EVALUATION OF IMMUNE CORRELATES OF PROTECTION AGAINST PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS CHALLENGE IN PIGS IMMUNIZED INTRANSALLY WITH ADJUVANTED VACCINES

A THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

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2012

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is an economically devastating disease of pigs, caused by PRRS virus (PRRSV), incurring estimated $664 million losses annually to the US pork industry. As prevention measure, widely used modified live PRRSV vaccine (PRRS-MLV) has failed to completely protect pigs against genetically variant strains, and it is unsafe to use the same in pregnant sows due to potential vertical and horizontal transmission of the mutated vaccine virus. In contrast, inactivated PRRSV vaccines are safe but they have failed to protect against reinfections by even homologous strains. Therefore, developing a safe and protective PRRSV vaccine remained as a challenge. The primary site of PRRSV infection is the lungs and the virus enters through respiratory mucosal surfaces. Mucosal immunization strategy has the potential to elicit protective local and systemic immunity, while parenterally administered vaccines elicit weak mucosal immunity. Therefore, the aim of our research was to develop protective mucosal vaccines against PRRS. In this report, we are presenting results of two independent studies on live attenuated and killed PRRSV vaccines.

Reactive oxygen species (ROS) are produced predominantly by phagocytic cells. Optimal levels of ROS have potent antimicrobial properties, while excessive production of ROS induces apoptosis/necrosis of infected as well as bystander cells, resulting in inflammatory pathology. Previously, we have shown that co-inoculation of pigs with PRRS-MLV with a potent mucosal adjuvant Mycobacterium tuberculosis whole-cell lysate (M. tb WCL), intranasally, induces superior cross-protective immunity. In this
study, we have demonstrated that peripheral blood mononuclear cells (PBMCs) and bronchoalveolar lavage fluid (BAL) cells of PRRS-MLV plus M. tb WCL vaccinated PRRSV challenged pigs secreted reduced (but optimum) levels of ROS compared to unvaccinated virus challenged pigs, which secreted significantly higher amounts of ROS, associated with increased lung pathology. Thus, protective mucosal immunity induced using PRRS-MLV plus M. tb WCL protects pigs against RSO mediated lung pathology. Further, in our endeavor to develop a safe and protective killed vaccine, we reinforced the killed PRRSV vaccine in two ways; firstly by coupling it with M. tb WCL, and secondly to achieve the sustained vaccine delivery the killed viral antigens and adjuvant M. tb WCL were entrapped in PLGA [poly(lactideco- glycolide)] nanoparticles. Subsequently, the vaccine formulation was analyzed in vivo in pigs using different vaccine and adjuvant combinations. Our results indicated that, PLGA entrapped PRRSV killed vaccine adjuvanted with unentrapped M. tb WCL had a better cross-protective immunity to a virulent heterologous PRRSV strain MN184 challenge. The immune correlates of protection were demonstrated at both mucosal sites and systemically, characterized by: (1) higher levels of PRRSV specific IgG and IgA antibody response; (2) upregulated PRRSV specific neutralizing antibody titers; (3) complete clearance of viremia and replicating PRRSV from the lungs; and (4) enhanced frequency of IFN-γ secreting cells in the lungs. Results of our study are useful in developing a safe and protective PRRSV vaccine. In conclusion, our study for the first time, has demonstrated that co-administration of PLGA nanoparticles entrapped adjuvanted killed PRRSV vaccine with a potent mucosal adjuvant is capable of eliciting cross-protective immunity against PRRS in pigs.
Acknowledgements

I wish to offer special thanks to my adviser Dr. Renukaradhya J Gourapura for his support, intellectual guidance, encouragement, and great patience throughout my degree program. Without his sincere support, guidance, and help, this work would not have been possible. I am deeply indebted to him for giving me an opportunity at a critical stage of my life to realize my longstanding dream to pursue graduate studies abroad. I wish also to thank my other graduate committee members, Dr. Daral J. Jackwood and Dr. Chang Won Lee for accepting to be on my advisory committee.

I am very grateful to Dr. Juliette Hanson, Kingsly Berlin, Todd Root, and Andrew Wright for their support with animal studies. I want to thank my colleagues Varun Dwivedi, Cordelia Manikam and Kang Ouyang for their generous support and help. I sincerely appreciate the help of Ruthi Patterson for her tireless and organized assistance in animal study as well as in laboratory. Many thanks to all the staff members in Food animal Health Research Program for their assistance in my studies. Special thanks to my friends, Rajkumar Noubade for introducing me to Dr. Aradhya and for constant guidance, and Rajeswaran Mani for his warmth, moral and academic support during the coursework in Columbus.

I owe my greatest appreciation to my wife Rashmi for sacrificing her promising career to join me here in USA and for making my life much easier than I thought. I am blessed with an amazing son, Mayank, whom I appreciate for not demanding my time to spend
with him. I thank my wife very much for taking care of my son and sparing my time to devote it for my studies. Finally, I am highly indebted to my beloved mother for allowing me to leave my homeland even when my stay with her was needed.

I would like to thank editor of the journal 'Viral Immunology' for permitting me to republish the data of my master's research results in this thesis.
Dedicated to

All those turns and twists in my life, for taking me to places.
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Conference/Abstracts/Presentation:
Binjawadagi, B., V. Dwivedi, C. Manickam, J. B. Torrelles, and G. J. Renukaradhya (2011), “Intranasal delivery of an adjuvanted modified live porcine reproductive and respiratory syndrome virus vaccine reduces the ROS induced lung pathology” in CRWAD Meeting, December 4-6, 2011, at Chicago, IL, USA


FIELDS OF STUDY
Major Field: Comparative and Veterinary Medicine
Studies in Mucosal Immunology and Vaccine development
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CHAPTER 1

LITERATURE REVIEW

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an important chronic viral disease of pigs that is prevalent worldwide. PRRS causes an annual economic loss of an estimated $664 million to the U.S. pork industry (Holtkamp & Kliebenstein, 2011). According to the Animal and Plant Health Inspection Service (APHIS) report of January 2009, 49.8% of unvaccinated pigs are seropositive to PRRSV in the US. This is based on a collective data from 94 percent of pork producers in 17 states, suggesting the widespread prevalence of PRRS in the US. With greater than 50% of sites from all the four regions in the US (North, South, West Central, and East Central) had anti PRRS virus antibodies and approximately 60% of pig samples in northern and southern regions were found positive for anti-PRRSV antibodies. PRRS is manifested in two forms, respiratory and reproductive diseases (Christopher-Hennings et al., 1995; Collins et al., 1992), with major losses from reproductive failure in sows, such as stillbirths, mummifications, weak born piglets and high preweaning mortality (Rowland, 2007). PRRSV is excreted from all the body secretions at low levels or perhaps intermittently in saliva, nasal secretions, urine, milk, colostrum, feces, and also in semen of infected pigs (Christopher-Hennings et al., 1998). Under natural conditions in the absence of any intervention, transport and transmission of PRRSV by fomites and personnel occur
between swine populations (Pitkin et al., 2009). In addition, airborne transport of PRRSV may occur over long distances with no change in the infectivity of the virus (Otake et al.; 2010). Therefore, control of PRRSV transmission within and between swine herds is a major challenge to swine industry.

PRRS is caused by PRRS virus (PRRSV), which is a positive-stranded RNA virus, belongs to the family Arteriviridae (Wang et al., 2007). PRRSV is divided broadly into two distinct genotypes, type I (European) and type II (North American). Each genotype contains several subtypes and strains, which are genetically highly diverse and therefore display significant differences in their virulence and pathogenicity (Kim et al., 2007). Different PRRSV field isolates within the North American genotype have genetic diversity ranging from 84 - 100% based on the amino acid homologies of ORFs 2 - 6 relative to VR2332 strain, a prototype North American strain of PRRSV (Kim et al., 2007). Therefore, immunity to the initial infecting genotype of the PRRSV may provide partial or no protection to reinfection, implying not only the complexity of genetic but also immunological variation among the strains (Botner et al., 1997; Li et al.; 2010).

Currently, commercial modified live virus (MLV-PRRS) and inactivated PRRSV vaccines are licensed for use; although the MLV-PRRS offers good protection versus homologous virus isolates, there continue to be questions regarding the vaccine efficacy against antigenically variant heterologous field strains (Botner et al., 1997; Martelli et al., 2009). The efficacy of available killed PRRSV vaccines is inadequate to protect pigs against even antigenically closely related PRRSV, and none of the current vaccines prevents respiratory infection and pig-to-pig transmission of PRRSV (Kimman et al.,
2009). Administration of either field isolates of PRRSV or vaccine including MLV-PRRS by the parenteral or intranasal route suppresses the innate Natural killer (NK) cell cytotoxic function and IFN-α production (Albina et al., 1998; Renukaradhya et al., 2010). The most efficient and rapid host response against viruses consists of production of type I IFNs (IFN-α/β), an essential part of the antiviral innate immune system (Thiel & Weber, 2008). The IFN production is triggered by the first contact of cells with the virus, subsequently secreted IFN slows down or even blocks virus multiplication, aiding in establishment of an adaptive immune response (Thiel & Weber, 2008). The NK cell is a lymphocyte subset that can be activated to mediate significant levels of innate anti-viral cytotoxic activity and also produce high levels of IFN-γ and chemokines (Biron et al., 1999). Thus, innate immune response mediated through type I IFNs and NK cells is critical for induction of protective immunity. PRRSV experts collectively agree that only replicating PRRSV vaccines have the promise to reduce the PRRS incidence in the field (http://vetmed.illinois.edu/news/PRRSwhitepaper.pdf). However, the live vaccines have been implicated in the spread of vaccine virus to non-immunized animals of the same and/or nearby herds. On the other hand, it is proposed that, inactivated PRRSV vaccines could elicit protective immunity, if they undergo proper inactivation processes and added with an appropriate adjuvant (Darwich et al., 2010; Vanhee et al., 2009). Since the 1990s development of a safe and effective vaccine that induces broadly protective immunity to PRRS remains as a principal goal of researchers and the swine industry.

