reproductive and respiratory syndrome virus [3]

Abstract: Porcine reproductive and respiratory syndrome virus (PRRSV) is prevalent in swine farms worldwide and is a major source of economic loss and animal suffering. Rapid genetic variation of PRRSV makes it difficult for current vaccines to confer protection against newly emerging strains. We recently demonstrated that a novel peptide nanofiber hydrogel (H9e) could act as a potent adjuvant for killed H1N1 vaccines. Therefore, the objective of this study was to evaluate H9e as an adjuvant for PRRSV modified live virus (MLV) vaccines. Pigs were vaccinated with Ingelvac PRRSV MLV with or without H9e adjuvant before being challenged with the VR-2332 (parental vaccine strain) or MN184A (genetically diverse strain) PRRSV. Pigs vaccinated with MLV+H9e had higher levels of circulating vaccine virus. More importantly, pigs vaccinated with MLV+H9e had improved protection against challenge by both PRRSV strains, as demonstrated by reduced challenge-induced viremia compared with pigs vaccinated with MLV alone. Pigs vaccinated with MLV+H9e had lower frequency of T-regulatory cells and IL-10 production but higher frequency of Th/memory cells and IFN-γ secretion than that in pigs vaccinated with MLV alone. Taken together, our studies suggest that the peptide nanofiber hydrogel H9e, when combined with the PRRSV MLV vaccine, can enhance vaccine efficacy against two different PRRSV strains by modulating both host humoral and cellular immune responses.

4.1. Introduction

Pork is one of the most widely consumed meats in the world, accounting for more than a third of meat production worldwide. Infectious diseases remain the biggest threat to the pork industry, resulting in billions of dollars in economic losses [1]. One particularly devastating disease known

[3] Chapter 4 was reproduced from X. Li, et al, Peptide nanofiber hydrogel adjuvanted live virus vaccine enhances cross-protective immunity to porcine reproductive and respiratory syndrome virus, Vaccine (2013) 1-12.

to lead to the dramatic decline of swine herds and increased pork prices is porcine reproductive and respiratory syndrome (PRRS) [1]. Clinical features of PRRS include massive abortion in sows and weight loss, respiratory disease, and mortality in young pigs. PRRS outbreaks continue to emerge rapidly and with increased virulence; if left untreated, outbreaks will endanger swine industries worldwide.

PRRS is caused by the PRRS virus (PRRSV). Field isolates often differ significantly in the degree of virulence and pathogenicity, presumably due to a high degree of genetic variation among strains [2]. PRRSV can be broadly divided into two distinct genotypes, Type 1 (European) and Type 2 (North American). Each genotype also contains several subtypes, which are also genetically diverse and lead to immunity limited to the initial infecting genotype, with only partial or no protection from reinfection by other subtypes [3]. Due to genetic diversity and the rapid evolution rate of PRRSV, development of a broadly protective PRRSV vaccine is challenging, but vaccination remains the most effective way to control PRRS. Several types of commercial vaccines, including killed or modified live vaccines, have been widely used [4]. Current killed vaccines are largely ineffective in preventing both PRRSV infection and disease, so most farms vaccinate their herds with modified live vaccines to control PRRS outbreaks. Modified live vaccines are shown to reduce disease caused by genetically similar strains, but they provide very limited or no protection against genetically unrelated field isolates [5]. Therefore, broad cross-protection against genetically dissimilar PRRSV strains should be the main consideration for the design of improved PRRSV vaccines.

Adjuvants including oil-in-water emulsions, polymers, and bacterial antigens have been tested in combination with modified live vaccines in an effort to reduce the antigenic load and improve vaccine efficacy [6,7]. Results from these studies suggest that addition of adjuvant to MLV PRRSV vaccines can lead to increased protection to PRRSV challenge. Peptide hydrogels also might be a promising delivery system for vaccines due to their high water content, polymer network and reversible sol-gel (solution to gel) formation. Peptide hydrogels have been well studied as drug delivery systems, for tissue engineering applications, and in 3-D cell culture and show promising results [8,9]. We recently developed a novel peptide that can form a flexible nanofiber hydrogel (H9e) and functions as a potent adjuvant for killed H1N1 influenza vaccines

[10]. To further characterize the capabilities of the H9e hydrogel, we evaluated H9e as an adjuvant for PRRSV MLV vaccines. Results show that the addition of H9e to MLV enhanced protection of pigs to both homologous and heterologous strains of PRRSV. Compared with pigs vaccinated with MLV alone, animals vaccinated with MLV+H9e developed earlier and more robust PRRSV-specific neutralizing antibodies as well as increased PRRSV-specific Th1 cytokine IFN-γ and reduced immunosuppressive cytokine IL-10. Together, these results suggest that PRRS MLV vaccine formulated with H9e adjuvant may increase vaccine efficacy against genetically diverse PRRS viruses.

4.2. Materials and Methods

4. 2.1. Cells, virus and adjuvant preparation

MARC-145 cells were maintained in modified Eagle's medium (MEM) supplemented with 7% fetal bovine serum (FBS) containing penicillin (100U/ml) and streptomycin (100 µg/ml) at 37 °C with 5% CO_2. For virus infection and titration, MEM supplemented with 2% FBS was used. Ingelvac PRRS® modified live virus vaccine (MLV) was purchased from Boehringer Ingelheim Vetmedica Inc (St. Joseph, MO). PRRSV MN184A was a kind gift from Dr. Kay Faaberg (National Animal Disease Center, USDA-ARS, Ames, IA). PRRSV strains VR-2332 (ATCC, Manassas, VA) and MN184A were prepared and titered in MARC-145 cells and stored in aliquots at -80 °C until use. H9e peptide was prepared as previously described with a final concentration of 17.5 mg/ml [10]. PRRS MLV vaccine was resuspended in 50 ml vaccine diluent, provided by the manufacturer, to yield a 2-fold concentrate of vaccine virus. MLV-alone vaccine was then mixed 1:1 with vaccine diluent. A solution of 6 mg H9e with 1.2% porcine serum in diluent/MEM medium was added 1:1 with 2x MLV to prepare MLV+H9e vaccine.

4.2.2. Pigs, vaccination and PRRSV challenge

Thirty-five female/unvaccinated (3 weeks old) Large White-Duroc crossbred PRRSV-free pigs were divided into 7 groups (n = 5) and housed in separate pens within the Large Animal Research Center (LARC) at Kansas State University. These piglets were confirmed sera-negative for antibodies to PRRSV by ELISA and PRRSV-free in serum by RT-PCR. Pigs were

immunized intramuscularly on day 0 with placebo, PRRS-MLV ($1x10^6$ $TCID_{50}$/ pig), or PRRS-MLV+H9e ($1x10^6$ $TCID_{50}$ + 6 mg H9e/ pig). Twenty-eight days post vaccination (DPV), the pigs were challenged with either homologous PRRSV VR-2332 ($1x10^6$ $TCID_{50}$) or heterologous MN184A ($5x10^5$ $TCID_{50}$). Body weight measurements and blood samples were collected weekly (0, 7, 14, 21, 28 DPV and 7, 14 DPC). Pigs were also monitored daily for rectal temperature and clinical signs after challenge. All pigs were humanly euthanized 15 days post challenge (DPC). All animal experiments were approved by the Institutional Animal Care and Use Committee at Kansas State University.

4.2.3. Analysis of serum virus titer

Total RNA was extracted from serum and one-step SyBR Green real-time PCR (Bio-Rad, Hercules, CA) was performed to evaluate the PRRSV ORF7 expression level as previously described [11]. For quantification, total RNA of a known $TCID_{50}$ of virus was 10-fold serially diluted and were used to generate a standard curve. The virus quantities of unknown samples were determined by linear extrapolation of the Ct value plotted against the standard curve.

4.2.4. PRRSV-specific and virus neutralizing antibody titration

PRRSV-specific ELISA antibody titers were measured using the Herdcheck Porcine Reproductive and Respiratory Syndrome X3 Antibody Test (IDEXX Laboratories, Westbrook, ME) as described by the manufacturer. Virus neutralizing antibody titer in the serum was analyzed as previously described [11]. Briefly, serum samples were heat-inactivated and serial dilutions were mixed with PRRSV VR-2332 or MN184A viruses. After incubation, the mixtures were transferred to MARC-145 cells and incubated for 72 hours. Cytopathic effect (CPE) was used to determine the end-point titers that were calculated as the reciprocal of the highest serum dilution to neutralize >90% CPE induced by 200 $TCID_{50}$ of PRRSV in duplicate wells per sample.

4.2.5. Analysis of cytokine responses

Pig sera were collected at 7 DPC to evaluate IL-4, IL-8, IL-10, IFN-γ, and TNF-α cytokine secretion profiles by ELISA. Procedures were performed as per the manufacturer's instructions (Invitrogen, Carlsbad, CA). Additionally, at necropsy (15 DPC), 10^6 tracheobronchial lymph node (TBLN) mononuclear cells (MNCs) and lung MNCs were restimulated with 200 $TCID_{50}$ of the respective challenge PRRSV similar to that described in Ferrari et al. [12]. Cell culture supernatants were analyzed by ELISA for IL-10 cytokine secretion (Invitrogen).

4.2.6. Flow cytometry analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by Ficoll-Hypaque gradient centrifugation using Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO). Flow cytometry analysis was performed to determine different lymphocyte populations based on the cell surface marker phenotype: T-helper cells ($CD3^+CD4^+CD8^-$), cytotoxic T lymphocyte ($CD3^+CD4^-CD8^+$), Th/memory cells ($CD3^+CD4^+CD8^+$), T-regulatory cells ($CD4^+FoxP3^+CD25^+$) and γδ T cells ($CD8^+$ $TcR1N4^+$). The mouse anti-pig TcR1N4 antibody was purchased from VMRD (Pullman, WA), and all other antibodies were purchased from BD Biosciences (San Jose, CA). Immuno-stained cells were acquired using a FACS Caliber (BD Biosciences) flow cytometer. Frequencies of individual lymphocytes were analyzed by 100,000 events using FlowJo software (Tree Star, Inc., Ashland, OR).

4.2.7. Statistical analysis

All data were expressed as the mean value of five pigs ± SEM. The differences in the level of humoral response, body weight, cytokine production and viremia among each group were determined by the one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test using Sigmaplot 11 software (Systat Software Inc., San Jose, CA). Differences were considered statistically significant when $p<0.05$.

4.3. Results

4.3.1. H9e adjuvant enhances cross-protection efficacy of MLV to heterologous PRRSV infection in pigs

Our previous studies showed that H9e hydrogel can be a safe, efficacious adjuvant for the killed H1N1 swine influenza vaccines, resulting in significantly higher hemagglutination inhibition titers and antibody titers to swine influenza than immunization with antigen alone [10]. Since H9e acts as a potent adjuvant for killed subunit vaccines, we hypothesized that H9e hydrogels could also work as an adjuvant for a modified live PRRS vaccine.

H9e solution is a free-flowing solution at ambient temperature, and forms an injectable hydrogel at physiological conditions. H9e was easily mixed with MLV and showed no virucidal effects on the vaccine virus (data not shown). Pigs were vaccinated with Ingelvac PRRS MLV vaccine alone (MLV), Ingelvac PRRS MLV vaccine adjuvanted with hydrogel H9e (MLV+H9e), or PBS (mock). Twenty-eight days post vaccination (DPV), pigs were subjected to virus challenge with low virulence homologous VR-2332 (1×10^6 $TCID_{50}$/pig) or moderately virulent heterologous MN184A (5×10^5 $TCID_{50}$/pig) strains of PRRSV. The mean body temperature of unvaccinated pigs challenged with VR-2332 or MN184A was 0.3 or 1.0^oC higher than vaccinated pigs, respectively, with no difference between vaccinated groups (data not shown). Interestingly, pigs vaccinated with MLV gained significantly less weight than unvaccinated and MLV+H9e vaccinated pigs at 28 DPV (Fig. 4.1A, B), suggesting that the un-adjuvanted MLV vaccine virus may cause sub-clinical disease in pigs.

To determine if vaccinated pigs were protected from homologous or heterologous virus challenge, titers of circulating virus were measured 7 days post challenge (DPC). We found that the pigs vaccinated with MLV+H9e were able to significantly clear both the VR-2332 and MN184A strains circulating in the blood 7 days post challenge (7 DPC). Pigs vaccinated with MLV alone were able to significantly clear the homologous VR-2332 virus strain (Fig.4.1C). The pigs vaccinated with MLV alone had reduced viral load in the blood after MN184A challenge; however, it was not statistically significant from that in the unvaccinated-challenged

group of pigs (Fig. 4.1D). Taken together, these results suggest that the addition of H9e adjuvant to PRRSV MLV vaccines can enhance protection against genetically distinct stains of PRRSV.

4.3.2. Pigs vaccinated with MLV+H9e have increased vaccine virus circulation in the blood after vaccination

H9e rapidly forms a gel once it is formulated with MLV. Therefore, we suspect that the H9e nanofiber network may act as a scaffold to prolong vaccine virus entry into the blood or enhance its replication within the host to enhance the vaccine's protective effects. To determine if H9e adjuvant affects the viral load of the vaccine strain of PRRSV in vaccinated pigs, we measured circulating viral load in the serum of all pigs weekly. Interestingly, pigs vaccinated with MLV+H9e started to develop significantly higher levels of circulating virus two weeks after vaccination and reached an average maximum of 5.5-fold (9,057±402 $TCID_{50}$ equivalent/ml) higher virus titers than that in pigs vaccinated with MLV alone (1,638± 625 $TCID_{50}$ equivalent /ml) on 21 DPV (Fig. 4.2A). By 28 DPV, the MLV virus was eliminated from the blood of pigs in all groups. Therefore, our results suggest that H9e may act to stabilize the MLV virus within the host and thus to increase the exposure of antigen to the host immune system.

4.3.3. H9e-MLV vaccinated pigs show enhanced PRRSV-specific antibodies and PRRSV neutralizing antibodies

To determine whether increased antigen exposure might lead to enhanced humoral and cellular immune responses in vaccinated pigs, we first evaluated antibody responses of pigs vaccinated with PRRSV MLV in the presence or absence of H9e. Serum samples were analyzed by commercial IDEXX PRRSV-specific antibody ELISA. By 14 DPV, 9 out of 10 pigs vaccinated with MLV+H9e were positive for PRRSV-specific antibodies (as defined by manufacturer at S/P ratio ≥ 0.4), compared with only 5 out of 10 pigs in MLV alone groups (Fig. 4.2B). Therefore, these results suggest that addition of H9e adjuvant results in the earlier onset of PRRSV antibodies than MLV alone. By 21 DPV, all vaccinated pigs had seroconverted to anti-PRRSV antibody positive.

PRRSV MLV vaccination is characterized by generation of early non-protective antibodies specific to the nucleocapsid protein (as measured by IDEXX ELISA) and delayed generation of protective virus neutralizing antibodies. To determine if the H9e-mediated prolonged viremia affects the production of neutralizing antibodies as well, the PRRSV neutralizing antibody titers (VN titers) were analyzed. On 28 DPV, pigs vaccinated with MLV+H9e had similar VN titers to both VR-2332 and MN184A as that in pigs vaccinated with MLV-alone (Fig.4.2C). After homologous VR-2332 viral challenge, pigs vaccinated with MLV+H9e had significantly higher VN titer to VR-2332 and comparable VN titer to MN184A compared with pigs vaccinated with MLV alone (Fig. 4.2D). However, all vaccinated pigs developed similar levels of VN titers after heterologous MN184A viral challenge (Fig. 4.2E). Therefore, our results show that the addition of H9e hydrogel adjuvant can induce early on-set and enhanced antibody production over vaccinating pigs with MLV alone.