**PRRSV modulates host immune responses:**

**Innate Immunity:**
Regardless of the route of entry, productive infection occurs predominantly in alveolar macrophages of the lung. *In vitro* the virus can grow in primary cultures of alveolar macrophages (Murtaugh et al., 2002). In addition, the virus infects other cells of the monocyte/macrophage lineage, notably pulmonary intravascular macrophages, subsets of macrophages in lymph nodes and spleen, and intravascular macrophages of the placenta and umbilical cord (Duan et al., 1997; Lawson et al., 1997). Remarkably the virus does not replicate in non-activated monocytes, but it is unknown completely which molecular pathways should be activated to sustain continuous virus replication in permissive cells (Thanawongnuwech & Suradhat, 2000). Pigs exposed to PRRSV may demonstrate prolonged viremia and may continue to shed virus for long periods, but the virus does not enter a latent stage. The virus replication and persistence in tonsils, lungs, and lymphoid organs are responsible for chronic disease (Lamontagne et al., 2003). Novel phenotypic variants of PRRSV emerge during the prolonged infection, but neither the selective pressures that drive their emergence are not defined nor the mechanism of virus persistence (Rowland et al., 1999).

One of the most remarkable features of PRRSV infection is that in the lungs of infected pigs the virus fails to elicit critical inflammatory cytokine response such as type I interferons (an important step in innate immunity), interleukin (IL)-1, and TNF-α (Thanawongnuwech et al., 2001; Van Reeth et al., 1999). PRRSV does not induce a generalized suppression of host gene transcription. Since the transcript copies do not correlate with IFN-α protein levels, post-transcriptional mechanisms of suppression induced by PRRSV are likely involved in immunoregulation (Lee et al., 2004). The
downregulation of type I IFN, in particular IFN-α, is probably a crucial step in the pathogenesis, because IFN-α has been shown to inhibit PRRSV replication (Albina et al., 1998). The suppressive effect on IFN-α production was lost when the virus was inactivated by UV light (Vanhee et al., 2009), hence, it is likely that inactivated vaccines do not downregulate IFN-α production. Although IFN-β may also protect macrophages against PRRSV replication, it is probably not sufficient to elicit adaptive immune response (Loving et al., 2007). The virus can also infect mature and immature porcine monocyte derived dendritic cells (DCs) in vitro, but the infection is less productive in this cell type than in alveolar macrophages. PRRSV infection of DCs interferes with many of their functions (Flores-Mendoza et al., 2008; Park et al., 2008). It was found that PRRSV replication in pig monocyte-derived DCs induces apoptosis, down-regulate the expression of CD11b/c, CD14, and costimulatory molecule CD80/86, and major histocompatibility complex (MHC) class I and II molecules, resulting in reduced allogeneic stimulation of T cells. In addition, PRRSV infection up-regulates the expression of proinflammatory cytokines, IL-12 and TNF-alpha as well as immunosuppressive cytokine, IL-10 (at mRNA and protein levels) (Genini et al., 2008; Park et al., 2008). The cytokine IL-10 is a major regulator of immune responses to pathogens. Upregulation of IL-10 may contribute to downregulation in the production of inflammatory cytokines and the Th-1 response during PRRSV infection. Bone marrow-derived (myeloid) immature DCs (BM-imDCs) were also permissive to productive replication of PRRSV. Lung DCs are not permissive (Wang et al., 2007). PRRSV appears to have a differential influence on IFN-α and IFN-β production, and likely exploits several mechanisms to interfere with the expression and function of these type I IFNs. PRRSV infection activates the signal transduction
components of NF-κB and AP-1, but not of interferon regulatory factor 3 (IRF3), an essential IFN-β transcription factor. Furthermore, PRRSV infection significantly blocked synthetic dsRNA-induced IFN-β production and IRF3 nuclear translocation (Luo et al., 2008; Pichlmair et al., 2006). Therefore, both the type I IFNs production were influenced, but primarily IFN-α production is affected by PRRSV.

Another factor that could contribute to viral persistence is the lack of incorporation of viral proteins into the plasma membrane of PRRSV-infected macrophages. Because PRRSV particles, like other arteriviruses, assemble within the host cell by budding of nucleocapsids into the lumen of the endoplasmic reticulum and/or golgi compartments. Viral antigenic proteins are retained in and accumulate at the budding site. As a result, infected cells are invisible to PRRSV-specific antibodies and refractory to antibody- and complement-mediated cell lysis (Costers et al., 2006).

A characteristic consequence of PRRSV infection is apoptosis. However, responsible viral sequences, mechanisms and consequences of apoptosis are unknown. Interestingly however, apoptosis is observed both in PRRSV-infected and uninfected bystander cells (Miller & Fox, 2004). Both GP5 of PRRSV and apoptogenic inflammatory cytokines, notably TNF-α, have been implicated in the induction of apoptosis of uninfected bystander cells. Expression of GP5 in monolayer of cells, using a vaccinia virus expression vector induced apoptosis, while the vaccinia vector alone did not (Suarez et al., 1996). In addition to apoptosis-induced cell death, PRRSV-infected cells undergo
necrosis, perhaps even more than apoptotic cell death (Choi & Chae, 2002; Miller & Fox, 2004).

Like many other viruses, PRRSV appears to trigger activation of caspase cascades, known to induce apoptosis. In addition to these mechanisms, reactive oxygen species (ROS)-mediated oxidative stress might be involved in inducing apoptosis by PRRSV (Lee & Kleiboeker, 2007). PRRSV infection induces oxidative stress in cells by generating ROS, whereas antioxidants inhibited NF-κB DNA binding activity in PRRSV-infected cells. These findings suggest that ROS may be a mechanism by which PRRSV infection activates NF-κB. NF-κB activation enhances the expression of matrix metalloproteinase (MMP)-2 and MMP-9 in PRRSV-infected cells. These MMPs are known to promote infiltration of inflammatory cells, implying that NF-κB activation is a biologically significant aspect of PRRSV pathogenesis (Lee & Kleiboeker, 2005). Interestingly, there appears to be no direct relation between virus load and severity of lesions (Yoo et al., 2004).

Inhibition of the innate immune response by PRRSV appears to be crucial and it is an important property of PRRSV, similarly other viruses, such as Hepatitis A virus, FMDV, HSV-1 etc., have evolved similar mechanisms to avoid induction of the host innate immune response (Johnson et al., 2008; Paulmann et al., 2008). The Th1 cytokine IFN-γ plays a critical role in the disease, and its serum levels negatively correlate with viral load (Loving et al., 2008). In addition to IFN-α, IFN-γ inhibited PRRSV replication in vitro (Bautista & Molitor, 1999; Gaudreault et al., 2009). Early production of IFN-γ might
result from activation of NK cells and alveolar macrophages. Altogether, PRRSV dampens the innate immune responses by altering the cytokine production of macrophages and DCs, as well as by modifying the expression of molecules involved in antigen presentation. As a consequence, NK cell activation and mobilization of cells from the acquired arm of the immune system are likely delayed by PRRSV, resulting in slow and delayed neutralizing antibody, lymphoproliferative, and IFN-γ responses (Butler et al., 2008; Chang et al., 2008). Thus, the initial innate immune response to PRRSV may be considered as weak and possibly contributes to the long survival of the virus in infected pigs.

**Acquired Immunity:**

Adaptive immunity against PRRSV infection develops in infected pigs, but it is significantly delayed to reach the peak levels (upto 2 - 3 months), and does not appear to be sufficient to prevent reinfection and infection caused by heterologous viral strains (Murtaugh et al., 2002; Zuckermann et al., 2007). It was also demonstrated that, induction of virus specific memory immune responses were highly variable, resulting in incomplete protection against heterologous viral strains (Lager et al., 1999). Thus, immunodominent conserved epitopes are necessary in protection against both European and American type PRRSV strains. However, due to broad genetic and antigenic variations of simultaneously circulating PRRSV field strains, the efficacy of humoral and cell-mediated immune responses is clearly insufficient to provide protection under field circumstances (Darwich et al., 2010). Ability of PRRSV vaccines or field strains to
induce protective immunity depends on their capacity to induce a strong cell-mediated and Th1 cytokine (IFN-γ) response (Diaz et al., 2006).

**Humoral Immunity:** Pigs mount a rapid antibody response to PRRSV infection which is detectable from day 5 post-infection, but these early viral antibodies are mainly directed to the N- and M-proteins, and are of non-neutralizing nature. Neutralizing antibodies begin to appear at about 7–10 days post-infection, but their titers remain low, and substantial variation in the neutralizing antibody response has been reported (Loemba et al., 1996; Plagemann, 2006). Thus, the humoral immune response to PRRSV in pigs is characterized by early production of non-neutralizing antibodies followed by the delayed appearance of neutralizing antibodies between 2 and 4 weeks post-infection, which persist at low levels (Darwich et al., 2010). The protective capacity of neutralizing antibodies is debated, as there may be concurrent circulation of neutralizing antibodies and the virus *in vivo*, while *in vitro* neutralizing antibodies (as well as non-neutralizing antibodies) may enhance PRRSV replication in macrophages. Such antibodies may coat the virus and thus enhance the internalization of viral particles into macrophages, by a mechanism called antibody dependent enhancement (ADE) of viral replication. Likewise, viremia may be resolved in the absence of detectable levels of neutralizing antibodies (Diaz et al., 2006).