4.3.4. Pigs vaccinated with MLV+H9e hydrogel have increased pro-inflammatory cytokines and reduced immunosuppressive cytokine secretion profiles

Because we found that H9e can improve the humoral immune responses of pigs to the PRRS MLV vaccine, we next assayed the effects of H9e adjuvant on MLV-elicited cytokine profiles. In doing so, sera at 7 DPC were analyzed for the presence of IL-4, IL-8, IL-10, IFN-γ and TNF-α. As shown in Fig. 4.3, the levels of IFN-γ, but not TNF-α, in the sera from MLV+H9e vaccinated pigs was significantly higher than that in pigs vaccinated with MLV-alone after challenge (Fig4.3A,B). The levels of IL-4 and IL-8 in sera from pigs vaccinated with MLV+H9e were significantly higher than that from pigs vaccinated with MLV-alone when the pigs were challenged with VR-2332 PRRSV (Fig. 4.3C, D). Conversely, the secretion of immunosuppressive cytokine IL-10 in sera of MLV+H9e vaccinated pigs was reduced compared with that in the MLV-alone vaccinated pigs after challenge with both VR-2332 and MN184A (Fig. 4.3E). IL-10 expression levels of lung and TBLN MNCs also were analyzed at necropsy (15 DPC). As shown in Fig. 3e, after these cells were re-stimulated with either VR-2332 or MN184A *in vitro*, reduced IL-10 cytokine levels were observed in the supernatant of lung and lymph node MNCs of the pigs vaccinated with MLV+H9e. Therefore, our results suggest that addition of H9e to MLV vaccine alters cytokine expression profiles.

4.3.5. Pigs vaccinated with MLV+H9e display decreased T-regulatory and increased Th/memory lymphocyte subpopulations

To verify if the change in cytokine expression patterns was associated with changes in lymphocytes population, the frequencies of T-helper cells, cytotoxic T lymphocyte, Th/memory cells, T-regulatory cells and γδ T cells in blood, lung, and lymph nodes were evaluated using flow cytometry analysis. As shown in Fig. 4.4, a significant decrease of the T-regulatory (Treg) lymphocyte population (Fig. 4.4A) and increase of the Th/memory lymphocyte population (Fig. 4.4B) was observed in the blood of pigs vaccinated with MLV+H9e than that in pigs vaccinated with MLV alone 4 weeks after vaccination. The decrease of Treg lymphocyte population and increase of Th/memory lymphocyte population were also observed 14 DPC in pigs challenged with homologous VR-2332 or heterologous MN184A PRRSV in blood, TBLN, and lung MNC samples (Fig.4.4C, D). Additionally, we examined Th cells, CTL, γδ T cells and NK cell population before and after challenge and found no significant changes in any groups (data not shown).

4.4. Discussion

Current commercial vaccines, both killed virus and modified live, are deficient in protecting swine herds from the consistently evolving field isolates of PRRSV [13]. One approach to improving PRRSV vaccine efficacy is the addition of immunomodulatory adjuvants including water-oil emulsions, aluminum, bacterial components, and polymers to killed or live modified PRRSV vaccines [14]. Interestingly, a new class of adjuvants, nanoparticles, has been shown to increase the cross-protection efficacy of killed PRRSV vaccines. In a recent study by Dwivedi et al, PLGA nanoparticle-entrapped killed PRRSV vaccine induces a cross-protective immune response against heterologous PRRSV challenge via enhanced innate and PRRSV-specific adaptive responses [15]. However, further studies are needed to reduce the cost and complexity of nanoparticle production before nanoparticle-based vaccines can be widely used as commercial products. Some more cost-effect commercial water-in-oil emulsion and polymers adjuvants, such as Montanide^{TM} ISA 15A and Gel01 ST, have also been utilized in live modified PRRSV vaccines [6]. Deville et al. showed that pigs vaccinated with adjuvanted MLV vaccine containing

50% of the antigen load had equivalent protection as pigs vaccinated with full dose of vaccine without the adjuvant.

We recently reported that a biodegradable hydrogel could act as an adjuvant for killed swine influenza vaccines [10]. These previous results show that when H9e hydrogel was used in place of the supplied adjuvant of commercially available FluSure XP (Zoetis Inc), the H9e-adjuvanted vaccine led to significantly higher HAI titers and equivalent IgG antibody responses than the standard formulation of FluSure. Based on these results, we explored the ability of H9e hydrogel to act as an adjuvant for PRRS modified live virus vaccine and here we demonstrated that H9e hydrogels enhanced the vaccine's protective effects for both homologous and heterologous PRRSV infection.

H9e hydrogel forms a biodegradable nanofiber network under physiological conditions [16]. Peptide-based nanofiber networks have been shown to aid in the controlled release of growth factors, therapeutics, and viruses [17-19]. Therefore, we hypothesized that this nanofiber network could create pockets that the vaccine virus could occupy and thus act as an antigen depot such that PRRS virus is presented slowly to the host immune system. We show here that the H9e had no virucidal effects and was able to facilitate increased PRRS vaccine virus presentation to the host, as shown by enhanced vaccine virus circulation in the blood (Fig. 4.2A). These results suggest high vaccine virus titers in the blood induced by MLV+H9e vaccination may facilitate the generation of an early and robust PRRSV immune response and provide better protection against genetically diverse strains of PRRSV.

In addition to high circulating vaccine virus, pigs vaccinated with MLV+H9e had earlier on-set of PRRS-specific ELISA antibodies and enhanced neutralizing antibody titers to homologous virus. Previous reports have shown that PRRSV-specific antibodies can appear as soon as one week post-vaccination or challenge, however seroconversion is often observed between 14-21 days post exposure [6,20]. Our results are consistent with these reports and we found that addition of H9e adjuvant reduced the time that most pigs became positive for PRRSV-specific antibodies (Fig.4.2B).

In order to gain insight into the immunologic mechanisms employed by the hydrogel adjuvant, cytokine expression levels after PRRSV challenge were compared among vaccinated groups of pigs. We found that the Th1-related cytokine IFN-γ in the sera of pigs vaccinated with MLV+H9e was significantly higher than that of pigs vaccinated with MLV alone after both homologous and heterologous challenges (Fig.4.3A, B). IFN-γ is a key cytokine that is associated with host cell-mediated immunity (CMI) response, which is secreted by natural killer cells and several different T cell subpopulations, and its expression is often decreased by PRRSV infection [21,22]. These studies suggest that decreased IFN-γ expression allows PRRSV to evade the host immune response and result in chronic PRRS infections. Interestingly, a recent study using two different PRRSV strains reported that systemic enhancement of IFN-γ further activates natural killers and T cell subpopulations creating a positive feedback loop for the rapid clearance of PRRSV [23]. Therefore, the elevated production of IFN-γ observed in the pigs vaccinated with H9e+MLV could explain the increased PRRS viral clearance and improved protective immune response we observed.

The expression of inflammatory cytokine IL-8, but not TNF-α, was increased in pigs vaccinated with H9e-MLV when pigs were challenged with homologous VR-2332 virus (Fig. 4.3B and D). In previous studies, low serum IL-8 levels are related to persistent PRRSV infection, and elevated IL-8 levels in serum is correlated with the clearance of PRRS virus [24,25]. Although our results also indicated IL-8 may play a role in vaccination-induced clearance of PRRS virus, further experimentation is required to fully characterize the ability of H9e adjuvant to modulate IL-8 expression levels.

The Th2-related cytokine IL-4 was increased in the sera of pigs vaccinated with MLV+H9e compared with the pigs vaccinated with MLV alone only after homologous VR-2332 challenge. IL-4 expression has been shown to control macrophage inflammatory activities in the pig [26]. While IL-4 expression levels in PRRSV-infected pigs can remain unaltered [27], recent studies suggest that natural PRRSV infection can significantly induce the expression of IL-4 [28], suggesting that PRRSV-mediated IL-4 induction may be strain dependent. In our hands, the increased IL-4 expression after VR-2332 challenge correlated well with enhanced protection of

pigs vaccinated with MLV+H9e than that of pigs vaccinated with MLV-alone. This indicates IL-4 may play a positive role in the immune response to PRRSV infection.

PRRSV infection or vaccination has been shown to induce a strong immunosuppressive response characterized by promoting the secretion of IL-10 to antagonize the protective Th1 immune response [29,30]. In our study, we found that the concentrations of IL-10 in the serum and tissues of pigs vaccinated with MLV alone were consistently higher than that from pigs vaccinated with MLV+H9e (Fig. 4.3E). IL-10 is mainly produced by a small subpopulation of T lymphocytes termed T-regulatory cells [31]. Consistent with IL-10 levels, the frequency of T regulatory cells in MLV+H9e vaccinated pigs was dramatically reduced in blood, lung MNCs, and TBLNs post infection (Fig. 4.4C). Therefore, it is likely that the reduced T-regulatory cell population and production of IL-10 may contribute to the enhanced Th1 response and efficient elimination of PRRSV after challenge in the pigs vaccinated with MLV+H9e.

$CD4^+CD8^+$ T cells, which include T-helper, cytolytic, and memory properties, are a major type I IFN-γ cytokine secreting population [32]. In our study, pigs vaccinated with MLV+H9e generated significantly higher Th/memory cell populations both before and after challenge compared with the unvaccinated and MLV vaccinated pigs. This result is consistent with the observation that IFN-γ production is elevated in pigs vaccinated with MLV+H9e. The high frequency of functional T memory cells may contribute to rapid recall response for the quick elimination of subsequent PRRS virus exposure [33]. The ability of H9e adjuvant to shift MLV vaccine from mainly humoral, to a response having both humoral and cell-mediated immune responses suggest that CMI may be important for increased vaccine protection potential. Our results support the notion that MLV+H9e may act to enhance IFN-γ and reduce IL-10 production via increasing Th/memory and decreasing Treg lymphocyte populations, thereby causing a shift to a Th1-type immune response to provide a better protection against PRRSV infection.

We have previously shown that flexible polymer adjuvants such Montanide™ Gel01 ST also can enhance the protective effects of modified live PRRSV vaccines: however, their enhanced protective effects are limited to homologous re-infection [11]. Interestingly, it was demonstrated that the addition of Gel01 adjuvant to MLV vaccine could enhance vaccine-induced antibody-

mediated immunity but did not promote a stronger cellular-mediated immunity. Furthermore, Gel01 adjuvanted MLV did not show improved efficacy in reducing heterologous challenge-induced viremia as compared with MLV alone. Thus, these results and our previous work suggest that the vaccine's ability to generate a cellular-mediated immune response may be essential to mediate its cross-protective efficacy against PRRSV infection.

Author Disclosure Statement

The authors declare that they have no competing financial interests.

4.5. References

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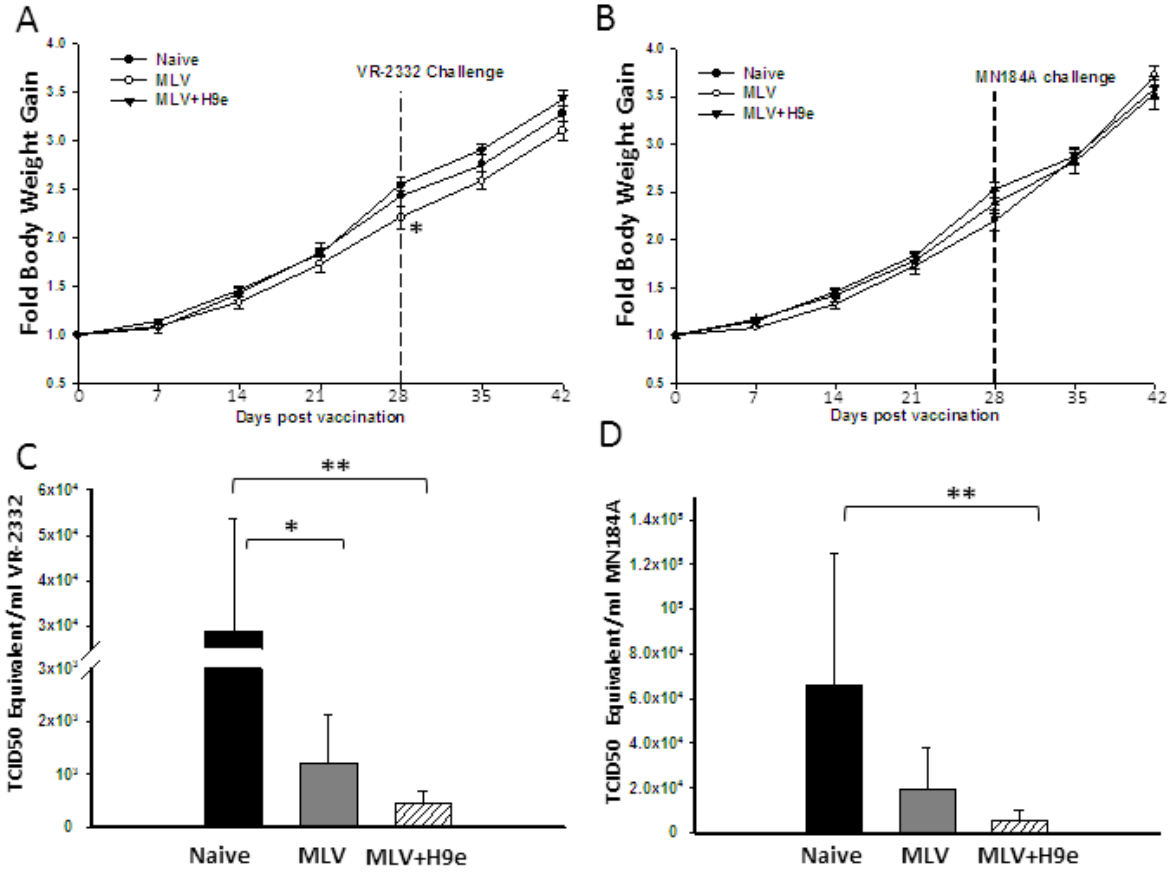
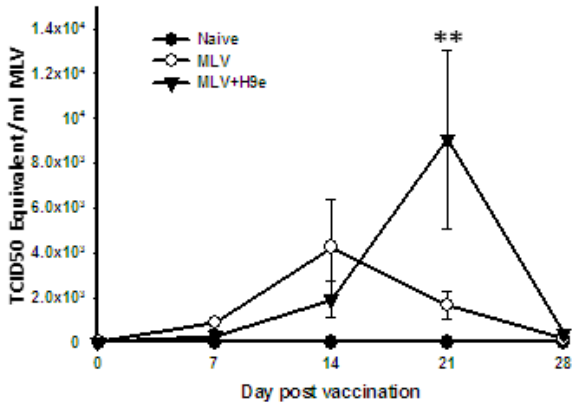


Figure 4.1. H9e adjuvant enhances protection efficacy of MLV to homologous and heterologous PRRSV infection in pigs. Pigs (3-week-old) were vaccinated with MLV or MVL+H9e and challenged with the VR-2332 or MN184A strain of PRRSV 28 days post vaccination. (A, B) Fold body weight gain during the duration of the experiment was determined. (C, D) Viral RNA in the serum ($TCID_{50}$ equivalent /mL) was measured on 7 days post challenge (DPC) by RT-PCR. Viremia data are shown as means ± SEM (n=5). Bracketed groups were compared and * denotes $p < 0.05$, ** denotes $p < 0.01$.