However, pigs receiving an amount of neutralizing antibodies sufficient to reach a serum titer of 8 consistently did not develop viremia, whereas serum titers of 32 produced sterilizing immunity (Lopez & Osorio, 2004; Osorio et al., 2002). However, other authors
do not report such a strong correlation between neutralizing antibodies and the absence of viremia (Jiang et al., 2007; Zuckermann et al., 2007). Confocal analysis showed that neutralizing antibodies may block infection through reduction in virus attachment and internalization (Delputte et al., 2004). Viral neutralizing epitopes (NE) capable of inducing neutralizing antibodies appear to reside on the M, GP2a, GP3, GP4, and GP5 proteins (Kim & Yoon, 2008; Yang et al., 2000). Of these, neutralizing antibodies to GP5 appear to be most relevant for protection. Plagemann and co-workers used peptide mapping to show that the major neutralization epitope of PRRSV is located at the middle of the GP5 ectodomain (aa 36–52) (Plagemann, 2006). However, decoy epitopes near NE of GP5 are attributed to be one of the responsible factors for development of non-neutralizing antibodies (Fang et al., 2006; Ostrowski et al., 2002).

Another mechanism of host immune evasion strategies is the glycosylations of sequences in or around the NE of GP5, making a glycan shield around the NE leading to production of non-neutralizing antibodies. There are variations between the genotypes of PRRSV. For genotype I strains, up to 3 glycosylations may be found in or flanking regions of the GP5 NE, while for genotype II strains, there are 4 potential glycosylation sites (Faaberg et al., 2006). Taken together, these findings support the suggestion that natural PRRSV infection induces immune evasion in which few neutralizing antibodies along with large amounts of non-neutralizing antibodies are generated.

**Cell-mediated immunity:** Sufficient protective CMI response is detected approximately 4 weeks after PRRSV infection and directed mainly against GP5, M and N proteins of the
virus; among them M protein is a strong inducer of lymphoproliferation response (Bautista & Molitor, 1997). Proliferative T cell response is characterized by type I cytokine secretion (IFN-γ and IL-2), detected between 4 and 12 weeks after infection. However, the establishment of long-term persistence of the virus in the host suggests that CMI response is not sufficient enough to restrict the virus infection (Batista et al., 2004; Lowe et al., 2005). But still IFN-γ ELISPOT analysis remains as the reference assay for measuring CMI response against PRRSV. Normally, following wild-type PRRSV infection or PRRS-MLV, 3 - 4 times lesser IFN-γ secreting cells (ISCs) spots are detected compared to pseudorabies virus (Meier et al., 2003). In addition, PRRSV-specific ISCs appear 2–3 weeks post-infection (PI). ISCs are mainly CD4 CD8 double postivie T cells with a few CD4*/CD8α+ T cells (Meier et al., 2003). Although, proportion or the numbers of CD8+ cells increase in blood or tissues in the first few weeks PI (Shimizu et al., 1996), CD8 positive cells did not display cytotoxic activity (Costers et al., 2009). However, a live attenuated PRRSV vaccine that induced high ISCs frequencies protected pigs against viremia. Levels of IL-10 seemed to inversely correlated with interferon-γ response. These results may indicate a strong involvement of T cell immunity, IFN-γ, and possibly of IL-10, in the development of cell-mediated immunity against PRRSV (Diaz et al., 2006). However, the virus appears to utilize multiple strategies for its survival within its host, such as quasispecies variation, minimal IFN-α response in infected cells, lack of expression of viral proteins in infected cells, ADE, decoy epitopes, glycan shielding of NE etc., (Darwich et al., 2010).
Current vaccination strategies against PRRSV infection:

Both attenuated live and inactivated PRRSV vaccines have been licensed for use. Attenuated live vaccines have been widely used and have shown some efficacy in reducing the disease occurrence and severity, as well as the duration of viremia and virus shedding (Labarque et al., 2003; Murtaugh et al., 2002). It is generally considered that PRRS-MLV confers protection against clinical disease induced by homologous strains but failed to completely prevent respiratory infection, transplacental transmission, as well as pig-to-pig transmission of the virus (Osorio et al., 2002). Experimentally, the vaccinated gilts exhibited secondary neutralizing antibody response upon arrival to the endemic PRRSV-infected sow herds with evidence of reproductive failure, suggesting that such a recall response was not sufficient to provide full protection. In addition, the potential of spontaneous spreading of either NA or EU genotype PRRS-MLVs should be considered while planning control and eradication programs (grosse Beilage et al., 2009).

There have been several field reports concerning the use of live vaccines in PRRSV endemic areas resulting in emergence of mutated recombined viruses, which raised concern about safety. For example a vaccination program has led to an epidemic of the NA genotype PRRSV vaccine in the previously unaffected Danish pig population (Nielsen et al., 2001), and the existence of the vaccine-derived viruses in the Thai swine farms (Amonsin et al., 2009). And the capacity of the MLV to shape PRRSV evolution by homologous recombination with circulating virus in China (Li et al., 2009) has been reported. Genetic and phenotypic characterization of isolated field strains suggest that reversion to virulence is not a rare event. Furthermore, viruses genetically indistinguishable from vaccine and wild-type parental viruses have been detected in
clinical PRRS cases, clearly raising the need for a better differential tool (Kim et al., 2008).

Several inactivated PRRSV vaccines have also been developed to induce virus neutralizing antibodies but the vaccines offer only partial protection upon challenge (Kim et al., 2011; Vanhee et al., 2009). While PRRSV-neutralizing antibodies are believed to prevent infection and transplacental infection of pregnant sows, a killed vaccine that induced neutralizing antibodies failed to protect pigs against an in vivo challenge (Zuckermann et al., 2007). A commercial type I PRRSV inactivated vaccine, though reduced preweaning mortality of the piglets born to the vaccinated sows, failed to prevent the clinical signs associated with the PRRSV infection, viremia to a heterologous viral challenge and transplacental infection of their piglets (Scortti et al., 2007). Therefore, available killed-virus PRRSV vaccines are considered ineffective or of limited efficacy at best, even against homologous challenge (http://www.cvm.uiuc.edu/news/PRRS whitepaper.pdf).

One of the unknowns to be solved in the area of PRRSV immunopathology is the role of NA. It seems that NA may provide protection against the homologous challenge if present, but it also seems that NA contributes only partially to viral clearance. It is evident that a fully protective inactivated vaccine would be ideal, first, because of its safety, and second, because it would probably allow the differential diagnosis between vaccinated and infected animals. The basic question is whether it is possible to generate NA titers sufficient to be effective with the help of inactivated vaccines. A strategy to
enhance antibody response would be by the use of adjuvants. Recently, it has been shown that it is possible to produce NA responses when the vaccine virus underwent a formerly optimized inactivation procedure (UV irradiation or treatment with binary ethylenimine) which warrants neutralizing epitope structure preservation, and such treated vaccine candidate was combined with an incomplete Freund's adjuvant (Vanhee et al., 2009).

Several other approaches have been used to develop a more effective PRRSV vaccine, including DNA vaccines, recombinant DNA vector vaccines. de Lima and co-workers have developed a modified live vaccine strain carrying a deletion of an immunodominant B-cell linear epitope in the nsp2 gene. Removal of this 15-mer nsp2 epitope had no effect on immunogenicity, growth properties or virulence of the mutant virus. As expected, pigs vaccinated such a virus did not develop antibodies to the selected epitope (de Lima et al., 2008), but that candidate vaccine virus was not examined for its protective efficacy. Pseudorabies virus (PRV) has been used as a vaccine vector for expressing PRRSV immunogens (Qiu et al., 2005). Although a live attenuated vaccine-based PRV recombinant (strain Bartha) expressing the envelope protein GP5 of PRRSV failed to induce neutralizing antibodies, it conferred partial protection against clinical disease, and reduced pathogenic lesions and the duration of viremia in PRRSV challenged pigs. Replication-defective adenovirus vector vaccines containing PRRSV GP3, GP4, and GP5 recombinants was examined for its immunogenicity in mice (Jiang et al., 2008), but not tested for its protective efficacy in pigs. Another group cloned all open reading frames of a Danish isolate of PRRSV in DNA vaccination vectors (Barfoed et al., 2004), but immunization with such plasmids failed to inhibit virus persistence and shedding of
PRRSV (Pirzadeh & Dea, 1998). Together, all these studies suggested the need of a killed vaccine which could induce protective anti-PRRSV cell-mediated immunity and capable of complete clearance of the challenge virus.

Emergence of mucoadhesive PLGA [poly(lactide-co-glycolide)] nanoparticles as delivery systems for mucosal vaccines:

Mucosal vaccination partly relies on the transport of the antigen across mucosal barriers followed by uptake and presentation of vaccine antigens by professional antigen presenting cells (APCs). Hence, strategies to optimize mucosal vaccine delivery through the use of bioadhesives/mucoadhesive nanoparticles which protect the vaccine antigens and help in its sustained release at mucosal sites are required (Baudner & O'Hagan, 2010). Mucoadhesion appears to require a highly expanded and hydrated polymer network, which promotes an intimate molecular contact between the delivery system and the mucus layer (O'Hagan et al., 1993). Mucoadhesive delivery systems contribute to the immune response obtained due to one or more of the following reasons; (a) increased duration of retention at the mucosal site, (b) greater interaction with the epithelium, (c) enhanced absorption, and (d) sustained release from particulate delivery systems.