A



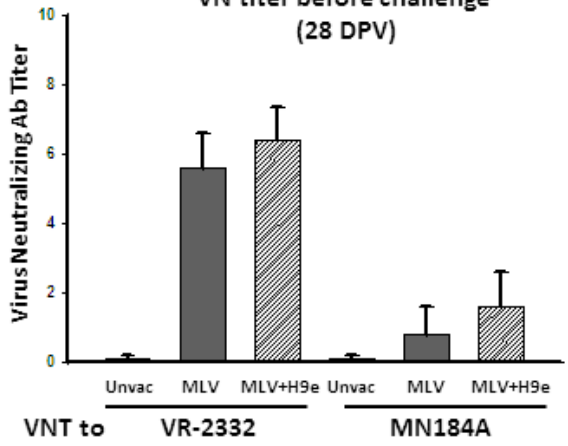
B

PRRSV Ab Seroconvertion

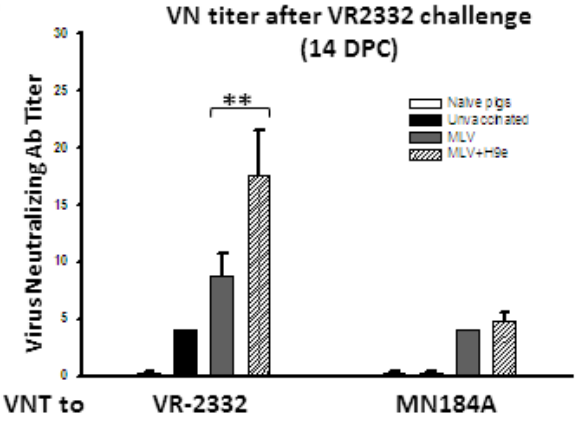
Group/DPV	7	14	21	28
Naïve pigs	0/15	0/15	0/15	0/15
MLV	3/10	5/10	10/10	10/10
MLV+ H9e	4/10	**9/10**	10/10	10/10

*Individual pigs are positive when s/p ratio >0.4

C



D



E

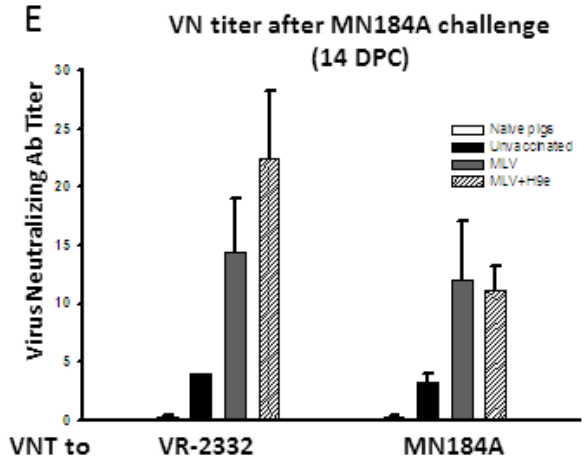


Figure 4.2. Pigs vaccinated with MLV+H9e have increased vaccine virus circulation and produce an earlier on-set of PRRSV-specific antibodies. (A) Viral RNA of MLV vaccine virus in the serum ($TCID_{50}$ equivalent /mL) was determined by RT-PCR weekly after vaccination with MLV, or MLV+H9e. Data were shown as mean ± SEM (n=5) ** $p < 0.01$. (B) Serum from vaccinated pigs was assayed for PRRSV-specific antibodies with IDEXX HerdCheck ELISA kit. The threshold for seroconvertion was set at a sample-to-positive (s/p) ratio of 0.4 according to manufacturers' instructions. (C-E) Serum samples were titrated individually in MARC-145 cells for the levels of anti-PRRSV neutralizing antibodies 28 days post vaccination (DPV) or 14 days post challenge (DPC) determined as the highest dilution that inhibited CPE. Data are shown as mean ± SEM (n= 5). Bracketed groups were compared and ** denotes $p < 0.01$.

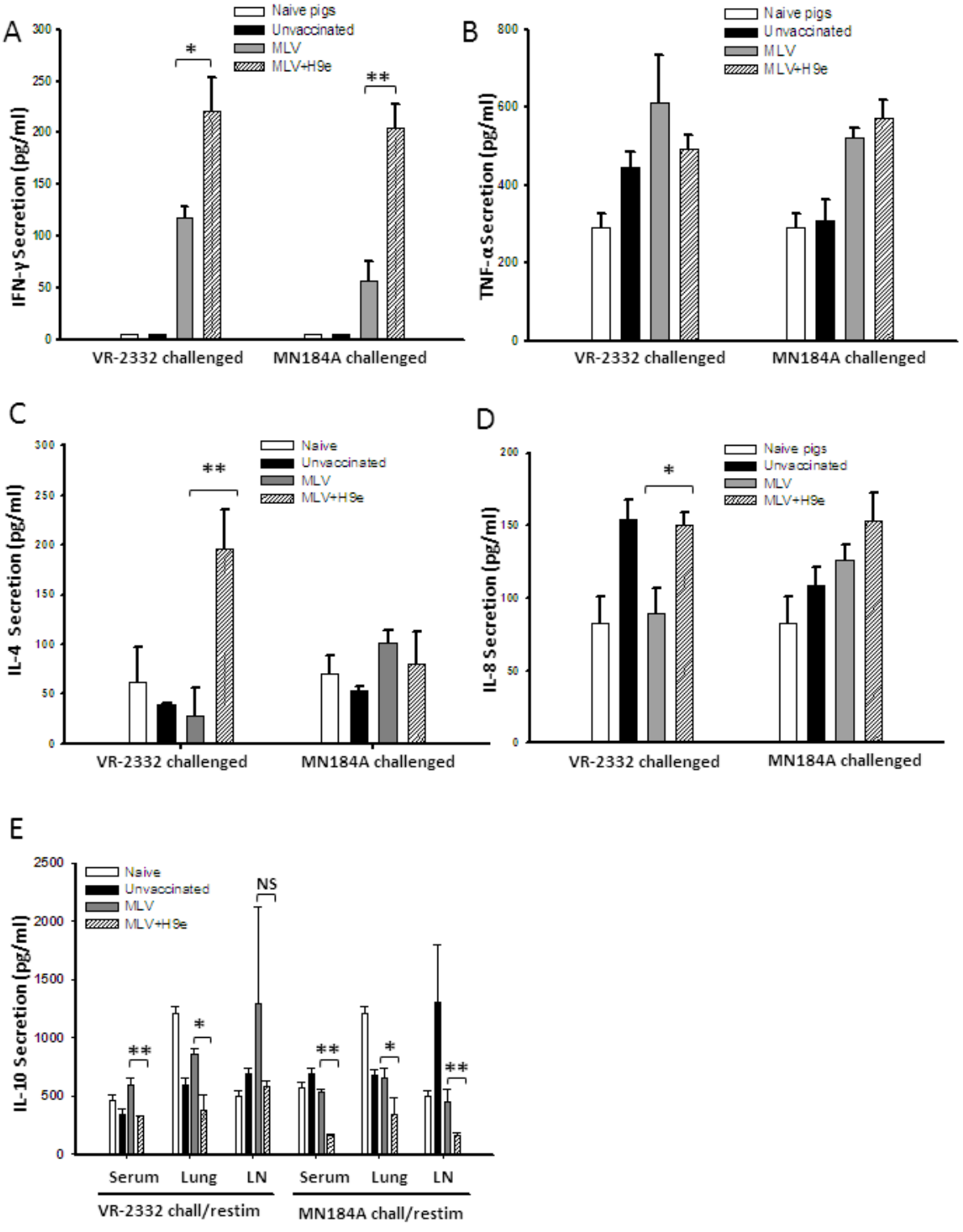

A
Naive pigs
Unvaccinated
MLV
MLV+H9e
IFN-γ Secretion (pg/ml)
VR-2332 challenged
MN184A challenged
B
TNF-α Secretion (pg/ml)
C
Naive
IL-4 Secretion (pg/ml)
D
IL-8 Secretion (pg/ml)
E
IL-10 Secretion (pg/ml)
NS
Serum
Lung
LN
VR-2332 chall/restim
MN184A chall/restim

Figure 4.3. Pigs vaccinated with MLV+H9e have increased PRRSV-specific IFN-γ, IL-4, IL-8 and reduced IL-10 cytokine secretion. Cytokine expression profiles in the sera of pigs 7 days post challenge (DPC) were examined by quantitative ELISA, (A) IFN-γ (B) TNF-α (C) IL-4 and (D) IL-8. (E) IL-10 concentrations in serum samples and supernatants of PBMCs and lung MNCs which were collected at necropsy (15 DPC) and restimulated with corresponding PRRSV strains. Bracketed groups were compared and * denotes p <0.05, ** denotes p <0.01, and NS=no difference

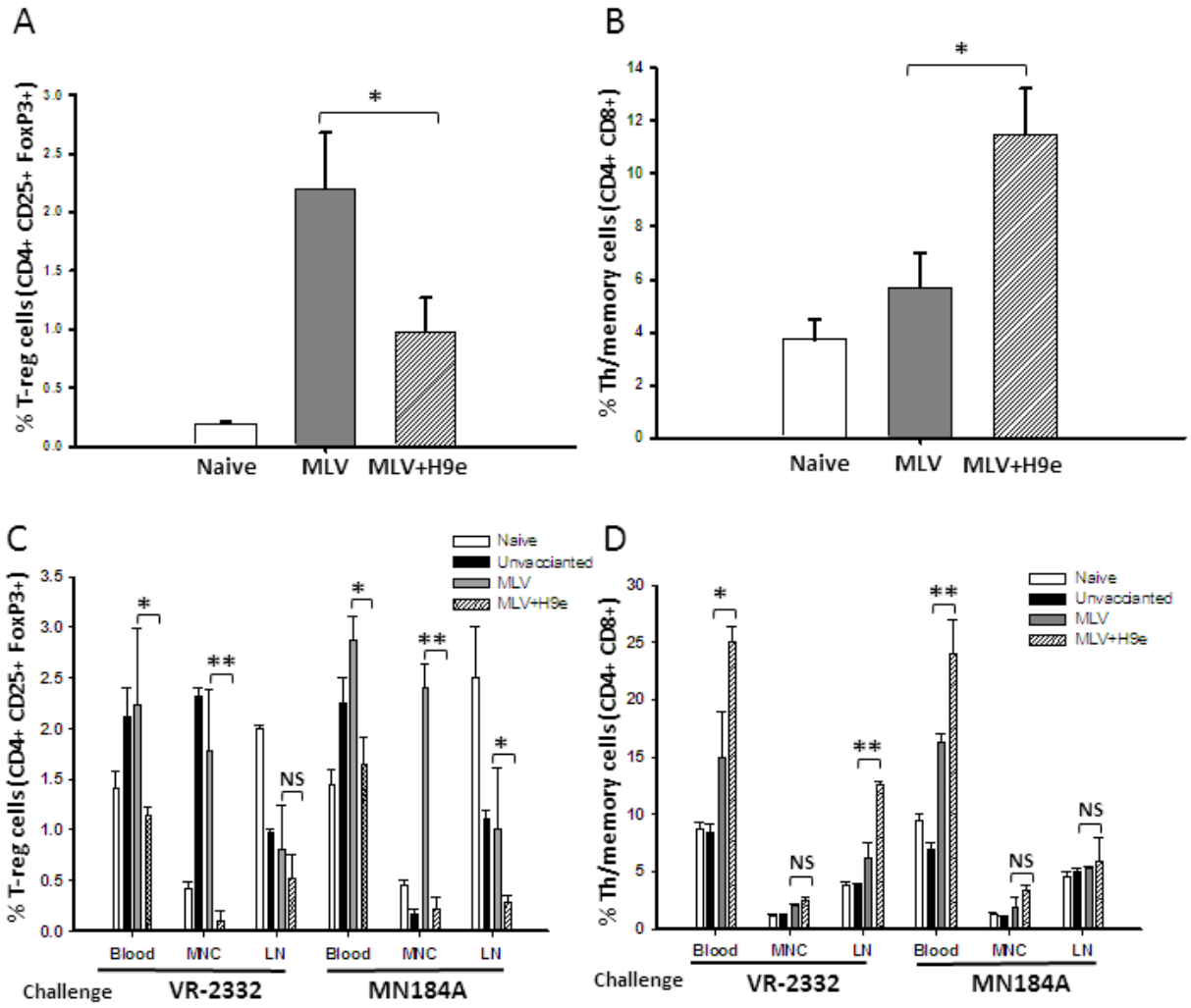


Figure 4.4. Pigs vaccinated with MLV+H9e have decreased T-regulatory and increased Th/memory lymphocyte subpopulations**.** Whole blood was collected and stained for CD4, CD8,

FoxP3, and CD25. (A) Shown are the percentages of T-reg cells that were triple-positive for CD4/FoxP3/CD25 28 days post vaccination (DPV) and (C) 14 days post challenge (DPC). (B) Also shown are the percentages of Th/memory cells that were double-positive for CD4/CD8 on 28 DPV and (D) on 14 DPC. Data is shown as mean ± SEM (n=5). Bracketed groups were compared and * denotes $p < 0.05$, ** denotes $p < 0.01$, and NS=no difference.

Chapter 5 - Comparison of immune responses between infection with a Chinese highly-pathogenic strain of PRRSV vs the US NADC-20 strain

Abstract: The differences in pig immune responses elicited by a Chinese highly-pathogenic strain of PRRSV (HP-PRRSV) and the American NADC-20 strain were compared in this study. Pigs infected with HV-PRRSV, rescued from an infectious clone of Chinese HP-PRRSV, exhibited significantly higher fevers, more body weight loss, and more severe histopathogical lung lesions than infection with U.S. NADC-20 strain. The HV-PRRS virus showed significant higher proliferation ability *in vivo* than NADC-20 evidenced by more than 10-fold increased viral load in the serum at 9 day post infection (DPI). However, the high proliferation ability of HV-PRRSV did not enhance the induction of PRRSV-specific ELISA antibodies. NADC-20 infected pigs showed significantly higher Ab titers than HV-PRRSV infected pigs at 9 DPI. Infection with HV-PRRSV induced a significantly higher levels of TNF-α and IL-10 in both serum and lung tissues and significantly higher IFN-α and IFN-γ in the serum. Flow cytometry analysis showed that HV-PRRSV infected pigs generated significantly higher frequencies of NK cells in the peripheral blood and Th/memory, CTLs, and T-reg cells in the lung tissue as compared with NADC-20 infected pigs. Thus, this study demonstrates that different immunity profiles were elicited by HV-PRRSV and NADC-20, and these differences may contribute to the better understanding of the pathogenesis of HP-PRRSV.

5.1. Introduction

Highly-pathogenic PRRS virus (HP-PRRSV) belongs to type 2 genotype (North American, prototype strain VR-2332) of PRRSV, which is a member of the genus *Arterivirus*, family *Arteriviridae*. HP-PRRSV is characterized by high fever and high rates of morbidity and mortality [1]. This novel and highly virulent variant of PRRSV, which first emerged in China in 2006, has rapidly spread to most countries in Southeast Asia [2]. HP-PRRSV exhibits more extensive tissue tropism than classic PRRSV [3]. Besides lymphoid tissues, IHC examination showed that HP-PRRSV antigen can also be detected in the tissues including trachea, esophagus, liver, kidney, cerebellum, stomach, and intestine, which proves its highly pathogenicity to pigs [4].

Compared with the prototype of type 2 genotype strain VR-2332, HP-PRRSV can elicit strong immune responses by the evidence of a striking elevation in the level of cytokines associated with both innate and adaptive immunity in HP-PRRSV infected pigs [5]. However, VR-2332, which was first isolated in 1987, only leads to mild clinical symptoms and does not circulate in the field any more [6]. NADC-20 is a virulent North American PRRSV strain, which was first isolated in an "atypical PRRSV abortion storm" in 2001 [7]. It has been widely used for viral challenge to evaluate the efficacy of PRRSV vaccines in the United States [8]. Compared with the other strains of PRRSV in the U.S., NADC-20 can lead to clinical fever (≥ 40°C) and more robust immune responses after infection of pigs [8]. Therefore, analysis of the host immune responses elicited by two virulent strains of PRRSV will contribute to better understanding of the pathogenesis of highly pathogenic PRRSV and facilitate more effective vaccine development. In this study, 7-week old pigs were infected with the HV-PRRSV or NADC-20 strain of PRRSV and the clinical symptoms and the profiles of host immune response were compared.

5.2. Materials and Methods

5.2.1. Cells and virus

MARC-145 cells were maintained in modified Eagle's medium (MEM) supplemented with 7% fetal bovine serum (FBS) containing 100U penicillin/ml and 100ug streptomycin/ml at 37 °C

with 5% CO2. For virus infection and titration, MEM supplemented with 2% FBS was used. HV-PRRSV was rescued from highly pathogenic PRRSV infectious clone [9], and propagated on MARC-145 cells for three passages before use. PRRSV NADC-20 was a kind gift from Dr. Lager Kelly (National Animal Disease Center, USDA-ARS, Ames, IA).

5.2.2. Experiment design

Fifteen conventional Large White-Duroc crossbred weaned specific-pathogen free piglets (7 weeks of age) were tested to be PRRSV negative by ELISA and RT-PCR and were divided into 3 groups. Five pigs were infected with $2x10^5$ $TCID_{50}$/pig NADC-20 and housed for 10 days before the necropsy within the Large Animal Research Center facility (Bio-safety Level 2) at Kansas State University. Another 10 pigs were divided into two groups (n=5/group) and housed in separate rooms within Biosafety Research Institute (Bio-safety Level 3) Kansas State University. One group of pigs was infected with $2x10^5$ $TCID_{50}$/pig HV-PRRV on day 0, and another group of pigs received MEM medium and worked as uninfected controls throughout the study. Weight measurements and blood samples were collected every 3 days and rectal temperature and clinical signs were monitored daily. All pigs were humanly euthanized at 10 days post infection (DPI). Thymic and lung tissues were weighed and compared with total body weight to evaluate thymic atrophy and lung inflammation induced by the viral infection. Serum samples were used to measure viral load, PRRSV-specific antibodies, and cytokine expression. All animal experiments were approved by the Institutional Animal Care and Use Committee at Kansas State University.