A range of both synthetic and natural materials has been tested to prepare polymer based nanoparticles. Ideally, these compounds must be non-toxic, biocompatible, free of leachable impurities and readily processible. Mucoadhesive formulations, such as liposomes and selectively engineered micro/nanoparticles may serve these purposes and their use has been reviewed extensively elsewhere (Baudner & O'Hagan, 2010; Mishra et
Synthetic polymers have the advantage of a sustained release of the therapeutic agent over a period of days to several weeks as compared with natural ones with a relatively short duration of drug release. On the contrary, they are usually limited by the need of organic solvents and harsh formulation conditions (Kumari et al., 2010; Park et al., 2005). It is likely that improved formulation and controlled release of potent immune potentiators will limit toxicities and improve efficacies (O'Hagan & Valiante, 2003). In general, the degree of mucosal penetration also dictates the induction of mucosal (IgA) and peripheral immune response (Gourley et al., 2004).

It has been suggested that small microparticles (MPs) (<10 µm) are taken up by APCs, while the larger ones (>10 µm) serve as an antigen depot, slowly releasing the antigen over an extended period of time (Heegaard et al., 2011). It was demonstrated that after oral delivery, particles smaller than 5 µm were rapidly translocated to the lymphatics and disseminated to the systemic lymphoid organs, while MPs larger than 5 µm remained in the Peyer’s patches with a very slow translocation to the efferent lymphatics (Kim et al., 2002; Zho & Neutra, 2002). Therefore, small MPs may induce a systemic as well as a mucosal immune response, whereas larger MPs may only induce a local response.

The most successful strategy to prolong circulation and to alter the bodily distribution of polymeric nanoparticles (100 to 1000 nm) is the modification of their surface by the incorporation of hydrophilic compounds. A strategy to render hydrophilic nature on the surface of nanoparticles is by coating their surface with surfactants. For example, coating of poly(methyl methacrylate) nanoparticles with poloxamine 908 dramatically reduces
their concentration in liver upon intravenous injection (Araujo et al., 1999). Efficient vaccine delivery systems alone are not able to elicit sufficient immune responses. Moreover, to be distinguished from commensal microorganisms, adjuvants (immune-potentiators) that activate innate signaling pathways (“danger signals”) in the epithelial cells and/or in the underlying APCs should be included in the vaccine formulation. Within mucosal vaccine delivery, it is most likely that antigen formulations will require both a delivery system and an immune-potentiator. Therefore, delivery systems should be coupled with appropriate adjuvants, and further improvements of present technologies are needed (Brayden, 2001; Lavelle & O'Hagan, 2006). A successful combination of TLR ligands entrapped in nanoparticles resulted in multiple TLR triggering, associated with enhanced persistence of germinal centers and plasma cell responses, early programming towards B-cell memory, and improved antibody responses by direct triggering of TLRs on B-cells and DCs, as well as T-cell help (Kasturi et al., 2011).
CHAPTER 2

INTRODUCTION

Objective 1: Evaluation of ROS mediated oxidative stress in pigs vaccinated with *M. tb* WCL adjuvanted PRRS-MLV and challenged with homologous or heterologous PRRSV

Primary target cells of PRRSV are alveolar and interstitial macrophages of infected pigs (Wang *et al.*, 2007). Both innate and adaptive immune responses are modulated by PRRSV in infected pigs (Renukaradhya *et al.*, 2010; Wang *et al.*, 2007; Xiao *et al.*, 2004). PRRSV-infected cells undergo apoptosis/necrosis, and to the large extent virus-mediated apoptosis of uninfected bystander cells was also observed. The bystander effect in the lungs of infected pigs is due to virus-induced aberrant and sustained secretion of proinflammatory cytokines and chemokines, which leads to excess production of reactive oxygen species (ROS), released by phagocytic cells (Sirinarumitr *et al.*, 1998; Xiao *et al.*, 2010).

ROS refer to differential oxidation and excitation states of molecular oxygen, such as superoxide, singlet oxygen, hydroxyl radical, hydrogen peroxide, and hypochlorites. ROS are an integral part of cell-signaling; however, at higher concentrations they cause severe
tissue damage (Chvanov et al., 2005). They play an important role in physiological events like host defense, mitogenesis, hormone biosynthesis, apoptosis, and fertilization. During active infection, ROS are produced by phagocytic cells in large quantities at the local mucosal infection sites. Several enzymatic activities are involved in the production of ROS, like the membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Quinn & Gauss, 2004), and the xanthine oxidase in the mitochondria of phagocytic cells (Droge, 2002; Green & Reed, 1998), that convert singlet oxygen to superoxide anions (O$_2^-$). Furthermore, superoxides are converted to hydrogen peroxide (H$_2$O$_2$) by the action of superoxide dismutase. In neutrophils, H$_2$O$_2$ can further originate hydroxyl radicals (OH$^-$), and/or it can combine with chloride ions (Cl$^-$) to generate hypochlorite (HCLO$^-$), mediated by myeloperoxidase. ROS are also produced at lower levels by non-phagocytic cells (Donko et al., 2005).

A steady-state production of pro-oxidants in cells and organs is balanced by an equal rate of consumption by antioxidants. Oxidative stress results from the imbalance in equilibrium between pro-oxidants and antioxidants, and unfortunately, it has always been in favor of pro-oxidants (Suarez et al., 1996). Extreme non-physiological concentrations of oxidants cause tissue destruction. Oxidative tissue damage caused by ROS have been detected in a large number of infectious settings, including viral pneumonitis (Fang, 2004; Steiner et al., 2006; Wang & Weinman, 2006). Peroxynitrate (ONOO$^-$) is a product of the reaction between nitric oxide (NO$^-$) and O$_2^-$ in specific phagocytes. Influenza virus induced lung pathology is mediated by the production of ONOO$^-$ in alveolar macrophages (Akaike et al., 1996; Kash et al., 2004; White et al., 2005). Pneumonia in
Hantavirus cardiopulmonary syndrome (HCPS) (Davis et al., 2002) and in herpes simplex virus-1 (HSV-1) disease is mediated through ROS (Adler et al., 1997). ROS are among the most important mediators of diffuse PRRSV-mediated pathology in the lungs and lymph nodes in infected pigs (Sirinarumitr et al., 1998). In this study, we quantified ROS production in immune cells present in the lungs and blood of PRRSV challenged unvaccinated or mucosally vaccinated (PRRS-MLV+ M. tb WCL) pigs. Our results identified the reduced production of ROS in mucosally vaccinated PRRSV challenged pigs, which supported our previously reported results of protective immunity associated with reduced lung pathology compared to mock control virus challenged pigs.

**Objective 2: Evaluation of immune correlates of protection against PRRSV in M. tb WCL adjuvanted PLGA nanoparticle based PRRSV inactivated vaccine immunized and virus challenged pigs.**

With a collective surface area of about 400 m² (in humans), mucosal surfaces are the major sites of entry for most pathogens. Mucosal vaccine administration offers several advantages, such as needleless delivery and less labor intensive, which results in improved vaccine compliance and avoids problems of blood transmissible infections (Levine, 2003). The quality and quantity of the adaptive immune response is dependent on the quality and quantity of the innate response. Cellular components involved in the innate immune response are found abundantly at mucosal inductive and effector sites, such as APCs (DCs, macrophages, and B cells) and other cells such as, mast cells, neutrophils, and lymphocytes (Brandtzaeg, 2009).
Mucosal immunization elicits strong mucosal immune response even at remote mucosal sites. The existence of a common mucosal immune system has been confirmed in large animals, and in addition, a systemic immune response was also detected following mucosal immunization depending on the nature and size of the vaccine particles (Scicchitano et al., 1984). There are several advantages in triggering protective mucosal immunity since greater than 90% of pathogens enter and initiate infection at the mucosal surfaces (Gerdts et al., 2006). Immunoglobulin A is the principal mucosal secretory protein responsible to mediate protective response, and parenteral routes of immunization cannot induce that efficiently. Systemic (parenteral) vaccination only triggers incomplete protection against mucosal infections (Bowersock et al., 1998).

Adjuvants are functionally defined as components added to vaccine formulations that enhance the antigenicity of the immunogen in vivo (Pashine et al., 2005). Given the latest understanding of innate immune mechanisms, this functional definition needs refinement and adjuvants now should be divided into two classes (delivery systems and immune potentiators) based on their dominant mechanisms of action. Immune potentiators activate innate immunity directly (for example, cytokines) or through pattern recognition receptors (PRRs) such as bacterial components, whereas delivery systems (for example, cationic microparticles) may concentrate and display antigens in repetitive patterns, target vaccine antigens to APCs and help to co-localize antigens and immune potentiators (O'Hagan & Valiante, 2003). Adjuvants must enhance both antigen specific immune responses and increase immunological memory, thus help to improve protective
immunity (Heegaard et al., 2011). Adjuvants per se are not specific to use at mucosal surfaces, but they facilitate the entry of the antigen into the mucosal lymphoid tissues and in addition deliver the required ‘danger’ signal. Once introduced into a mucosal tissue site, the microenvironment is conducive to determine the induction of mucosal immune response resulting in secretory-IgA secretion (Meeusen et al., 2004).

Dendritic cells isolated from mucosal tissues and lymph nodes also display a distinct mucosal cytokine profile and preferentially induce IgA switching and the expression of mucosal homing receptors such as α4β7 and CCR9 in vitro (Heegaard et al., 2011). The polarized immune microenvironment is likely to determine the mucosal bias of the immune response against any pathogen or antigen that breaches its surface (Felder et al., 2000; Goldammer et al., 2004). However, the initial innate stimulation following pathogen invasion or by a strong adjuvant is likely to induce activation of Th1 and IgG secreting cell response which migrate to peripheral environments and contribute to a systemic immune response (Dory et al., 2005). If the mucosal environment dictates the development of a mucosal immune response, vaccination strategy should target the vaccine antigens to a mucosal tissue (Meeusen et al., 2004). Mucosal vaccines consist of live or attenuated organisms co-administered with a suitable adjuvant/delivery system have shown to be effective in inducing mucosal immunity and protection against pathogens. However, at present, no licensed defined adjuvant/delivery formulation exists for mucosal delivery in either human or animal vaccine or medicine (Choe et al., 2005; Harpin et al., 1999).
Several approaches have been adopted to develop an effective PRRSV vaccine, which includes recombinant DNA vector vaccines, inactivated, and modified live vaccines, but none of these approaches have met with overwhelming success. Adjuvanticity of several adjuvants on the protective efficacy of a commercially available live attenuated PRRSV vaccine has been tested (Ingelvac PRRS MLVR) but with limited success. In one particular study, co-administration of IL-12 with MLV-PRRS effectively enhanced the cell-mediated immune response, but that did not significantly reduce the clinical disease (Charerntantanakul et al., 2006). Moreover, intranasal vaccination has shown several advantages over other mucosal routes because of its relative accessibility, high permeability of the local lymphoid tissues, less acidic pH, and lower levels of enzymatic activity compared to the gut lymphoid tissues (Stanley et al., 2001). Further, enhancement of mucosal immune responses may be achieved by optimizing the antigen dose and composition of the vaccine, by means of improving the cell targeting, and incorporating relevant adjuvants and immune modulators (Vanhee et al., 2009).

We have established the mucosal adjuvanticity of whole cell lysate (WCL) of Mycobacterium tuberculosis (M. tb) co-administered with PRRS-MLV vaccine (Dwivedi et al., 2011a; Dwivedi et al., 2011b). Components of WCL such as heat shock protein-70 (HSP-70) and PE (Pro-Glu)/ PPE (Pro-Pro-Glu) have shown potent adjuvant activity driving Th1 (Bansal et al., 2010; Harmala et al., 2002) and Th17 biased immune responses. Efficient Th1-immunity-inducing adjuvants are in demand, and such adjuvants promote cell-mediated immunity against subunit/inactivated vaccines (Heegaard et al., 2011).
We have also studied an innovative inactivated PRRSV vaccine delivery system, by entrapping inactivated PRRSV antigens in PLGA (50:50) nanoparticles, administered intranasally once in the absence of any adjuvant. The aim of this approach was to elicit protective mucosal immunity in the respiratory tract of pigs against PRRSV, and the results of that study were encouraging (manuscript in preparation). In the present study, we investigated a similar nanoparticle-based PRRSV vaccine delivery but with certain modifications to further improve the vaccine efficacy. Such as co-administration of 75:25 PLGA nanoparticles with a potent mucosal adjuvant, *M. tb* WCL (Dwivedi *et al.*, 2011a; Dwivedi *et al.*, 2011b) and administered a booster dose. We hypothesized that there would be more pronounced and protective PRRSV targeted innate and adaptive immune responses, leading to significant clearance of the challenged heterologous PRRSV associated with reduced lung pathology and a strong memory immune response. To test our hypothesis, we analyzed the immune correlates of protection in immunized virus challenged pigs using both recombinant PRRSV proteins (GP5, matrix and nucleocapsid) and total viral proteins specific IgG and IgA antibodies, NAs profiles and quantification of virus from the lungs and circulation. Further, we evaluated the cell-mediated immunity by measuring the frequency of ISC. To summarize, in pigs intranasally vaccinated with *M. tb* WCL adjuvanted PLGA nanoparticle-based inactivated PRRSV vaccine and challenged with a virulent PRRSV challenge, we examined the viral load in the lungs and circulation and analyzed various immune correlates of protection.
MATERIALS AND METHODS

Objective 1: Evaluation of ROS mediated oxidative stress in pigs vaccinated with PRRS-MLV with \textit{M. tb} WCL and challenged with homologous or heterologous PRRSV

\textbf{Pigs and inoculations:}\ Conventional Large White-Duroc crossbred specific pathogen-free piglets weaned at 3–4 wk of age were transported from the swine herd free from PRRSV, porcine respiratory corona virus, transmissible gastroenteritis virus, and porcine circo virus 2, to the animal facilities of the Food Animal Health Research Program at the Ohio Agricultural Research and Development Center, Wooster, Ohio. The blood samples collected from piglets on arrival were tested to be free from PRRSV antibodies by ELISA. The animals were maintained in our large animal BSL2 facility. Throughout the duration of the study animals received food and water \textit{ad libitum}. All the inoculations including PRRSV challenge were performed by intranasal route. Pigs were maintained, samples collected, and euthanized as per the approved protocol of the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC), The Ohio State University. Forty pigs were allocated to one of five groups: group 1, mock pigs (n=4) inoculated with vehicle (normal saline) and DMEM; for the other four groups (n=9 per group), groups 2 and 3 were unvaccinated, and groups 4 and 5 were vaccinated (PRRSV-MLV + \textit{M. tb} WCL). Groups 2 and 4 were challenged with wild-type homologous PRRSV VR2332 (2 × 10^6 TCID\textsubscript{50} per pig), and groups 3 and 5 were
challenged with virulent heterologous PRRSV MN184 (21) (1 X 10^6 TCID\textsubscript{50} per pig) in 2 ml on day 21 post-immunization (DPI). Three pigs each from groups 2 to 5 were euthanized at 15, 30, and 60 days post-challenge (DPC). Mock inoculated pigs (n=4) were euthanized separately prior to euthanizing any challenged animals.

Isolation of peripheral blood mononuclear cells (PBMCs): PBMCs were isolated from blood collected in acid citrate dextrose (ACD) anticoagulant solution from euthanized pigs as previously described (Renukaradhya et al., 2010; VanCott et al., 1993). Briefly, 35 ml of blood collected in a 60cc syringe containing 15 ml of ACD solution was gently mixed to prevent clotting of blood. Transferred the content to a 50 ml falcon tube and centrifuged at 400 xg for 20 minutes at room temperature (RT) with brake off. Pipetted out the plasma and collected the white buffy coat present over the red blood cells (RBCs) with the help of a sterile Pasteur pipette, and transferred to a 50 ml falcon tube and made up the volume to 50 ml with HBSS. Transferred 10 ml of suspension to each of 15 ml capacity five falcon tubes and gently underlayed with 5 ml of warm Ficoll-paque with the help of 35 ml syringe fitted with 18g 6 inch long needle. Centrifuged the tubes for 30 minutes at 900 xg at RT with brake off. Collected the interface using sterile cotton-plugged Pasteur pipette and placed in a 50 ml falcon tube containing 10 ml of HBSS and then made up the volume to 50 ml with HBSS. Centrifuged the tubes at 400 xg for 20 minutes at 4\textdegree C and the cell pellet was subjected for RBCs lysis by hynmonic shock using 5 ml sterile distilled water by mixing 3-5 times to resuspended the pellet and rapidly brought the volume to 50 ml with HBSS (in less than 10 seconds). Centrifuged at 400 xg for 15 minutes at 4\textdegree C and repeated the washing step again. Finally, resuspended the pellet
in 5-10 ml of enriched RPMI [RPMI-1640, 10% fetal bovine serum, gentamicin (100 µg/ml), ampicillin (20 µg/ml), 20 mM HEPES, 2 mM l-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 50 µM of 2-ME] and counted the viable cells by tryphan blue (0.05%) exclusion method using hemocytometer. Cell suspension volume equivalent to 20 million cells per cryogenic vial was taken and centrifuged at 400 xg for 5 minutes at 4°C, the cell pellet was resuspended in one ml of ice-cold freezing media (10% DMSO in fetal bovine serum) and vials were frozen in a liquid nitrogen tank.

**Isolation of bronchoalveolar lavage fluid (BAL) cells:** BAL cells were isolated as previously described (Kuroki *et al.*, 2003; Loving *et al.*, 2007; Renukaradhya *et al.*, 2010). Briefly, about 200 ml of BAL was collected by lavaging the lung airways with sterile PBS containing 0.03% EDTA. Centrifuged the lavage at 400 xg for 15 minutes at 4°C. Discarded the supernatant, added 45 ml of 1X HBSS and 450 µl of 1M DTT (to clear the mucus content). Incubated the tube with gentle shaking for one hour at 37°C and washed by centrifugation at 400 xg for 15 minutes at 4°C. The cell pellet was washed twice in 50 ml HBSS and subjected to RBCs lysis (if the pellet had RBCs) and cells were frozen as described above.