5.2.3. Collection of blood samples for analysis

Blood was collected from each pig every 3 days. Serum was separated from clotted blood and preserved at -20°C. Serum was used for evaluation of viral titer and PRRSV-specific ELISA antibody titers (Herdchek Porcine Reproductive and Respiratory Syndrome Antibody test Kit, IDEXX Laboratories) as previously described [10]. Pig serum at 7 DPI and the supernatant of lung homogenates were used to analyze cytokine expression [11]. IFN-α and IFN-β ELISA kits were purchased from Abcam (Abcam, Cambridge, MA). IL-4, IL-8, IL-10, IFN-γ and TNF-α were purchased from Invitrogen (Life Technologies, Carlsbad, CA). Procedures were performed as per the manufacturer's instructions. For a given sample, the OD_{450} was then transformed to

concentration by applying a linear regression formula calculated from the results of the standards provided in each kit.

Total RNA was extracted from serum and One-step Taq-Man qPCR was performed to calculate PRRSV RNA copy number in the serum sample according to the brochure of manufacture (EZ-PRRSV^{TM} MPX4.0 Real Time RT-PCR, Tetracore Inc., Rockville, MD). A standard curve was constructed by preparing serial dilutions of an RNA control, supplied in the RT-PCR kit, and virus quantities of unknown samples were determined by linear extrapolation of the Ct value plotted against the standard curve.

Hepatized whole blood was subjected to flowcytometry analysis to determine different lymphocyte populations based on the cell surface marker phenotype: T-helper cells ($CD3^+CD4^+CD8^-$), cytotoxic T lymphocyte ($CD3^+CD4^-CD8^+$), Th/memory cells ($CD3^+CD4^+CD8^+$), T-regulatory cells ($CD4^+FoxP3^+CD25^+$) and γδ T cells ($CD8^+TcR1N4^+$) [11]. The mouse anti-pig TcR1N4 antibody was purchased from VMRD (Pullman, WA), and all other antibodies were purchased from BD Biosciences (San Jose, CA). Immuno-stained cells were acquired using a FACS Caliber (BD Biosciences) flow cytometer as described previously[11]. Briefly, PBMC was treated with 2% pig serum to block Fc receptors. Cells were then stained with an appropriate Ab which was either directly conjugated to a specific fluorochrome or with a purified Ab to pig specific immune cell surface marker (TcR1N4). For cells stained with a purified Ab, labeled cells were treated with anti-species isotype specific secondary Ab conjugated with fluorochrome. Finally, cells were fixed with 1% paraformaldehyde before flow cytometer reading. Percentages of each lymphocyte population were analyzed by 100,000 unique events using FlowJo software (Tree Star, Inc., OR, USA).

5.2.4. Histopathological analysis

Pigs were humanely euthanized at 10 DPI as approved by the Kansas State University Institutional Animal Use and Biosafety Committee. The lungs were macroscopically and microscopically evaluated as previously described [12]. Briefly, the dorsal and ventral surfaces of each lung lobe were given a score representing the approximate proportion that was

consolidated. Individual lobe scores were used to determine an overall lung score representing the percentage of lung with pneumonia. Sections of each of the 4 lobes of the right lung were fixed in 10% buffered neutral formalin, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H & E). Scoring of microscopic lung pathology was done in a blinded fashion by a veterinary pathologist in the Kansas State Veterinary Diagnostic Laboratory. Grading was on a 4 point scale as previously described [12].

5.2.5. Statistical analysis

All data were expressed as the mean value of five pigs ± SEM. The differences in the level of humoral response, body temperature and body weight, viral titer, lung score, cytokine production, and percentage of lymphocyte subpopulations among each group were determined by the one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test using Sigmaplot 11 software (Systat Software Inc., San Jose, CA). Differences were considered statistically significant when $p<0.05$.

5.3. Results

5.3.1. Pigs infected with HV-PRRSV had significantly higher fever and less body weight gain as compared with NADC-20 infected pigs

HP-PRRSV infection is characterized by high fever, high percentage of morbidity and mortality in pigs [1]. Thus, the rectal temperature of pigs was monitored daily. The average body temperature in HV-PRRV infected pigs was above 40°C, the cutoff of clinical fever throughout the study, and it was significantly higher than that in NADC-20 infected pig except at 7 DPI (Fig.5.1A). The NADC-20 infected pigs developed clinical fever only at 1 and 7 DPI, with the mean body temperature on these two days being 40.5°C. One pig within the HV-PRRV infection group died at 3 DPI and two other pigs were euthanized due to severe weakness and moribund condition at 6 DPI (Fig. 5.1B). The clinical signs of HP-PRRSV-infected pigs included dehydration, respiratory distress, shivering, and inability to bare weight on front limbs. Two of the dead pigs developed cutaneous hemorrhages and cyanotic extremities on the edges of their ears. None of pigs in the NADC-20 infection group or control group died or had to be euthanized. Pigs in the NADC-20 infection group showed transient fever, but no other clinical

symptoms were observed. HV-PRRSV infected pigs rapidly lost their body weight as compared with the naïve and NADC-20 infected pigs, but their average body weight returned to the starting weight by 10DPI (Fig.5.1C).

5.3.2. HV-PRRSV infection led to severe thymus atrophy and lung inflammation in pigs

Severe lesions including marked interstitial pneumonia, lymphadenopathy and thymic atrophy were observed in HV-PRRSV infection pigs. Postmortem finding include pulmonary edema, hematoma, pleural adhesion, peritoneal and pericardial effusions, and renal petchia. Pigs in HV-PRRSV infection group showed more severe and extensive pneumonia than NADC-20 infected pigs, and the macro- and histo- pathological lung scores in this group were significantly higher than NADC-20 infected group (Fig.5.2A, B). No pathologic lesions were identified in control pigs.

HV-PRRSV was previously reported to lead to thymus atrophy [3]. To confirm this, the ratio of thymus/body weight was calculated to evaluate the thymus atrophy at necropsy. The ratio of thymus/total body weight of pigs in HV-PRRSV infection group was significantly lower as compared with NADC-20 infection group (Fig.5.2C), which supports that severe thymus atrophy occurs in HP-PRRSV infected pigs. In contrast, the thymus weights of pigs in the NADC-20 infected group showed the similar average weight to the naïve pigs. The ratio of lung/total body weight was used to evaluate the inflammation status after virus infection. The ratio was significantly higher in HV-PRRSV infected pigs than NADC-20 infected pigs (Fig.5.2D), and there was no difference in the ratio between NADC-20 infected pigs with naïve pigs. The above data showed that HV-PRRSV infection lead to significant thymus atrophy and lung inflammation as compared with NADC-20 infection in pigs.

5.3.3. HV-PRRSV infection showed enhanced viral titers in pigs but did not elicit earlier or higher PRRSV-specific IDEXX ELISA Antibodies than NADC-20 infected pigs

HP-PRRSV was previously reported to have higher proliferation ability than the classic PRRSV strains [5]. Indeed, the virus RNA copy number in the serum was higher in HV-PRRSV infected

pigs than NADC-20 infected pigs at 3 DPI, but the difference was not significant (Fig.5.3A). At 6 DPI, the viremia in the blood was similar (average $2.5x10^6$ RNA copy number/μl) in both challenge groups. By 9 DPI, the viral titer in NADC-20 infected pigs dropped 10 fold, whereas the serum virus copy number of HV-PRRSV infected pigs increased to $3x10^6$ RNA copy number/μl. The above results showed that HV-PRRSV has significantly higher proliferation ability than NADC-20.

PRRSV-specific antibodies elicited by the two strains of PRRSV were measured by IDEXX ELISA kit. The high proliferation ability of HP-PRRSV did not elicit earlier or higher titer of PRRSV-specific Ab. Indeed, at 9 DPI, the average ELISA antibody titer in NADC-20 infected pigs was significantly higher than that in HV-PRRSV infected pigs (Fig.5.3B).

5.3.4. Cytokine expression in the serum and lungs was up-regulated by HV-PRRSV compared with NADC-20 infection

Sera collected at 7 DPI and the supernatant of lung homogenates collected at 10 DPI were analyzed for innate cytokine (TNF-α, IFN-α, IFN-β, and IL-8) and adaptive cytokine (IL-10, IL-4, and IFN-γ) expression. As for the innate cytokines, HV-PRRSV infection induced significantly higher TNF-α level in both serum and lung samples from the pigs (Fig.5.4A, B). HV-PRRSV infection also induced significantly higher IFN-α in the serum but significantly lower IFN-α in the lung samples as compared with NADC-20 infected pigs. There was no significant difference between the two infected groups for the expression of IFN-β and IL-8 in serum and lung samples. As for the adaptive cytokines, HV-PRRSV infection elicited significantly higher IL-10 and IFN-γ in the serum of pigs, and significantly higher IL-10 in the lung samples sample as compared with NADC-20 infected pigs (Fig.5.4A, B).

5.3.5. Higher-frequency of NK cells, Th/memory, CTLs and Treg cells, but reduced total T cells were observed in HV-PRRSV infected as compared with NADC-20 infected pigs

The frequency of various lymphocyte populations after infection was monitored by flow cytometry. In the blood, the frequency of total T cells and NK cells in HV-PRRSV infected pigs were significantly higher than the NADC-20 infected pigs (Fig.5.5A). In contrast, HV-PRRSV

infection significantly decreased the Th/memory cell population in the blood samples of pigs as compared with NADC-20 infected pigs. There were no significant differences among the groups for all other cell populations assayed. In the lung, the frequency of Th/memory, CTLs, and T-reg cells in HV-PRRSV infected pigs were significantly higher than that in NADC-20 infected pigs (Fig.5.5B). However, the total T cell population in HV-PRRSV infected pigs was significantly lower than that in NADC-20 infected pigs. There was no significant difference for the percentage of T-helper cells and γδ T cells between two infected groups in the lung samples.

5.4. Discussion

Classic PRRSV causes mild clinical symptoms and leads to abortion in sows and death of piglets. In contrast, highly pathogenic strains of PRRSV lead to death at all ages of pigs with 100% morbidity and 20% of mortality [1]. Several research groups reported that high fever and increased tissue atrophy were associated with the high mortality rate caused by HP-PRRSV [3,14,15]. HP-PRRSV was also reported to elicit elevated expression of inflammatory cytokines, which may partially explain the high fever developed after viral infection [5]. However, the difference of host immune responses induced by HP-PRRSV and classic PRRSV was seldom explored. In one study, HP-PRRSV was reported to replicate in swine with at least 100-fold increased kinetics over U.S. strain VR-2332, and elicit a striking elevation in the levels of cytokines associated with both innate and adaptive immunity [5]. VR-2332 was isolated in 1987 and it is the parental strain of one of the current PRRS modified live vaccines. This virulent strain of PRRSV can only cause mild clinical symptoms and moderate lung damage. NADC-20 was first isolated in an "atypical PRRSV abortion storm" in 2001, and it can lead to high fever (≥ 40°C) and severe lung and lymph node tissue damage [7]. Therefore, in this study, we compared the host immune responses elicited by Chinese HP-PRRSV and US NADC-20.

The HP-PRRSV was reported to induce high fever, loss of body weight, severe respiratory symptoms and high mortality. In our study, the pig body temperature in HV-PRRSV infected pigs was higher than 40°C during the duration of the infection, which may partially contribute to the dehydration and respiratory distress (Fig.5.1A). The HV-PRRSV infection led to significant pig body weight loss as compared with NADC-20 infected pigs. The HV-PRRSV infected pigs

lost an average of 10% of their body weight at 3 and 6 DPI, however, body weight returned to original weight by 10 DPI (Fig.5.1C). The body weight of NADC-20 infected pigs increased consistently after infection, although it was significantly lower as compared with the control pigs at 6 and 10 DPI (Fig.5.1C). Consistent with a previous report [5], HV-PRRSV infected pigs also showed more severe clinical symptoms including cutaneous hemorrhages and cyanotic extremities on the edge of ears ("blue ear") and higher mortality rate (3/5 pigs died).

HV-PRRSV led to significant thymus atrophy compared with NADC-20 infection. The ratio of thymus/total body weight was significantly lower in HV-PRRSV infected pigs as compared with NADC-20 infected pigs (Fig.5.2C). Thymus is the primary lymphoid tissue, in which T-lymphocytes mature and constitute the peripheral T-cell repertoire responsible for directing many facets of the adaptive immune responses. The malfunction/atrophy of thymus leads to the depletion of T lymphocytes, which was consistent with the significant loss of total T lymphocytes in the lung analyzed by the flowcytometry. In contrast, the ratio of lung/total body weight was significantly higher in HV-PRRSV infected pigs as compared with NADC-20 infected pigs (Fig.5.2D), which indicated more inflammatory responses after HV-PRRSV infection. Indeed, several T cell subpopulations which exert cytotoxic functions, such as CTLs ($CD3^+CD4^-CD8^+$) and Th/memory ($CD3^+CD4^+CD8^+$), were significantly higher after HV-PRRSV infection as compared with NADC-20 infection.

HP-PRRSV has showed higher proliferation ability than classic PRRSV both *in vitro* and *in vivo* [5]. In this study, both HV-PRRSV and NADC-20 showed similar proliferation ability within the first 6 days of infection. Interestingly, by 9 DPI the viremia in NADC-20 infected pigs declined while the viremia of HV-PRRSV infected pigs was still increasing (Fig.5.3A). In a study by Guo et al [5], the virus titer and virus load in the serum were significantly higher after rJXwn6 HP-PRRSV infection as compared with VR-2332 infection from 2 to 11 DPI. The discrepancy of the verimia level could be due to the different strains of PRRSV was used in each study, and the NADC-20 used in our study is more virulent than VR-2332. However, the high proliferation ability of HV-PRRSV did not correlate with higher titer of PRRSV-specific IDEXX ELISA antibody response, in that the average antibody titer in NADC-20 infected pigs was significantly higher than HV-PRRSV infected pigs at 9 DPI (Fig.5.3B). The IDEXX ELISA measures the

antibody response against N proteins of PRRSV, which has no protective ability to the PRRSV infection although it has been widely used for the diagnosis in the field [16]. The different ability to induce PRRSV IDEXX ELISA Ab between HP-PRRSV and classic PRRSV may contribute to the pathogenesis of viruses, and need further exploration.

TNF-α is a proinflammatory cytokine, which plays a very important role in regulation of immune responses, fever development (inflammation), and cell apoptosis [17]. Several studies showed that PRRSV down-regulated TNF-α production in the early stage of infection, which may be used by virus to circumvent infected cell apoptosis [18,19]. At the late stage of PRRSV infection, the peak of both apoptotic cells and viral antigen expression were observed in lymph nodes and tonsils of infected animals [20]. In our study, HV-PRRSV induced significantly higher TNF-α in both serum and lung samples at 7 DPI, and the high level of TNF-α expression correlates with the high level of viremia. The coincidence between high expression of TNF-α and high level of viremia at the late stage of PRRSV infection may indicate that PRRSV induces TNF-α mediated cell apoptosis to release virion progeny to infect other vulnerable cells.

Previous studies showed that infection with several classic strains of PRRSV virus induced delayed or failed production of detectable serum IFN-α level [21-23]. In contrast, HV-PRRSV infection induced significantly higher IFN-α in the serum of pigs but significantly lower levels in the lung samples. Working as a potent antiviral molecule, IFN-α was reported to significantly inhibit PRRSV replication and enhance cellular-mediated immunity (IFN-γ responses) [24,25]. However, the elevated serum IFN-α has no effect on virus clearance by the evidence of high level of viremia in HV-PRRSV infected pigs at 9 DPI (Fig.5.3A and Fig.5.4A). Also, the low level of IFN-α expression in the lung tissue after HV-PRRSV infection did not lead to the decreased IFN-γ production as compared with NADC-20 infected pigs. Therefore, the role of IFN-α in the pathogenesis of PRRSV and host immunity to combat PRRSV needs to be further explored.