**Revival of PBMCs and BAL cells:** Frozen BAL cells and PBMCs were revived a day before the planned experiment by rapid thawing of frozen vials at 37°C for 1-2 min. Transferred the cell suspension to a 15 ml falcon tube containing 10 ml of RPMI wash media and centrifuge the tube at 400 xg for 5 min at RT. The cell pellet was resuspended in enriched RPMI medium and incubated in a 37°C water bath overnight.
**Determination of ROS concentration by colorimetric assay:** A colorimetric assay based on the ability of ROS to reduce nitroblue tetrazolium (NBT) salt to a blue-colored formazan was performed as previously described (Choi *et al.*, 2006; Tunc *et al.*, 2010; VanCott *et al.*, 1993). The quantity of ROS produced is directly proportional to the optical density (OD) value measured at 650 nm. Briefly, BAL cells (0.5 X 10^6) and PBMCs (1 X 10^6) were suspended in enriched RPMI and restimulated in the presence or absence of killed crude PRRSV antigens (Ags) (50 µg/mL) at 37°C for 24 h to induce PRRSV-specific response. The cells were washed twice, resuspended in PBS (pH 7.2) and seeded in 48-well tissue-culture plates in quadruplicate. As controls, duplicate wells treated with 0.1% NBT and another duplicate wells treated with 0.1% NBT and 10 µM of diphenlyeneiodonium (DPI), a potent inhibitor of NADPH oxidase, were included. Cells treated with phorbol 12-myristate 13-acetate (PMA; 600 ng/mL) and 0.1% NBT were included as a positive control. Cells unstimulated with PRRSV Ags were included as a blank, and the plates were incubated for 45 min in a CO₂ incubator at 37°C. The reaction was stopped using ice-cold PBS (pH 7.2) (500 µL/well), and washed three times in PBS by centrifugation at 400 xg for 5 min. Subsequently, the cells were treated with methanol to remove excess un-utilized NBT, and air-dried at RT. The reduction of NBT by ROS into blue colored formazan was measured by solubilizing the cell membrane by addition of 2M KOH (120 µL/well), and subsequently DMSO (140 µL/well) to dissolve the formazan, and the mixture was gently mixed for 10min at 37°C. Harvested supernatant was transferred into a 96-well plate and read in a microplate reader at OD_{650} nm.
Flow cytometric analysis to determine the frequency of ROS producing cells: The principle of this assay is based on the ability of ROS to reduce NBT to formazan, which in turn quenches the cellbound immunofluorescent molecule, resulting in reduction in the frequency of fluorescence-positive cells. Therefore, the frequency of ROS-producing cells is inversely proportional to that of fluorescence-positive cells, as measured by flow cytometry. The assay was performed as previously described (Fattorossi et al., 1990) with a few modifications. Briefly, fluorescein isothiocynate (FITC)-conjugated concanavalin A (FITC-ConA) (Sigma Chemical Co., St. Louis, MO) was used as a cell surface probe. ConA is a plant mitogen that binds to cell surface receptors containing mannose, which are present on many cell types including phagocytes (Schmalstieg et al., 1986). ConA does not influence the phagocytic activity and cell proliferation seen under the conditions described in our assay, such as dose, time and temperature of incubation (McPhail et al., 1981). BAL cells and PBMCs suspended in enriched RPMI were either unstimulated or stimulated using inactivated PRRSV Ags (50 µg/mL) at 37 °C for 24 h. The cells were washed twice in PBS and labeled with FITC-ConA (25 µg/mL) on ice for 30 min. The cells were washed once each using RPMI and PBS to remove the unbound FITC-ConA, resuspended in PBS, and seeded onto a 96-well plate. Cells were treated with 0.1% NBT in PBS, and a control well with unlabeled cells not treated with NBT was included in the assay. Cells stimulated with PMA (150 ng/mL) and 0.1% NBT in the presence or absence of DPI (10 µM) were included as controls, and the plates were incubated for 45 min at 37°C in a CO₂ incubator. The reaction was stopped by addition of ice-cold PBS, and washed twice and resuspended in ice-cold PBS and stored at 4°C until analyzed by flow cytometry. Unlabeled cells were used to set up the forward and side-
scatter values and the frequency (percentage) of fluorescence-positive cells was recorded from a total of 20,000 acquired events using the flow cytometer (FACS Aria II; BD Biosciences, San Jose, CA) as previously described (Dwivedi et al., 2011a; Renukaradhya et al., 2010). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

**Statistical analysis:** All the data were expressed as the mean and standard error of the means (SEM) of three pigs. Statistical analyses were performed by a non-parametric Wilcoxon t-test (SAS software; SAS Institute Inc., Cary, NC). Statistical significance was set at $p < 0.05$, and only significant data are indicated in the figures.

**Objective 2: Evaluation of immune correlates of protection to PRRSV in M. tb WCL adjuvanted PLGA nanoparticle-based PRRSV inactivated vaccine immunized and virus challenged pigs.**

**Cells, PRRSV, and adjuvant:** A stable *Mycoplasma*-free MARC 145 cells (African Green monkey kidney cell line) which support the growth of PRRSV (Christopher-Hennings et al., 2001) was used to prepare PRRSV stocks and for immunological assays. Cells were maintained in Dulbecco’s minimum essential medium (DMEM, Lonza) with 10% fetal bovine serum (Atlanta Biologicals) at 37°C with 5% CO$_2$. For virus infection, DMEM supplemented with 2% horse serum was used. PRRSV MN184 (virulent strain) (Kim et al., 2007) was used as heterologous virus for challenge studies, was provided by Mike Murtaugh (University of Minnesota). *Mycobacterium tuberculosis* whole cell lysate
(M. tb WCL) was provided by Drs. Dobos and Belisle under NIH/NIAID funded contract HHSN266200400091c "TB Vaccine Testing and Research Materials" (Colorado State University).

**Preparation of PRRSV VR2332 vaccine antigens:** MARC 145 cells infected with PRRSV VR2332 strain (0.001 MOI) in roller bottles showing greater than 80% cytopathic effect (~3-4 days) were freeze-thawed three times. The harvested cell culture fluid was clarified to remove cell debris at 2000 xg for 30 minutes and then subjected to ultracentrifugation with 20% sucrose cushion at 107000 xg for 2 hr. Pooled crude viral pellet was resuspended in PBS and titrated to determine the live viral titer and found to be approximately 5x10^7 TCID$_{50}$/ml. Further, the pellet was UV treated (254 nm for 1 hr) to inactivate the virus, sonicated, and the protein content was estimated using BCA assay kit (Biorad), and found to be approximately 10 mg/ml.

**Entrapment of Inactivated PRRSV VR2332 lysate or M. tb WCL in PLGA nanoparticles:** PLGA (poly lactic-co-glycolic acid) nanoparticles entrapped with inactivated North American PRRSV prototype strain VR2332 crude total Ags or M. tb WCL were prepared by solvent evaporation/double emulsion method (also known as water-in-oil-in-water, w/o/w) as described earlier (Rajapaksa et al., 2010) with few modifications. Briefly, 4% PLGA (75:25) polymer solution was prepared by dissolving 0.18 gm of PLGA in 4.5 ml of methylene chloride/dichloro methane (DCM). Five mg of PRRSV Ags or M. tb WCL resuspended in 0.5 ml PBS was mixed in 0.25 ml of 2% polyvinyl alcohol (PVA) solution prepared in 10 mM HEPES buffer (pH 7.5), then 0.25
ml of 2% sucrose and 0.5 ml of 2% magnesium hydroxide were added to 4.5 ml of PLGA polymer solution and emulsified using probe sonication for 20 seconds (Branson-Sonifier 450) with a duty cycle of 30% and output control of 3. The resulting emulsion (w/o) was divided into two tubes and 11.5 ml of 2% PVA and 1 ml of 12.5% Poloxamer 188 solution were added to each tube and emulsified by sonication for 40 seconds to obtain the final w/o/w emulsion. Contents of both the tubes were combined in a 100 ml beaker and stirred for 20 hr with a magnetic stirrer at 400 rpm at 4°C to allow solvent evaporation. The nanoparticles were collected by centrifugation at 10976 xg (in Beckman-Coulter, FX6100 router) for 30 min and resuspended in sterile distilled water; the washing step was repeated three times with distilled water. The pellet of entrapped nanoparticles was resuspended in 5 ml of 5% sucrose solution and the tube was freeze-dried for 16-18 hr and the lyophilized powder was stored at -20°C.

**Determination of protein entrapment efficiency:** Total protein entrapment in the PLGA nanoparticles was estimated using BCA protein assay kit (Biorad). Approximately 5 mg of freeze-dried nanoparticles was added to 1 ml of 5% SDS in 0.1M NaOH and incubated with gentle shaking for one hour at 37°C. The solution was centrifuged at 11,000 xg for 5 minutes and the supernatant was collected and estimated for protein using BCA protein assay kit. The protein entrapment (w/w) was expressed as the amount of protein (in percentage) relative to the weight of the nanoparticles.

**Scanning Electron Microscopy:** The morphology of the protein-loaded PLGA nanoparticles was visualized using scanning electron microscopy. Briefly, the
nanoparticles were placed on a double-sided adhesive tape attached to an aluminum stub and sputter-coated with gold/palladium beam for 2 min. The coated sample was imaged using Philips XL30-FEG scanning electron microscopy at 10kV.

**Pigs and Inoculations:** Conventional Large White-Duroc crossbred, specific-pathogen-free piglets (N=30) were weaned at 16 to 20 days of age and transported to animal facilities at FAHRP, OARDC, The Ohio State University, Wooster, OH. The swine herd was confirmed sero-negative for antibodies to PRRSV, PRCV, TGEV, and PCV2. Piglets were bled on arrival, and the sera were tested to confirm the absence of PRRSV antibodies. Pigs were allowed to acclimate for an additional week before initiation of the experiment. Animals were maintained in our large animal BSL2 facility under the supervision of a veterinarian. Throughout the duration of the study animals were received food and water *ad libitum*. All the inoculations, adjuvant (*M. tb* WCL 1 mg/pig either nanoparticles (NP) entrapped or unentrapped) (Dwivedi *et al.*, 2011a; Dwivedi *et al.*, 2011b), inactivated VR2332 strain of PRRSV (PRRSV-K-Ag) [100 or 500 µg/pig of either NP entrapped or unentrapped] was co-administered intranasally twice at 2 weeks interval. Pigs were challenged on post-immunization day (PID) 28 using a virulent heterologous PRRSV strain MN184 (Kim *et al.*, 2007) (1 x 10⁶ TCID₅₀/pig). Both adjuvant and PRRSV vaccine were combined just before administration and delivered equally to each nostril. A total of 30 pigs were divided in one of the ten groups. The pig groups, type, and dose of vaccination are tabulated below.
<table>
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<tr>
<th>Groups</th>
<th>Experimental groups (n=3 per group)</th>
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<tbody>
<tr>
<td>1</td>
<td>Mock pigs</td>
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<tr>
<td>2</td>
<td>Mock+ challenged with PRRSV MN184</td>
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<td>3</td>
<td>100 µg K-Ag/pig+ PRRSV MN184</td>
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<td>4</td>
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<td>10</td>
<td>500 µg NP- K-Ag/pig + M. tb WCL+ PRRSV MN184</td>
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Pigs were euthanized on post-challenge day (PCD) 15. Mock inoculated pigs with PBS and DMEM medium were euthanized separately prior to euthanasia of any vaccinated virus challenged pigs.

**Collection of blood and lung samples for analysis:** For evaluation of viremia and PRRSV specific antibody response, 3 to 5 ml of heparinated blood was collected on the days of vaccine inoculation and DPC 0, 6, 10, and 15. Plasma was separated from the clotted blood and aliquots were preserved at −20 °C until used in assays. Pigs were monitored daily for the respiratory disease, and rectal temperature and body weight were recorded twice weekly.
Preparation of the lung homogenate/lysate: One gram of lung tissue was collected in 5 ml of DMEM from each pig and minced into tiny pieces and then homogenized using Stomacher 400 laboratory blender (Seward, Long Island, NY) (Renukaradhya et al., 2010) for one min. The clarified supernatant collected after centrifugation at 400 xg for 5 minute was aliquoted and preserved at -80°C until used in the assays.

Collection of BAL fluid: The pig lungs were lavaged with 20-30 ml of cold PBS containing 0.03% (w/v) EDTA and 100 U/ml penicillin and 100 ug/ml streptomycin sulphate depending on the size of the lung. Then lungs were gently massaged to facilitate collection of the cells and surface secretions in bronchi and alveoli into the lavage solution. Drained the fluid carefully through trachea to a sterile bottle. Centrifuged the lavage at 400 xg for 15 minutes at 4°C. Collected and aliquoted the supernatant and stored at -80°C until used.

Isolation of PBMCs and BAL cells: PBMCs and BAL cells were isolated as per the procedure described above. In this study, freshly isolated cells were used for assays. Aliquots of excess cells were also preserved in liquid nitrogen as described above for future experiments.

Isolation of Lung-mononuclear cells (lung-MNCs or LMNCs): LMNCs from individual pigs were isolated as per the procedure described previously (Calder et al., 2004; Khatri et al., 2010; Kuroki et al., 2003; Loving et al., 2007) with few modifications. Briefly, after euthanasia, lungs were collected and the pulmonary
vasculature was flushed with sterile PBS to remove the peripheral blood. The airways were lavaged to remove free cells as well as to collect BAL cells as described previously (Basta et al., 2000) using sterile ice cold PBS containing EDTA (0.03%). Subsequently, the lung tissue was collected in ice cold PBS, washed in sterile PBS, minced and suspended in PBS containing DNase (Sigma) (40 μg/ml) and Type II collagenase (Invitrogen) (1.5 mg/ml) and incubated on an orbital shaker at 37°C for 2 hr. Released cells were collected and fractionated using 43% and 70% Percoll density gradient centrifugation and the interface rich in lung-MNCs was collected. After RBCs lysis by hypotonic shock using sterile distilled water, cells were counted using a hemocytometer and the viability was tested by tryphan blue dye (0.05%) exclusion method. The viability of lung-MNCs harvested by this method was >95%.

Isolation of tracheobronchial lymph nodes mononuclear cells (TBLN-MNCs):
Procedure for isolation of TBLN-MNCs was followed as described previously (Dwivedi et al., 2011b). Briefly, TBLNs were collected in DMEM and cut into small pieces and homogenized using syringe plungers placed in stainless steel selectors. Homogenate was transferred into a 50 ml falcon tube and washed twice at 400xg for 15 min and the pellet was dissolved in RPMI containing 43% Percoll and centrifuged for 25 min at 1825 xg at 4°C, with brake off. RBCs in the cell pellet were lysed using sterile distilled water and cells were washed resuspended in enriched RPMI medium.
**PRRSV specific isotype antibody (IgG and IgA) analysis in lungs and blood:** Total PRRSV specific IgA and IgG antibodies in the lungs and plasma samples were analyzed as described previously (Mulupuri et al., 2008; Zhang et al., 2007). Briefly, ELISA plates were coated with pre-titrated crude killed PRRSV (MN184) antigens or with PRRSV recombinant proteins, GP5, M and N (5 μg/ml), in carbonate-bicarbonate buffer (pH 8.8) and incubated overnight at 4°C. The plates were washed with PBS containing 0.05% Tween-20 (PBST) and treated with 200 µl/well of sterile blocking buffer (2% BSA in PBST) and incubated for 2 hr at RT. Discarded the blocking buffer and plated serial ten-fold dilutions of concentrated BAL fluid or plasma samples (50 µl/well) and incubated for 2 hr at RT or at 4°C overnight. Plates were washed 4 times with PBST and the bound PRRSV isotype specific antibody was detected using 50 µl per well of goat anti-pig IgA and anti-pig IgG secondary antibodies conjugated with HRP (KPL) (1:2000 dilution). Incubated the plate at RT for 2 hr, washed 4 times and developed using a chromogen ABTS solution with 0.3% H₂O₂, and after 30 minutes the reaction was stopped using 1% SDS and read the plates at OD405 nm. For calculation of antibody levels in the BAL fluid samples, raw OD values were considered, as there was no dilution dependent reduction in OD values observed. In contrast, to determine the PRRSV specific antibody titer in the plasma samples, mean OD values from 30 PRRSV negative samples (i.e., before any treatment) plus two times the standard deviation at 1 in 100 dilution was considered as the positive-negative cut-off OD value. The reciprocal dilution which gave OD value greater than the positive-negative cut-off OD was considered as the end titer.
**Virus titration and Virus neutralization Assays:** PRRSV titer and virus neutralizing antibody titer in lung homogenates and plasma samples were analyzed by indirect immunofluorescence assay (IFA) as previously described (Christopher-Hennings *et al.*, 2001). Briefly, for virus titration, a confluent monolayer of MARC-145 cells in 96-well microtiter plate was treated with 10-fold dilution of test sample for 48 hr (100 µl/well). For VNT, sample was subjected to UV treatment for 45 minutes to inactivate any PRRSV and then subjected to heat inactivation at 56°C for 30 minutes to inactivate the complement function. Two-fold dilutions of test samples made in serum free DMEM (100 µl/well) were incubated with 50 µl of PRRSV (MN184) 250 TCID$_{50}$ units per well for 2 hr at 37°C, subsequently 100 µl of the suspension was transferred into 96-well microtiter plate containing confluent monolayer of MARC-145 cells and incubated for 2 hr at 37°C. Further, 100 µl/well of DMEM containing 2% horse serum (infection medium) was added to each well and incubated for 48 hr at 37°C in a CO$_2$ incubator. Pipetted out the contents from the plate and the cells monolayer was fixed with 80% acetone in water for 15 minutes and plates were allowed to dry in a fumehood for 20 minutes. Later cells were rehydrated with 100 µl/well of PBS for 3-5 minutes. Cytopathic effects in both the plates meant for virus titration and VNT were examined after treatment with 50 µl/well of mouse anti-PPRSV nucleocapsid protein specific mAb (SDOW-17) (1:5000) followed by Alexa-488 conjugated anti-mouse IgG (H+L) secondary antibody (1:3000), after each treatment plates were incubated at 37°C for 2 hr. Plates were washed 4 times in between the treatments and observed under an inverted fluorescent microscope after mounting the cell monolayer with glycerol-PBS in 6:4 ratio (50 µl/well). The PRRS
viral titers (in per gram of lung tissue and per ml of plasma respectively) and PRRSV specific VNT titers were determined.

Detection of PRRSV load in lungs by qRT-PCR: PRRSV RNA was extracted from lung homogenate using MagMax™-96 virus Isolation kit (Ambion/Applied Biosystems, Carlsbad, California, USA) as per the manufacturer's instructions. Briefly, approximately one gram of lung tissue was weighed and homogenized with 5 ml of RPMI-1640, and 50 µl of the sample was used for RNA extraction. Extracted RNA was reverse transcribed into complementary DNA (cDNA) using Quantitect Reverse Transcription kit from Qiagen. The cDNA was subjected to quantitative real time PCR reaction using primers against PRRSV ORF6 in PerfeCta SYBR Green Fast Mix. Standard curves were generated using serial ten-fold dilution of PRRSV VR2332 stock starting at 10^7 TCID_{50} per µl for viral RNA quantification.