HV-PRRSV also elicited a significant elevation of adaptive immunity cytokines in the serum samples, such as IL-10 and IFN-γ, and significantly higher IL-10 in the lung samples (Fig.5.4A and B). Induction of IL-10 following PRRSV infection is believed to be focal mechanism leading to the unique immunological outcomes and interference of PRRS vaccine efficacy. The

production of IL-10 in the early stage of PRRSV infection is associated with a wide array of PRRSV-induced immunomodulatory activities [24,26]. Consistent with previous studies, the expression of IL-10 in the serum and lung samples was significantly higher in HV-PRRSV infected pigs as compared with NADC-20 infected pigs [5]. The high level expression of IL-10 correlates with high titer of viremia in this study and PRRSV antigen gene expression in the lungs and tonsils of PRRSV infected pigs in previous studies [22]. Some strains of modified live PRRSV vaccines also induced IL-10 production in vaccinated pigs, which may partially contribute to the failure of PRRSV vaccination [24]. Therefore, how to circumvent the inhibitory effect of IL-10 in the early stage of PRRVS vaccination/infection could be a challenge for the PRRSV vaccine development.

IFN-γ is a key cytokine that is associated with host cell-mediated immunity (CMI) response, which is secreted by natural killer cells and several different T cell subpopulations. Significantly higher levels of IFN-γ in the serum was found in pigs infected with HV-PRRSV as compared with NADC-20 infected pigs (Fig.5.4A), which was associated with a significantly higher percentage of NK cells in the blood (Fig.5.5A). The coincidence of high levels of IFN-γ expression and the high percentage of NK cells may indicate that the production of IFN-γ at this stage might be a result of the innate immune response, most likely from antigen-stimulated NK cells [27]. However, the high level of IFN-γ in the serum did not lead to the decreased level of viremia. In contrast, the level of viremia in HV-PRRSV infected pigs was significantly higher than NADC-20 infected pigs. Therefore, the role IFN-γ plays in the protection to PRRSV infection at this stage is questionable.

Author Disclosure Statement

The authors declare that they have no competing financial interests.

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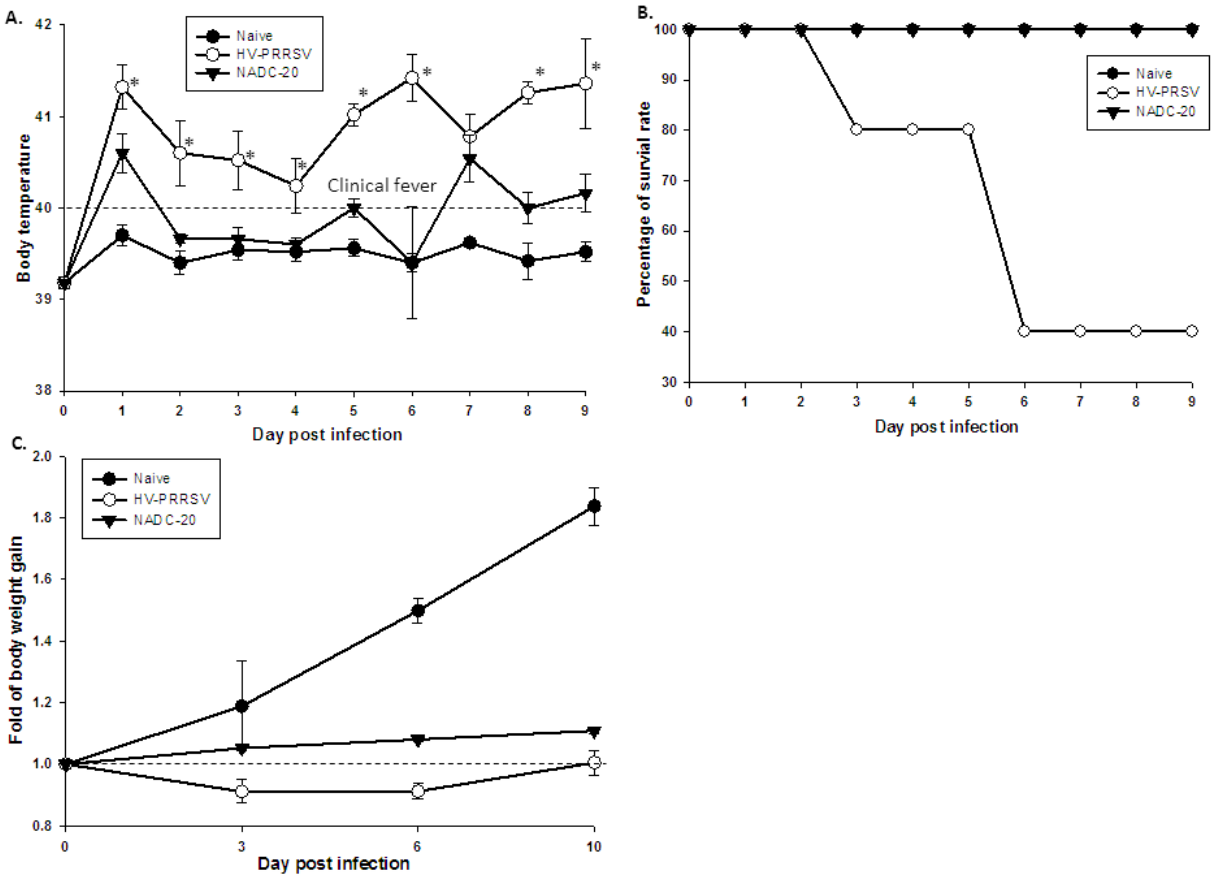

Figure 5.1. High fever, high mortality rate, and loss of body weight after HV-PRRSV infection. (A) Rectal temperature of all pigs was monitored daily after PRRSV infection. (B) Survival rate. (C) Fold total body weight gain during the duration of the experiment was calculated by considering the weight of the pig on day 0 as 1. Each bar represents the average of five pigs ± SEM. *$p<0.05$.

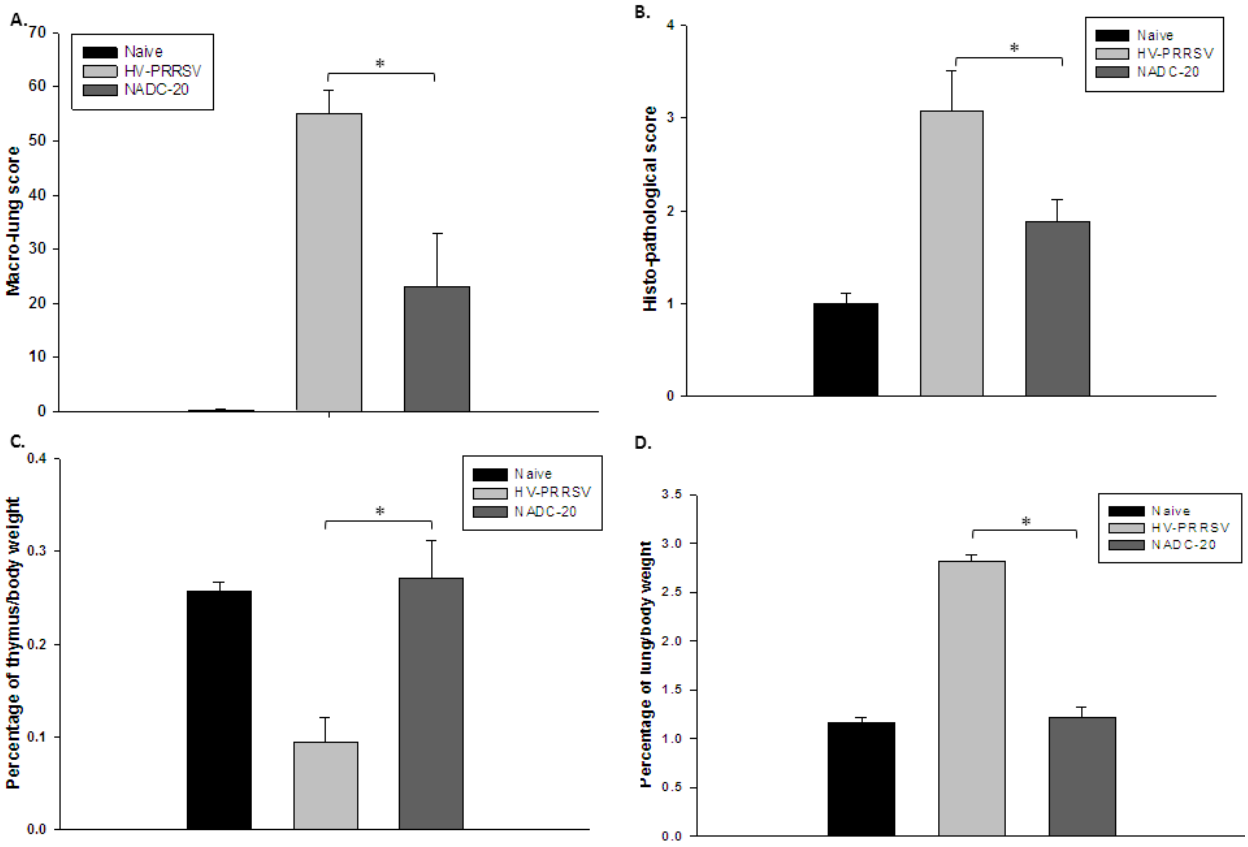

Figure 5.2. Thymus atrophy and lung inflammation caused by HV-PRRSV. Thymus weight and lung weight to body weight ratios of HV-PRRSV infected pigs showed pronounced thymus atrophy (A) and lung inflammation (B) as compared with NADC-20 infected pigs. Each bar represents the average of five pigs ± SEM. *$p<0.05$.

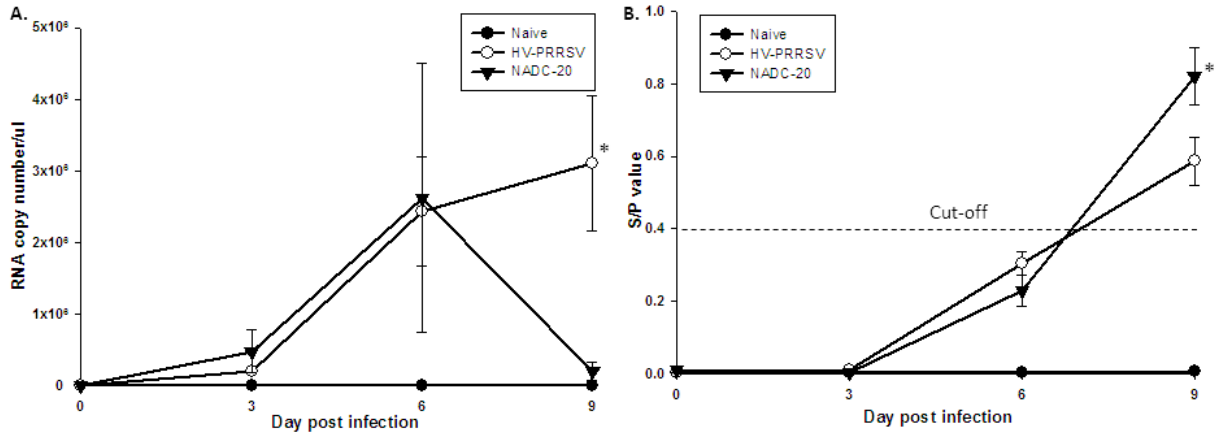

Figure 5.3. Viremia and PRRSV-specific ELISA Ab profiles after PRRSV infection**.** (A) PRRSV Viral RNA in the serum was determined by qPCR. (B) Pig serum was assayed for PRRSV-specific antibodies with IDEXX HerdCheck ELISA. The threshold for seroconvertion was set at a sample-to-positive (s/p) ratio of 0.4 according to manufacturers' instructions. Each bar represents the average of five pigs ± SEM. *p<0.05.

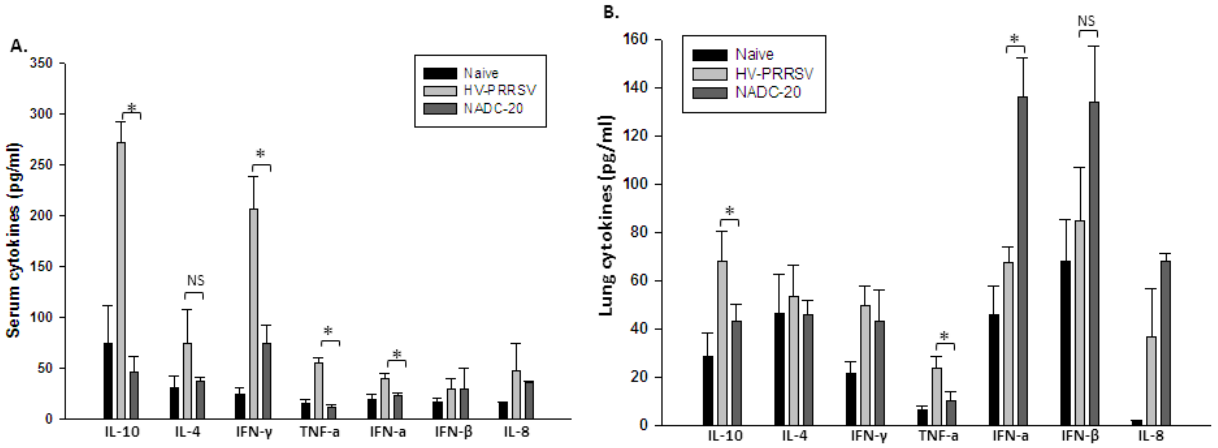

Figure 5.4. Serum immune cytokine profiles after PRRSV infection**.** Cytokine expression profiles in the sera of challenge pigs 7 days post infection (DPI) and supernatants of lung homogenates were tested by quantitative ELISA. Data were shown as mean ± SEM for 5 pigs per group. One asterisk denotes a statistically significant difference (P <0.05), and "NS" denotes there was no statistically significant difference.

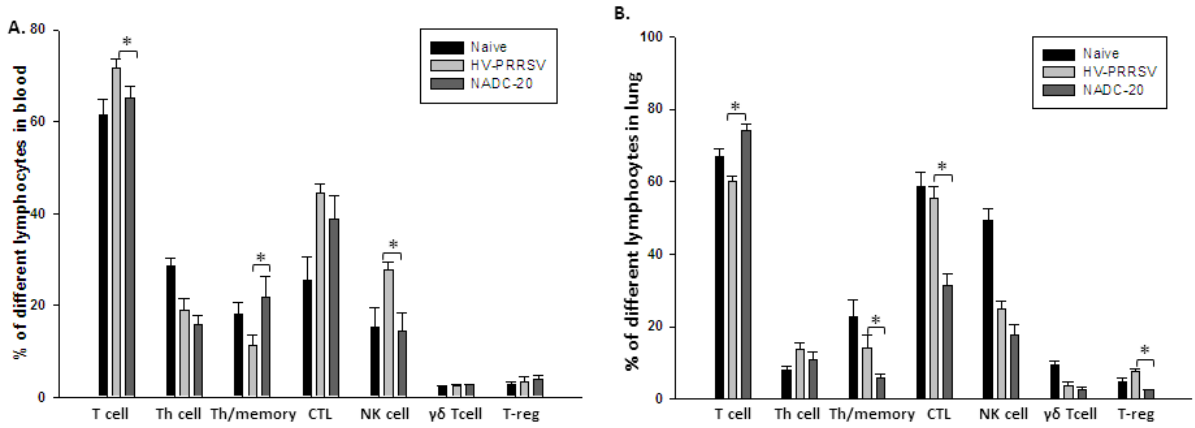

Figure 5.5. T lymphocyte population profiles after PRRSV infection**.** Whole blood and lung samples were collected at necropsy and were used to analyze the percentage of different T lymphocyte populations by flow cytometer according to their phenotypes. Data were shown as mean ± SEM for 5 pigs per group. One asterisk denotes a statistically significant difference ($P < 0.05$), and "NS" denotes there was no statistically significant difference.

Chapter 6 - Pigs immunized with Chinese high pathogenic PRRSV modified live vaccine are protected from challenge with North American NADC-20 PRRSV strain

Abstract: Porcine reproductive and respiratory syndrome (PRRS) causes huge economic loss to the swine industry worldwide, and vaccination is the most effective way to control the disease. Recently, strains of highly pathogenic PRRSV (HP-PRRSV) have appeared in China and Southeast Asia. Traditional type 2 modified live virus (MLV) vaccines developed in the United States offer no protection to these HP-PRRSV strains. Modified live vaccines specific to HP-PRRSV strains available in China are reported to provide protection to the Chinese strains of HP-PRRSV, however, the efficacy of Chinese HP-PRRSV vaccines to current circulating North American PRRSV viruses has not been reported. The aim of this study is to investigate whether pigs challenged with the North American NADC-20 strain are protected by vaccination with Chinese MLV HP-PRRSV vaccines. On day 0, pigs were vaccinated with Chinese JXA1-R-MLV vaccine or a mock vaccine. After 28 day post vaccination, pigs were challenged with $2x10^5$ $TCID_{50}$ NADC-20 PRRSV. The MLV-HP-PRRSV vaccinated pigs showed good protection to NADC-20 challenge as shown by reduced virus-induced-fever, reduced lung pathology scores, and lower NADC-20 virus load in the blood. PRRSV-specific Ab, as measured by IDEXX ELISA, appeared one week after vaccination and virus neutralizing Abs were detected 4 weeks post vaccination. Vaccinated pigs developed high titers of viral neutralizing Abs to NADC-20, JXA1-R, and HV-HP-PRRSV (a highly pathogenic strain of PRRSV). The secretion of innate cytokines IFN-α and IFN-β were elevated in the lung tissue at necropsy, but the level TNF-α was decreased in the lung tissue of MLV-HP-PRRSV vaccinated animals. In summary, our study provides the first evidence that Chinese HP-PRRSV vaccines confer protection to the North American PRRSV strain NADC-20. Therefore, the availability of Chinese HP-PRRSV vaccines in North America may not only act to increase the preparedness of possible transmission of HP-PRRSV to North America but also help protect pigs against PRRSV strains native to North America.

6.1. Introduction

Porcine reproductive and respiratory syndrome is caused by porcine reproductive and respiratory syndrome virus (PRRSV), which is a member of the genus *Arterivirus*, family *Arteriviridae*. PRRSV causes respiratory distress in pigs of all ages and reproductive failure in sows, and pigs infected with PRRSV have enhanced susceptibility to secondary microbial infections [1]. PRRSV is a highly devastating swine disease, which causes $664 million in losses within US annually, an increase from the $560 million annual cost estimated in 2005 [2]. Since 2006, highly pathogenic PRRSV (HP-PRRSV) has been reported in China in which infected pigs developed predominant clinical signs including high fever (≥42°C), anorexia, listlessness, red discoloration of skin, respiratory distress and very high morbidity and mortality rate [3]. So far, this virus has rapidly spread to most countries in Southeast Asia including Cambodia, Laos, Philippines, Bhutan, Myanmar, Thailand, South Korea and Russia [4]. The first HP-PRRSV isolate JXA1 (Genbank ID: EF112445.1) in 2006 shares 91% genome similarity with the prototype of type 2 genotype strain VR-2332 (Genbank ID: AY150564.1). However, during the prevalence of HP-PRRSV from 2006 to 2009, the commercial type 2 PRRSV vaccines failed to provide protection of pigs to HP-PRRSV infection until the first Chinese HP-PRRSV MLV JXA1-R was used in the field in 2009 [5,6]. Subsequently, two additional commercial HP-PRRSV MLV vaccines, TJM-F92 and HuN4-F112, were also launched into the Chinese market [7].

Currently, traditional type 2 PRRSV live attenuated vaccines including Ingelvac® PRRS MLV have been widely used in North American and European countries. Based on the fact that traditional PRRV MLVs failed to provide protection to HP-PRRSV, the availability of Chinese HP-PRRSV vaccines may not only act to increase the preparedness of possible transmission of HP-PRRSV to these countries, but also may help protect pigs against PRRSV strains native to them. However, the efficacy of HP-PRRSV vaccines to the circulating field PRRSVs outside China has never been explored. To answer this question, pigs were vaccinated with the HP-PRRSV-MLV vaccine and then challenged with the North American NADC-20 strain of PRRSV and the protection ability of the vaccine was evaluated.

6.2. Materials and Methods

6.2.1. Cells and virus

MARC-145 cells were maintained in modified Eagle's medium (MEM) supplemented with 7% fetal bovine serum (FBS) containing 100U penicillin/ml and 100ug streptomycin/ml at 37°C with 5% CO_2. For virus infection and titration, MEM supplemented with 2% FBS was used. NADC-20 PRRSV was a kind gift from Dr. Lager Kelly (National Animal Disease Center, USDA-ARS, Ames, IA), and JXA-1r HP-PRRSV vaccine was a kind gift from Guangdong Dahuanong Animal Health Product Co., Ltd.

6.2.2. Pigs, vaccination and challenge

Twenty conventional large White-Duroc crossbred weaned specific-pathogen free piglets (3 weeks of age) were divided into four groups within the Large Animal Research Center (LARC) facility, Kansas State University. These piglets were confirmed sera-negative for antibodies to PRRSV by ELISA and PRPSV-free in the blood by RT-PCR. Pigs were allowed to acclimate for an additional week before initiation of the experiment. Pigs were first divided into two groups (10 pigs /group) and kept in two separate pens.

Pigs in the first group were immunized intramuscularly on day post-vaccination (DPV) 0 with $1x10^6$ $TCID_{50}$ JXA-1r MLV HP-PRRSV vaccine Pigs in another group of pigs remained unvaccinated. After 4 weeks, 5 pigs in the vaccinated group were switched with 5 pigs in the unvaccinated group. Ten pigs (5 vaccinated and 5 unvaccinated pigs) were then challenged with NADC-20 ($2x10^5$ $TCID_{50}$/ pig), and other 10 pigs remained unchallenged. Necropsy was performed at 10 days post-challenge (DPC). Pigs were monitored for rectal temperature for the first 9 days after challenge and body weight once a week after vaccination and every 3 days after viral challenge.

6.2.3. Collection of blood samples for analysis

Pig blood was collected every 7 days after vaccination and every 3 days after viral challenge. Serum was separated from clotted blood and preserved at -20°C. Serum was used for evaluation of viral titer and PRRSV-specific ELISA antibody titers (Herdchek Porcine Reproductive and

Respiratory Syndrome Antibody test Kit, IDEXX Laboratories) as previously described [8]. Pig serum at 6 dpi and the supernatant of lung homogenates were used to analyze cytokine expression [9]. IFN-α and IFN-β ELISA kits were purchased from Abcam (Abcam, Cambridge, MA); and IL-4, IL-8, IL-10, IFN-γ, and TNF-α were purchased from Invitrogen (Life Technologies, Carlsbad, CA). Procedures were performed as per the manufacturer's instructions. For a given sample, the OD_{450} was then transformed to concentration by applying a linear regression formula calculated from the results of the standards provided in each kit.

Total RNA was extracted from serum and One-step Taq-Man qPCR was performed to calculate PRRSV RNA copy number in the serum sample according to the brochure of manufacture (EZ-PRRSV^{TM} MPX4.0 Real Time RT-PCR, Tetracore Inc., Rockville, MD). A standard curve was constructed by preparing serial dilutions of an RNA control, supplied in the RT-PCR kit, and virus quantities of unknown samples were determined by linear extrapolation of the Ct value plotted against the standard curve.

6.2.4. Virus neutralizing antibody titer

Serum samples were heat inactivated (56°C, 30min) and serially diluted before the titration. The serial dilutions of serum were mixed with equal volume of PRRSV strains: NADC-20, JXA1-R (parental strain of HP-PRRSV MLV vaccine) and HV-PRRSV (a Chinese HP-PRRV rescued from an infectious clone) containing 200 $TCID_{50}$ of virus. After incubation at 37°C for 1h, the mixtures were transferred to MARC-145 monolayers in 96-well plates and incubated for an additional 72h at 37°C in a humidified atmosphere containing 5% CO2. Cells were then examined for cytopathic effects (CPE). CPE was used to determine the end-point titers that were calculated as the reciprocal of the highest serum dilution to neutralize 200 $TCID_{50}$ of PRRSV in 90% of the wells.

6.2.5. ELISpot assay

Half million PBMCs were plated in enriched RPMI in a 96-well multiscreen plate (Millipore, Billerica, MA) pre-coated overnight with capture IFN-γ mAB (BD pharMingen, San Diego, CA). PBMCs were re-stimulated with NADC-20 at 0.1MOI for 24h at 37°C. IFN-γ-secreting cells were detected by biotinylated anti-pig IFN-γ detection antibody and visualized using the immunospot image analyzer (Cellular Technology, Cleveland, OH). The background values

were subtracted from the respective counts of the unstimulated cells and the immune responses were presented as the mean numbers of antigen-specific IFN-γ-secreting cells per 10^6 PBMCs from duplicate wells of each sample.

6.2.6. Histopathological analysis

Pigs were humanely euthanized on DPC 10 as approved by the Kansas state University Institutional Animal Use and Biosafety Committee. The lungs were macroscopically and microscopically evaluated as previously described [10]. Briefly, the dorsal and ventral surfaces of each lung lobe were given a score representing the approximate proportion that was consolidated. Individual lobe scores were used to determine an overall lung score representing the percentage of the total lung that was macroscopically pneumonic. Sections of each of the 4 lobes of the right lung were fixed in 10% buffered neutral formalin, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H & E). Scoring of microscopic lung pathology was done in a blinded fashion by a veterinary pathologist in the Kansas State Veterinary Diagnostic Laboratory. Grading was on a 4 point scale as previously described [10].

6.2.7. Statistical analysis

All data were expressed as the mean value of five pigs ± SEM. The differences in the level of body temperature, lung pathology score, humoral response, cytokine production and viremia among each group were determined by the one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test using Sigmaplot 11 software (Systat Software Inc., San Jose, CA).

6.3. Results

6.3.1. JXA1-R MLV vaccination protects pigs from hyperpyrexia induced by NADC-20 challenge but leads to decreased body weight gain

NADC-20 is a virulent North American PRRSV strain and was first isolated in an "atypical PRRSV abortion storm" in 2001 [11]. It has been used for viral challenge to evaluate the efficacy of PRRSV vaccines in the United States [12]. The clinical symptom induced by NADC-20 includes clinical fever (≥40°C), listless, anorexic and loss of body weight. To explore if JXA1-R MLV vaccination can reduce fever caused by NADC-20 infection, the rectal temperature of pigs

was monitored daily after NADC-20 challenge. As shown in Fig.6.1A, after NADC-20 challenge, the average of body temperature in vaccinated pigs was below clinical fever (≥ 40°C), and the body temperature of unvaccinated and NADC-20 challenged pigs developed high fever at 1, 5, 7, 8 and 9 DPC with a mean body temperature above 40°C, Therefore, the vaccination protect pigs from developing a clinical fever induced by NADC-20.

The body weight gain of pigs was monitored every week after vaccination and viral challenge. The body weight gain of vaccinated pigs was significantly lower than that of unvaccinated pigs three weeks post vaccination (Fig.6.1B). After NADC-20 challenge, the unvaccinated pigs showed slower growth rates, while vaccinated pigs had steady weight gain during the challenge period. Field HP-PRRSV was previously reported to lead to significant body weight loss [9], however, this report shows the first evidence that HP-PRRSV vaccine can also cause reduced body weight gain in pigs.

6.3.2 JXA1-R MLV Immunized pigs are protected from NADC-20 challenge and have reduced viremia and tissue damage compared with unvaccinated pigs

The PRRS viral load in the blood was measured every week after vaccination and every 3 days after challenge. Consistent with previous studies [13], vaccinated pigs developed the highest level of viremia at 14 DPV, and then virema went to undetectable levels by 28 DPV (Fig.6.2A). At 3 DPC, the circulating viral load in the serum was at similar levels in all pigs NADC-20 challenged at 3 DPC. However, by 7DPC, the viremia level in unvaccinated pigs was dramatically increased and was significantly higher than vaccinated pigs, in which viremia dropped to undetectable level. These results show that JXA1-R-MLV immunized pigs were protected from NADC-20 challenge as evidenced by reduced viremia.

The gross lung score was evaluated at necropsy. The pathology scores in unvaccinated and NADC-20 challenged pigs were significantly higher than vaccinated pigs (Fig.6.2B). Interestingly, the lung scores in unvaccinated/unchallenged control pigs were significantly higher compared with vaccinated groups (Fig.6.2B). We suspect that the control pigs were infected with vaccine strain of PRRSV when they were comingled with vaccinated pigs on 28 DPV (data not shown). Indeed, qPCR results showed a very low level of PRRSV RNA (~1000 copy number RNA/μl) in this unvaccinated/unchallenged control group (Fig.6.2A) at 3 DPC and undetectable

level of RNA at 6DPC. Consistent with qPCR result, ELISA antibody titer in this group became positive at 9 DPC (s/p≥0.4, Fig.6.3A) and the scores of histopathological lung and lymph node (Fig.6.2C, D) were also higher than vaccinated groups but were lower than unvaccinated and NADC-20 challenged pigs. Taken together, histopathological data show JXA1-R-MLV vaccinated pigs had significantly lower lesion scores as compared with unvaccinated and NADC-20 challenged pigs, which again showed that immunized pigs were protected from NADC-20.

6.3.3 JXA1-R MLV vaccination induces high PRRSV-specific IDEXX ELISA antibodies and NADC-20 strain-specific neutralizing antibodies

To determine the PRRSV-specific antibody response profiles after vaccination and challenge, commercial IDEXX PRRS antibody kits were used for the serum samples at each time point. Consistent with previous studies, PRRSV-specific antibodies can be detected at 7 DPV with a maximum s/p value occurring by 28 DPV (Fig.6.3A). After NADC-20 challenge, the antibody titers in vaccinated and NADC-20 challenged pigs increased slightly compared with the vaccinated unchallenged pigs, which decreased slightly. The unvaccinated, NADC-20 challenged pigs showed a quicker onset of PRRSV antibodies compared with these induced by vaccination virus. All serum samples from these pigs became PRRSV-positive with average s/p value of 0.5 at 6 DPC (34 DPV). Therefore, the above data show that NADC-20 can elicit earlier and higher PRRSV-specific antibodies than the JXA1-R MLV vaccine.

The titers of PRRSV neutralizing antibodies directed against NADC-20, JXA1-R (parental vaccine strain), and HV-PRRSV (HP-PRRSV strain) were determined at 10 DPC (Fig.6.3B-D). The JXA1-R MLV vaccinated pigs showed significantly higher viral neutralizing (VN) titers to all strains than unvaccinated pigs, which were undetectable. The vaccinated and NADC-20 challenged pigs also showed higher VN titers to all three strains of virus than vaccinated without challenged pigs, but only the VN titers to HV-PRRSV was significantly different between the two vaccinated groups (Fig.6.3D).

6.3.4 JXA1-R MLV vaccination increases IFN-β and IL-4 but decreases TNF-α secretion in pigs

Cytokines related to host innate (IFN-α, IFN-β, TNF-α, and IL-8) and adaptive immunity (IL-4, IL-10, and IFN-γ) in the serum at 6 DPC and supernatant of lung homogenates at necropsy (10DPC) were analyzed by commercial ELISA kits. For the innate cytokines, vaccinated pigs generated more IFN-α in the lung sample as compared with unvaccinated pigs, but the difference was not statistically significant (Fig.6.4A). Vaccinated pigs also generated significantly higher IFN-β but significantly lower TNF-α level in the lung sample (Fig.6.4B, D). The serum IFN-β in unvaccinated and NADC-20 challenged pigs was significantly higher than the other three groups, which had undetectable levels (Fig.6.4B). Unvaccinated control pigs that became infected with vaccine JXA1-R-MLV due to comingling with vaccinated pigs showed significantly higher IL-8 expression levels in the serum (Fig.6.4C), even though viral load was low.

As for the adaptive cytokines, vaccinated and NADC-20 challenged pigs developed significantly higher serum IL-4 levels than all other groups (Fig.6.5A), and serum IL-4 in unvaccinated but NADC-20 challenged pigs was also significantly higher than vaccination alone pigs. There was no significant difference for IL-10 and IFN-γ expression in the serum and lung samples (Fig.6.5B, C). In a recall response, the PBMCs were re-stimulated with NADC-20. Vaccinated and NADC-20 challenged pigs generated higher quantities of IFN-γ secreting cells among all pigs, but the difference was not statistically significant (Fig.6.5D).

6.4. Discussion

As one of the most prevalent diseases in swine, PRRS has caused vast economic losses to the pig industry worldwide, and the wide spread of HP-PRRSV in Southeast Asia has caused devastating losses to the Asian swine industry. In 2006, HP-PRRSV affected over 2 million pigs with about 400,000 fatal cases in China alone [3]. Therefore, increased knowledge about the pathogenesis and the development of vaccines against HP-PRRSV is necessary for HP-PRRSV-free countries in the event of possible transmission. Recently, Porcine Epidemic Diarrhea Virus (PEDV) was reported to be found in 12 US states, and the isolated PEDV strains share the highest identity (99%) to strains from China in 2012 [14]. Even though the transmission routes of this disease to

US remain unknown, the possibility of HP-PRRSV transmission to other countries outside China seems very likely. The availability of Chinese HP-PRRSV vaccines in North America may not only act to increase the preparedness of possible transmission of HP-PRRSV to North America but also help protect pigs against PRRSV strains native to North America. Therefore, in this study, we explored the protection ability of HP-PRRSV vaccine to the North American native PRRSV strain NADC-20.

JXA1-R MLV vaccine protected pigs from high fevers induced by NADC-20 as compared with the unvaccinated and challenged pigs. As a virulent PRRSV strain, NADC-20 can cause clinical fever (≥40°C) at the early stage of infection. Vaccinated pigs showed transient increased body temperature in the first four days after NADC-20 challenge (Fig.6.1A). Vaccinated pigs eliminated NADC-20 virus in the blood to undetectable levels by 6 DPC, whereas the unvaccinated pigs developed the highest level of viremia at this time point (Fig.6.2A). The results of gross lung evaluation and histopathology of lung and lymph nodes also showed that JXA1-R vaccine was able to protect pigs from NADC-20 challenge, resulting in reduced pathology scores (Fig.6.2B-D). Therefore, all above results show that vaccinated pigs were protected from NADC-20 challenge. However, vaccinated pigs also showed decreased growth rate as compared with unvaccinated pigs before NADC-20 challenge (Fig.6.1B). HP-PRRSV field isolates were reported to lead to loss of body weight after infection, but it is the first time that HP-PRRSV vaccine was found to decrease pig growth rate. Therefore, the slow body weight gain rate caused by vaccination should be taken into consideration before widespread use of the HP-PRRSV vaccines.

By 7 DPV, the antibody response against N proteins of PRRSV, as measured by IDEXX ELISA, was detected in the vaccinated pigs and increased before NADC-20 challenge (Fig.6.3A). After NADC-20 challenge, the IDEXX ELISA antibody titer remained constant, and unvaccinated pigs showed PRRSV positive antibodies at 6 dpi. Interestingly, the unvaccinated and NADC-20 challenged pigs showed earlier onset of ELISA antibody titers as compared with other pigs, which could be due to the more virulent character and higher proliferation ability of NADC-20 over the vaccine strain. Neutralizing antibodies (NA) did not emerge until 28 DPV in vaccinated pigs. The NA titer of pigs in the vaccinated and NADC-20 challenged group showed higher

neutralizing ability to NADC-20 and parental strain of vaccine JXA1-R than other groups at 37 DPV (Fig.6.3B, C). Interestingly, the NA titer of pigs in the vaccinated and NADC-20 challenged group showed significantly higher titers to HV-PRRSV (Fig.6.3D), a strain of Chinese HP-PRRSV rescued from an infectious clone of PRRSV [15]. The enhanced neutralizing ability to HV-PRRSV may be due to the disparity of PRRSV strains and the strategy of prime (vaccination) and boost (challenge) with another heterologous PRRSV strain could generate broader cross-protection to PRRSV field strains.

Cytokines play multiple important roles in modulating host immunity to PRRSV infection. Vaccination increased innate cytokine IFN-α and IFN-β expression in the lung tissue (Fig.6.4A, B). Working as potent antiviral molecules, IFN-α/β was reported to significantly inhibit PRRSV replication and enhance cellular-mediated immunity (IFN-γ responses) [16,17]. However, several studies showed that PRRSV had evolved several strategy of expressing viral proteins to circumvent type I IFN response, especially to IFN-β [18-20]. Therefore, how to overcome the suppression of type I IFNs caused by PRRSV infection is one of priority of PRRSV vaccine development. In this study, vaccinated and NADC-20 challenged pigs developed significantly higher IFN-β in the lung tissue as compared with unvaccinated pigs after NADC-20 (Fig.6.4B), which may partially contribute to less lung damage and the reduced viremia level in the blood circulation. In contrast, vaccination decreases TNF-α expression in the lung tissues (Fig.6.4D). TNF-α is a proinflammatory cytokine, which plays a very important role in regulation of immune responses, fever development (inflammation), and cell apoptosis [21]. The suppression of TNF-α expression in the lung correlated well with less lung damage caused by NADC-20, which also had significantly lower macro- and histopathological lung scores (Fig.6.2B and C).

As for adaptive cytokines, the expression of IL-4 in the serum was significantly higher in vaccinated pigs as compared with unvaccinated pigs after NADC-20 challenge (Fig.6.5A). The Th2 cytokine IL-4 is involved in the suppression of pathogen specific Th1 immune response [22]. Our previous results and results from others suggest that increased IL-4 expression may play a positive role in the immune response and clearance of PRRS virus [13,23]. Our results show that vaccinated pigs challenged with NADC-20 PRRSV had increased IL-4 expression as compared with unvaccinated pigs and this correlated well with the higher virus NA titers induced

by NADC-20 challenge. These results indicate that IL-4 may play a positive role in the humoral immune response to the PRRSV infection.

Unvaccinated pigs were infected with vaccine strain of PRRS virus when they were mingled with vaccinated pigs at 28 DPV, even though the viremia level in these vaccinate pigs at this time point was very low (~ 1000 PRRSV RNA copy number/μl). These results suggest the high transmission ability of PRRSV even at such low viremia level. These pigs developed similar PRRVS-specific ELISA antibody titers at 6 day post infection (34 DPV) as vaccinated pigs at 7 DPV (Fig.6.3A).Therefore, vaccination for PRRS via herd immunity should be further explored.

Author Disclosure Statement

The authors declare that they have no competing financial interests.

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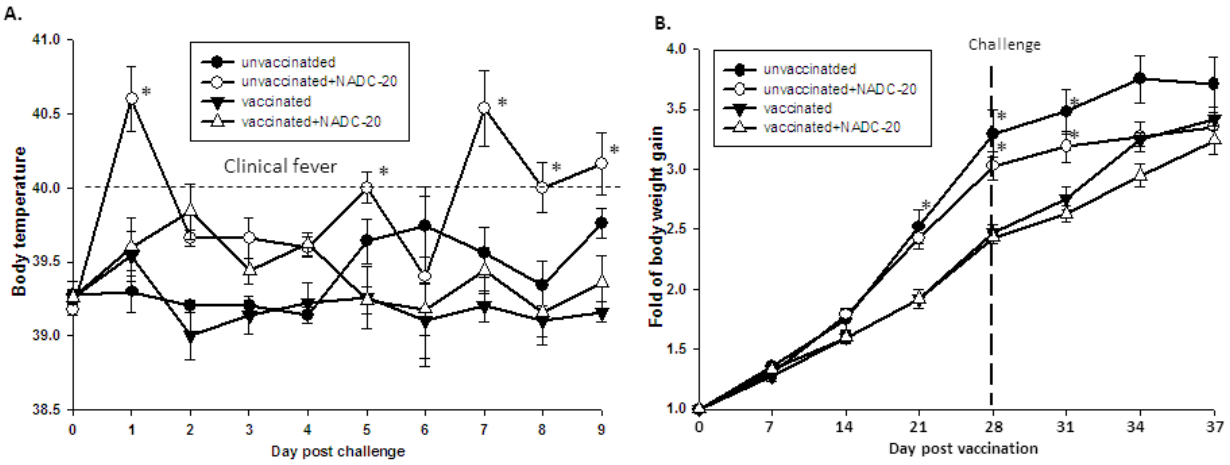

Figure 6.1. Body temperature and weight gain. (A) Rectal temperature of pigs was monitored daily after NADC-20 PRRSV challenge. (B) Fold body weight gain during the duration of the experiment was calculated by considering the weight of the pig on D0 as 1. Each bar represents the average of five pigs ± SEM. *$p<0.05$.

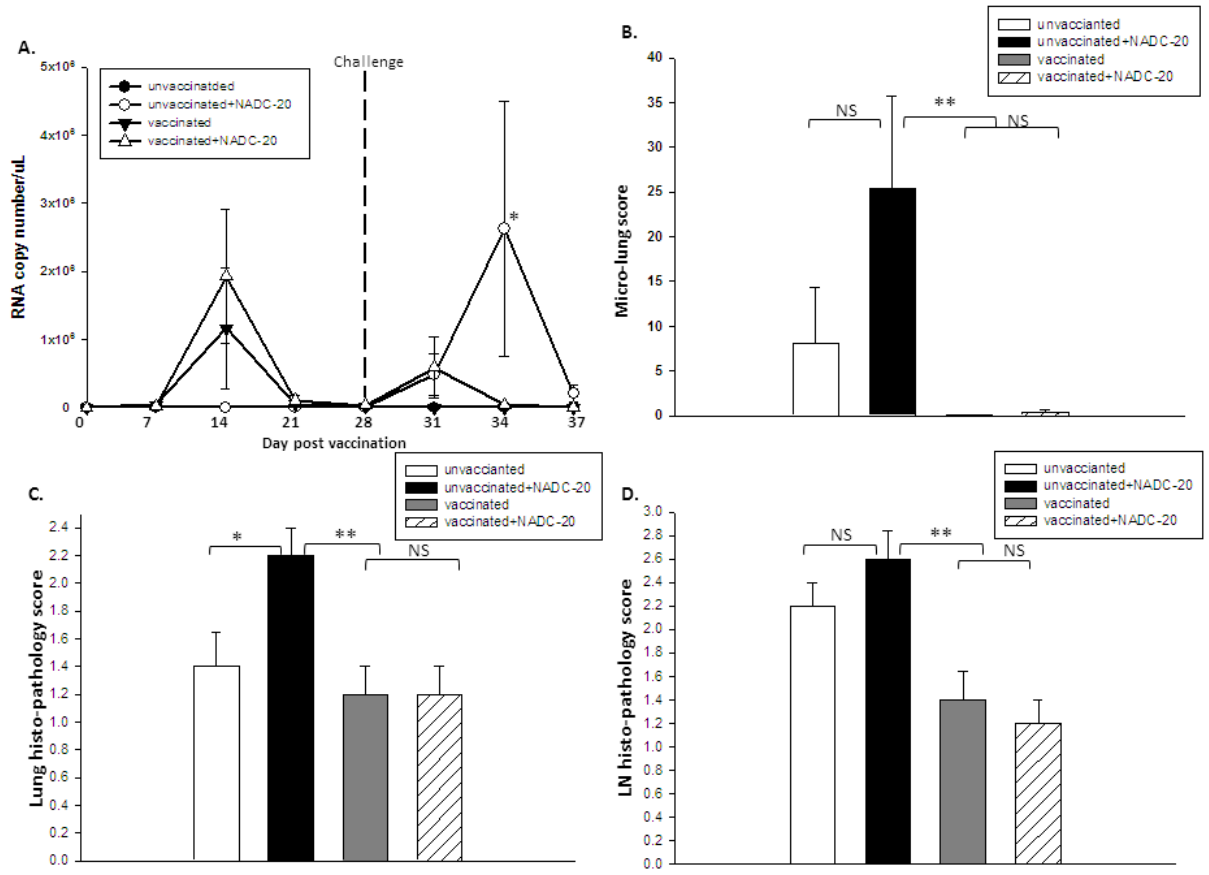

Figure 6.2. Viral load and pathological analysis after NADC-20 challenge. (A) PRRSV viral RNA in the serum was determined by qPCR. (B) Gross lung lesion scores present in all lung lobes on 10 DPC were scored using a 100 point scale. (C) The lungs were sectioned at necropsy (10DPC), blindly examined and given an estimated score of the severity of interstitial pneumonia from 0 to 4. (D) Lymph nodes were collected at 10 DPV and sections were blindly examined and given an estimated score according to the amount of hyperplasia from 1 to 3. One asterisk denotes a statistically significant difference ($P < 0.05$), and two asterisks denote a statistically significant difference ($P < 0.01$). "NS" denotes there was no statistically significant difference.

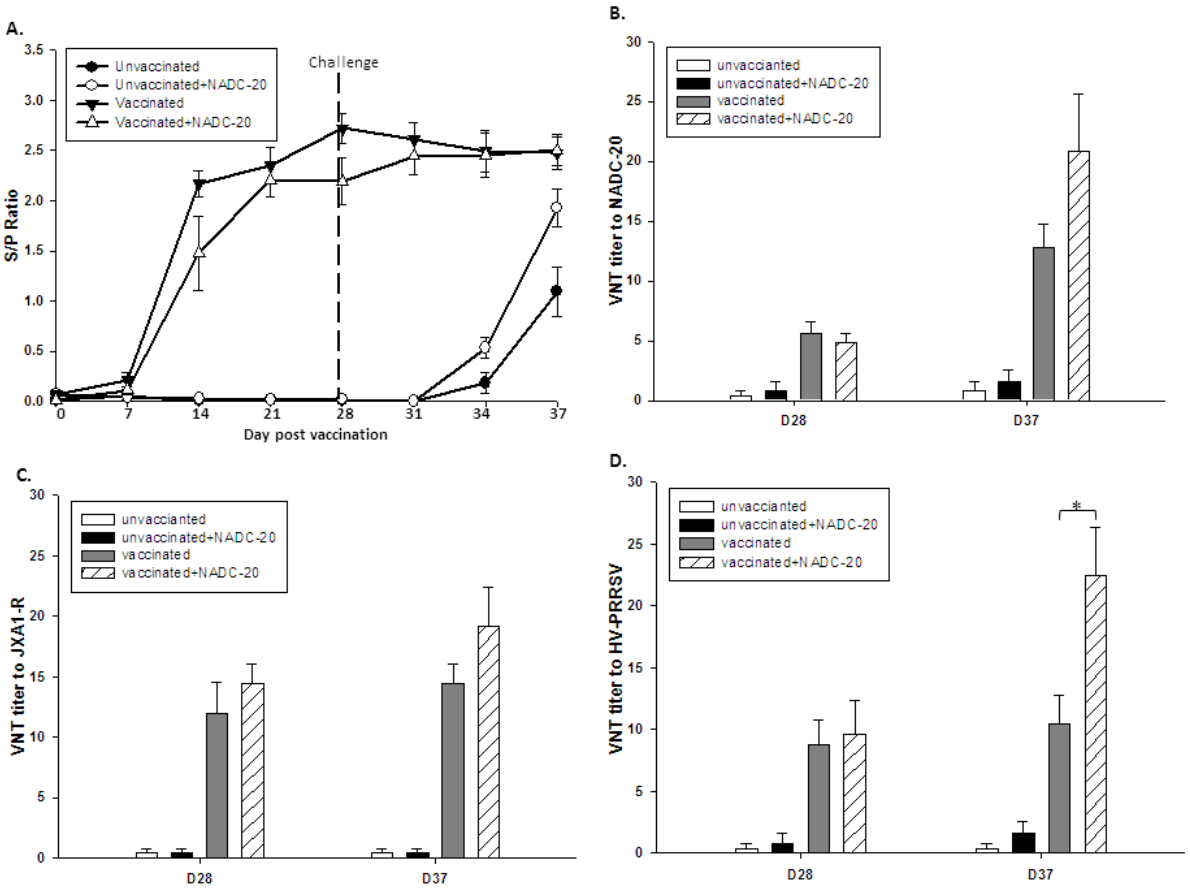

Figure 6.3. PRRSV-specific IDEXX ELISA antibody and neutralizing antibody profiles after vaccination and NADC-20 challenge. (A) Serum from pigs was assayed for PRRSV-specific antibodies with IDEXX HerdCheck ELISA. The threshold for seroconvertion was set at a sample-to-positive (s/p) ratio of 0.4 according to manufacturers' instructions. (B-D) Serum samples were titrated individually in MARC-145 cells for the levels of anti-PRRSV neutralizing antibodies on 28 days post vaccination (28 DPV) or 10 days post challenge (37 DPV) determined as the highest dilution that inhibited CPE. Data were shown as mean ± SEM for 5 pigs per group. One asterisk denotes a statistically significant difference ($P < 0.05$), and "NS" denotes there was no statistically significant difference.

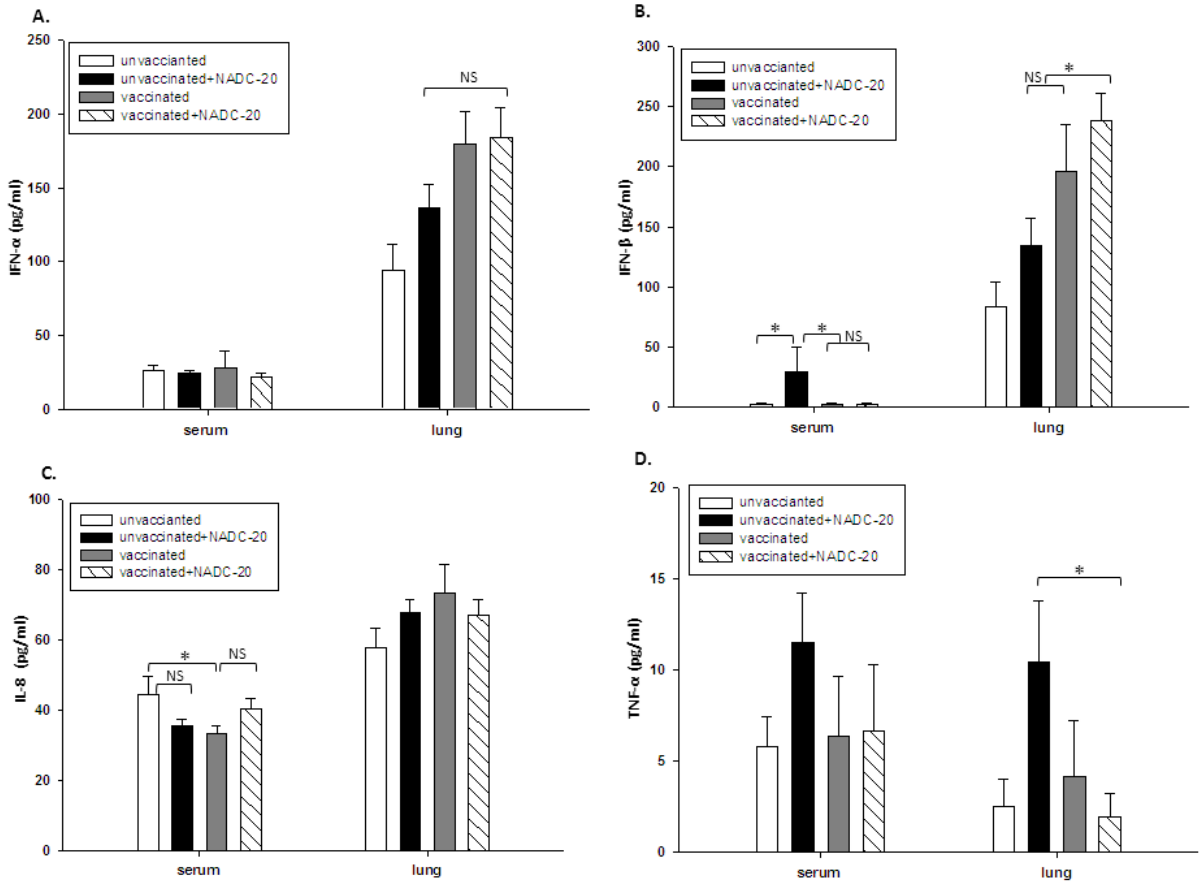

Figure 6.4. Innate cytokine responses after NADC-20 challenge. Cytokine expression profiles in the sera of challenge pigs 6 days post challenge (DPC) and supernatants of lung homogenates were tested by quantitative ELISA as shown in (A) IFN-α (B) IFN-β (C) IL-8 and (D) TNFα. One asterisk denotes a statistically significant difference ($P < 0.05$), and "NS" denotes there was no statistically significant difference.

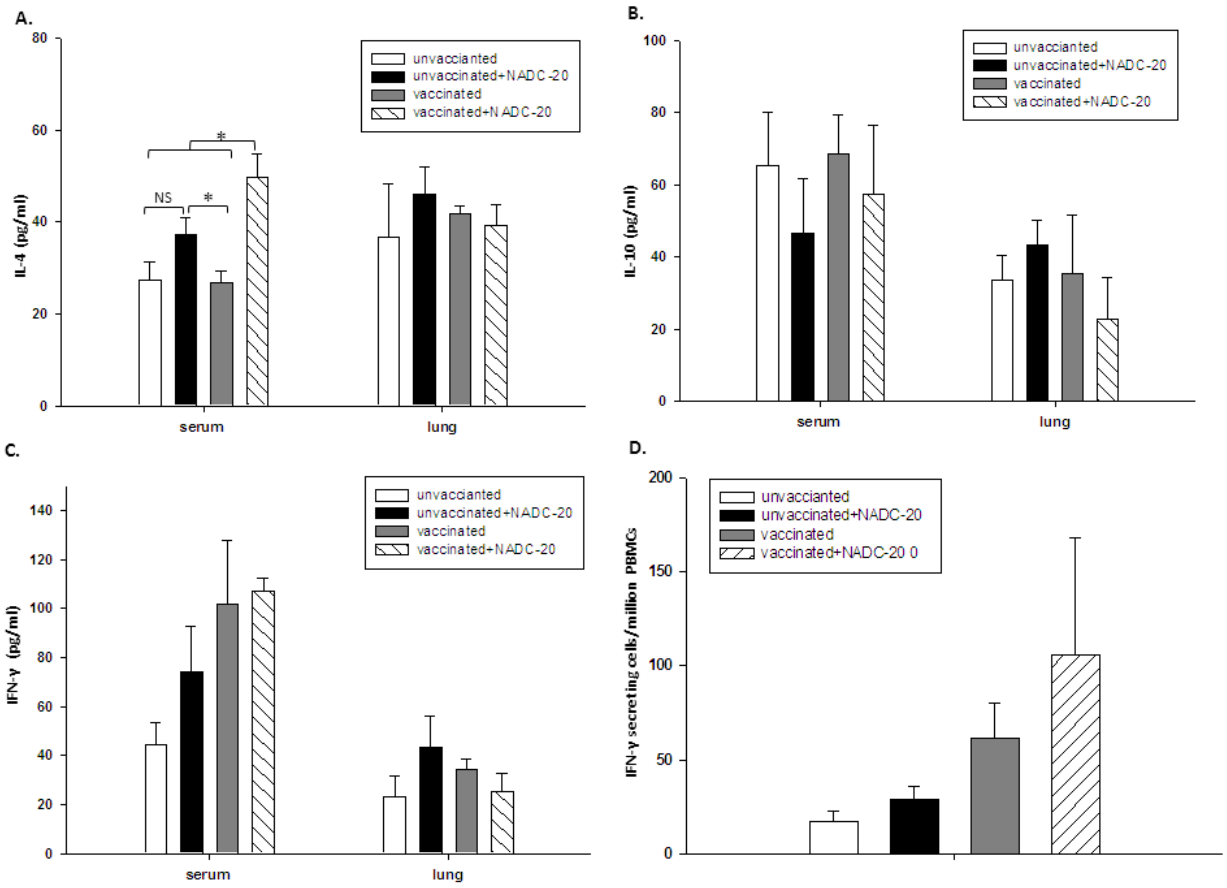

Figure 6.5. Adaptive cytokine responses after NADC-20 challenge. Cytokine expression profiles in the sera of challenge pigs 6 days post challenge (DPC) and supernatants of lung homogenates were tested by quantitative ELISA as shown in (A) IL-4 (B) IL-10 (C) IFN-γ. (D) PBMCs collected at 10 DPC were re-stimulated with NADC-20 strain of PRRSV. IFN-γ-secreting cells were then analyzed by ELISpot assay. Data were shown as mean ± SEM for 5 pigs per group. One asterisk denotes a statistically significant difference ($P < 0.05$), and "NS" denotes there was no statistically significant difference.

Chapter 7 - Conclusions and future directions

The rapid evolution rate of PRRSV worldwide generates countless genetically distinct field isolates, which pose a big challenge for current commercial PRRSV vaccines to provide broad protection. Studies on the difference of immune responses to homologous and heterologous challenge lay an important foundation for the development of effective vaccines and eradiation strategies. In chapter 2, by evaluating the differences of immune responses between vaccinated and unvaccinated pigs when challenged with homologous or heterologous PRRSV, we demonstrate that serum neutralizing antibody titers are associated with PRRSV vaccination-induced protection against homologous and heterologous challenge. PRRSV antibody IDEXX ELISA is widely used in the field to evaluate the antibody response after vaccination. These assays detect antibodies specific for the N protein of virus. In our study, we observed discrepancies between the level of protection, as shown by viremia/ lung damage results, and high titer of ELISA antibodies indicate anti-N protein antibodies are not predictive of PRRSV protection.

MLV vaccinated and KS-06 challenged pigs developed a higher level of VN antibodies to the heterologous NVSL97-7895 PRRSV strain, which supports the hypothesis that dual vaccination with differing PRRSV strains can generate higher neutralizing antibodies and therefore lead to broader cross-protection against diverse PRRSV field strains. Similar observations have been reported in influenza virus vaccination strategy studies [1]. Similar strategies have also increased protective neutralizing antibodies and cellular immunity in mouse and non-human primate models after live vaccine vaccination followed by a boost of DNA vaccine [2,3]. Therefore, a strategy involving consecutive vaccinations by two strains of PRRSV vaccines or two types of vaccines may increase the protection induced by vaccination.

In our study, we also found the PRRSV-dependent cytokine expression patterns are PRRSV challenge-strain specific as shown by increased serum IL-8 after VR-2332 viral challenge and increased IL-4 but decreased IFN-a/IL-10 after KS-06 challenge. The cytokine environment shapes the host immune responses to PRRSV infection and pigs which clear PRRSV infection have been shown to coordinate the expression of IL-1β, IL-8 and IFN-γ [4]. In our study, the

serum level of inflammatory cytokine IL-8 in vaccinated pigs challenged with homologous VR-2332 virus was the highest among all treatment groups, which is consistent with previous studies which have shown that low level of serum IL-8 is seen in persistent PRRSV infection, and elevated IL-8 levels in serum is correlated with the clearance of PRRS virus [5]. However, we did observe any changes to serum levels of IFN-γ among the four treatment groups and lower levels of IFN-γ was found in fully protected vaccinated pigs challenged with VR-2332 compared with partially protected vaccinated pigs challenged with KS-06 strain, which suggests that increased IFN-γ expression does not correlate with protection against PRRSV. Therefore, the role of IFN-γ in the protection to PRRSV infection needs to be further explored. We did not explore the role of IL-1β in mediating host immunity to PRRSV infection in this study.

Different types of adjuvants have been combined with modified live PRRSV vaccines to induce vaccination-mediated cross-protection against genetically dissimilar PRRSV strains. These adjuvants can enhance the immune responses by different mechanisms such as delivering the antigen slowly to the organism (depot effect), increased antigen uptake and presentation to antigen presenting cells, recruitment and activation of macrophages and lymphocytes, and stimulation of the production of cytokines and chemokines. In chapters 3 and 4, we evaluated the efficacy of PRRSV MLV vaccine combined with commercial Montanide^{TM} Gel01 and experimental H9e hydrogel peptide adjuvants.

In chapter 3, we found Gel 01-adjuvanted MLV was able to better protect pigs challenged with VR-2332 than pigs vaccinated with MLV alone and the protection was due to the higher neutralizing antibody titers induced by adjuvanted vaccinations. Additionally, pigs vaccinated with the MLV vaccine alone had increased IL-10 production as compared with unvaccinated animals and the addition of Gel01 adjuvant acted to decrease IL-10 to levels to at or below unvaccinated animals. IL-10 is a potent immunosuppressive cytokine that interacts with a wide array of immune cells to inhibit host innate and adaptive immunity [6]. Therefore, removal of PRRSV-induced immunosuppressive cytokine IL-10 should be taken into consideration for future vaccine and adjuvant development for PRRSV control.

In chapter 4, our results showed that H9e hydrogel is a promising adjuvant candidate for PRRSV MLV vaccines since it helps vaccine to improve both host humoral and cellular immune responses. Some self-assembling peptides have been successfully used as adjuvants to elicit strong antibody response and/or cellular response [7, 8]. As compared with these peptide adjuvants, H9e hydrogel has several significant advantages working as an adjuvant over the other peptide adjuvants. First, antigens can be encapsulated and protected for delivery by simply mixing with H9e hydrogel through various routes of administration, such as intranasal or intramuscular, and animal experiments show there were no injection site reactions [9]. Second, the release rate of antigen can be controlled by varying the concentration of H9e hydrogel. Altering H9e concentration from 0.17%-5% will create different sizes of porous networks, which will allow for different rates of antigen release. Third, the mixture of H9e hydrogel and antigen remains in liquid form and immediately converts into solid gel once it contacts serum components at body temperature. Fourth, its shear-thinning ability allows H9e hydrogel to be repeatedly converted from solid to liquid state within minutes, allowing for it to easily pass through a needle [10]. As shown in the literature [7-9], the hydrogel/vaccine formulation can improve host humoral and cellular immunity. However, the mechanism is hypothesized to involve an antigen-slow releasing (depot-effect of adjuvant) in the gel matrix. We measured the viral RNA copy numbers in the muscle injection sites and our preliminary data showed that PRRS virus did persist in the injection site longer when the virus was mixed with H9e. The other potential mechanisms of hydrogel working as an adjuvant need to be further explored. In order to gain mechanistic insight, we combined H9e with VV-OVA (recombinant vaccinia virus expressing ovalbumin). We found that mice immunized with H9e + VV-OVA generated increased pools of the OVA-specific memory CTL population than mice immunized with VV-OVA alone. Therefore, the hydrogel itself could act as an immune-stimulator or the hydrogel could activate certain cell signaling pathways to enhance immune responses. In the future, microarray or two-dimension electrophoresis techniques could be used to analyze differences in mRNA or protein which may be involved in the host immune responses.

Highly-pathogenic PRRS virus (HP-PRRSV) causes more severe clinical symptoms and more extensive tissue tropism than classic PRRSV, which leads to the death at all ages of pigs with 100% morbidity and 20% of mortality. The widespread prevalence of HP-PRRSV suggests

current PRRSV vaccines are ineffective and a lack of understanding the pathogenesis of viruses exists. In chapter 5, we compared the host immune responses elicited by a Chinese HP-PRRSV and a virulent American strain NADC-20, and the results showed that HV-PRRSV has significantly higher proliferation ability and induced significantly higher cytokine responses including IFN-α and IFN-γ, TNF-α, IL-10, which was consistent with a previous report from another research group [11]. In our study, we also found the high proliferation ability of HP-PRRSV was not associated with the kinetics of PRRSV-specific ELISA antibody response or the increase of antiviral cytokines such as IFN-α and IFN-γ. Therefore, PRRSV ELISA antibody titers, IFN-α and IFN-γ levels, which have been widely used to evaluate the protection provided by vaccination or infection in clinical trials, need to be examined further.

In chapter 6, we demonstrated that pigs challenged with the North American NADC-20 strain were protected by vaccination with Chinese JXA1-R MLV HP-PRRSV vaccines as evidenced by reduced level of clinical fever, reduced viremia, and less lung damage. The vaccinated pigs developed high titers of neutralizing antibodies to NADC-20. Also, vaccination increased the expression of innate cytokines IFN-α and IFN-β in the lung tissue and IFN-α/β was reported to significantly inhibit PRRSV replication and enhance cellular-mediated immunity [12, 13]. The above evidence of high viral neutralizing antibody titers and increased IFN-α and IFN-β cytokine expression may explain the protection provided by HP-PRRSV vaccination. However, the vaccinated pigs showed significant body weight loss after vaccination. Therefore, slow body weight gain rates caused by vaccination should be taken into consideration before use of the HP-PRRSV vaccines in the US.

Since Chinese JXA1-R MLV HP-PRRSV vaccines led to reduced body weight gain after vaccination, reduced vaccine load may eliminate the vaccine-induced loss of body weight gain. One of our pilot studies showed that pigs vaccinated with a half dose of PRRS MLV combined with H9e hydrogel had similar vaccine-induced protection to both homologous and heterologous PRRSV challenge (data not shown in the thesis). In our next experiment, we propose that, by adding H9e, we can further decrease the amount of PRRSV vaccines needed to provide equivalent protection without affecting pig growth rates, and the reduced dose of PRRSV

vaccines may not only reduce the cost of animal vaccination but also decrease risk of the vaccine reverting back to virulent strains.

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