ELISPOT assay to determine the frequency of PRRSV specific ISC: The levels of IFN-γ secreting cells in lung-MNCs were determined as described previously (Azevedo et al., 2006; Martelli et al., 2009). Briefly, lung-MNCs were plated (5×10^5 cells/well) in enriched RPMI-1640 in a 96-well MultiScreen plate (Millipore) pre-coated overnight with 10 µg/ml of mouse anti-pig IFN-γ mAb (BD Bioscience) at 4°C and subsequently blocked with enriched RPMI for 2 hr at RT. The plated cells were restimulated with killed PRRSV (MN184) antigens or recombinant PRRSV proteins (N, M and GP5) (10 µg/ml) for 24 hr at 37°C in a CO₂ incubator. Plates were washed six times with PBST followed by incubation with biotinylated anti-pig IFN-γ detection antibody for 1 hr at RT.
The plate was washed again six times and treated with streptavidin-HRP conjugate for 1 hr at 37°C and then developed using AEC substrate at RT for 1 hr. Finally, the plates were washed in tap water several times and air dried. Frequency of PRRSV specific IFN-γ secreting cells was counted using an AID® ELISpot Reader System. The background values from unstimulated cells were subtracted from the respective counts of the stimulated cells and the immune responses were expressed as the number of IFNγ secreting cells per million LMNCs. Cells stimulated with PHA and unstimulated were included as positive and negative control, respectively, in every plate.

**Statistical analysis:** All the data were expressed as the mean +/- SEM of three pigs. Statistical analyses were performed by one way ANOVA followed by Tukey's t-test using GraphPad PRISM (software version 5.0 for windows). Statistical significance was assessed as $P < 0.05$. Error bar represents the standard error. Comparisons were performed between different treatment groups and statistical significance between any two groups is indicated with alphabets 'a' through 'j' as follows:

a $\rightarrow$ Mock+Chal. V/s K-Ag (100 or 500 µg/pig) vaccinated + Chal. Group;
b $\rightarrow$ Mock+Chal. V/s K-Ag+ M. tb WCL+ Chal.;
c $\rightarrow$ Mock+Chal. V/s NP-K-Ag +NP- M. tb WCL+ Chal.;
d $\rightarrow$ Mock+Chal. V/s NP-K-Ag + M. tb WCL+ Chal.;
e $\rightarrow$ K-Ag + Chal V/s K-Ag + M. tb WCL+ Chal.;
f $\rightarrow$ K-Ag + Chal V/s NP-K-Ag + NP-WCL+ Chal.;
g $\rightarrow$ K-Ag + Chal V/s NP-K-Ag + M. tb WCL+ Chal.;
h $\rightarrow$ K-Ag + M. tb WCL+ Chal. V/s NP-K-Ag + NP- M. tb WCL+ Chal.;
i \rightarrow K-Ag + M. tb WCL+ Chal. V/s NP-K-Ag + M. tb WCL+ Chal.; and

j \rightarrow NP-K-Ag + NP- M. tb WCL+ Chal. V/s NP-K-Ag + M. tb WCL+ Chal.
RESULTS

Objective 1: Evaluation of ROS mediated oxidative stress in pigs vaccinated with *M. tb* WCL adjuvanted PRRS-MLV and challenged with homologous or heterologous PRRSV

**Determination of ROS concentration by colorimetric assay:** BAL mononuclear cells of unvaccinated PRRSV VR2332-challenged pigs produced a significant increase in the amount of ROS compared to vaccinated pigs at 30 and 60 DPC (Fig. 2.1A and Table 2.1A). In PBMCs a comparable increase in ROS production was also detected in unvaccinated compared to vaccinated pigs (Fig. 2.1B and Table 2.1B). Similarly, in unvaccinated compared to vaccinated and virulent heterologous PRRSV MN184 challenged pig BAL MNCs, a significantly increased production of ROS at an earlier time point (DPC 15) was detected (Fig. 2.1C and Table 2.1A). A significant increase in ROS production was also detected in PBMCs at DPC 15 and 60 (Fig. 2.1D and Table 2.1B).

**Flow cytometric analysis to determine the frequency of ROS producing cells:** In the PBMCs and BAL MNCs of unvaccinated compared to vaccinated PRRSV VR2332 challenged pigs, there was an increase in the frequency of ROS producing cells at DPC 15 and 60 (but not at DPC 30) (Fig. 2.2B and C and Table 2.2A and B). In unvaccinated, virulent PRRSV MN184 challenged pigs an increased frequency of ROS producing cells was detected in BAL cells at all DPC tested (Fig. 2.2D and Table 2.2A). In PBMCs a similar increase in ROS production was detected at DPC 30, and at DPC 60 the increase
in ROS-producing cells was statistically significant (Fig. 2.2E and Table 2.2B). The control bars and values in the figures and tables signify that the assays used in this study were specific to ROS production by porcine phagocytic cells. The control bars/values shown (Figs. 2.1 and 2.2 and Tables 2.1 and 2.2) indicate the difference between the cells stimulated using PMA (positive control), and cells stimulated using PMA but treated with DPI to inhibit ROS production (negative control).

**Objective 2: Evaluation of immune correlates of protection against PRRSV in *M. tb* WCL adjuvanted PLGA nanoparticle-based PRRSV inactivated vaccine immunized and virus challenged pigs.**

**Entrapment of inactivated PRRSV VR2332 lysate and *M. tb* WCL in PLGA nanoparticles:** The entrapment efficiency of crude PRRSV killed-antigens (K-Ag) in PLGA nanoparticles was approximately 55-60% of the amount of protein used. Likewise, entrapment efficiency was approximately 50-55% for *M. tb* WCL. Using the scanning electron microscopy, size of PRRSV Ags/WCL entrapped nanoparticles was determined and found to be ranging from 200 to 600 nm in diameter.

**PRRSV specific antibody response in the lungs of pigs:** BAL fluid samples collected on the day of necropsy [post-challenge day (PC) 15] was used to quantify PRRSV specific IgG antibodies produced in the lungs of immunized PRRSV MN184 challenged pigs. The virus specific antibody response against total viral protein (PRRSV K-Ag), an envelope glycoprotein protein (GP5), matrix and nucleocapsid proteins was determined.
In the 100 μg/pig vaccine dose category there was no significant difference among the treatment groups for IgG level against total viral proteins and GP5 was observed. However, against matrix protein, pigs belongs to group 6 (NP-PRRSV K-Ag+ M. tb WCL and virus challenged) showed significantly higher IgG antibody production compared to mock-challenged pigs (group 2) (Fig. 2.3A). In the 500 μg/pig vaccine dose group there was a significant increase in the matrix protein specific IgG response in pigs belong to group 6 compared to group 3 (K-Ag + Chal), group 4 (K-Ag + M. tb WCL), and group 5 (NP-K-Ag + NP- M. tb WCL) (Fig. 2.3B). While PRRSV specific IgA antibody response in the lungs of pigs against total viral protein was significantly higher in the lungs of pigs belonging to group 6 which received either 100 or 500 μg/pig vaccine dose compared to pig groups 2, 4, and 5 (Fig. 2.4A&B).

**PRRSV specific antibody response in blood:** In pigs received 100 μg/pig vaccine dose, total viral protein specific IgG response in the blood of group 6 pigs was consistently and significantly higher than mock-challenged pigs from PC 0 to 15. On PC 6 and 10, the virus specific IgG levels in group 6 pigs were significantly higher than groups 2, 3, and 5 (Fig. 2.5A). In the 500 μg/pig dose category significantly higher levels of IgG was detected to total viral proteins in pigs belong to group 6 compared to group 2 (mock-challenged pigs) from PC 0 to 15. In addition, specific IgG response in pigs belong to group 6 was significantly higher compared to group 3 on PC 0 and 6, group 4 on PC 6 and 10, and group 5 on PC 0, 10, and 15. Further, IgG levels in pigs of group 3 were higher than in mock-challenged pigs at PC 6 (Fig. 2.5B).
Pigs in group that received 6 of 100 µg/pig category showed significantly higher level of GP5 protein specific IgG response compared to all other groups except group 4 at PC 10 (Fig. 2.6A). In contrast, in the 500 µg/pig vaccine dose of group 6 and 3 (PRRSV K-Ag), comparable high levels of GP5 protein specific IgG production in the blood from PC 0 to 15 was detected (Fig. 2.6B). Matrix protein specific IgG response at 100 µg/pig vaccine dose groups was not significant among the pig groups (Fig. 2.7A). While in 500 µg/pig groups, group 6 showed significantly increased production of matrix protein specific IgG compared to in pigs of group 2 (mock-challenged) at PC 6 and 15. At PC 15 IgG levels were significantly increased in pigs of group 6 compared to group 5 (both PRRSV and WCL NP entrapped vaccine received pigs). Also at PC 0, group 5 showed significantly higher levels of matrix protein specific IgG than in groups 3 and 4 (Fig. 2.7B).

PRRSV nucleocapsid protein specific IgG antibody titer in blood of pigs of group 6 which received 100 µg/pig vaccine dose were significantly higher than groups 2 and 3 at all tested PCs (Fig. 2.8A). In addition, group 6 pigs had increased production of IgG levels than group 5 on PCs 0, 10, and 15, and group 4 on PCs 6 and 15. Group 4 also showed increased N protein specific IgG response compared to groups 2 and 3 pigs at PCs 0 and 6 (Fig. 2.8A). In pigs received 500 µg/pig vaccine dose, N protein specific IgG levels in group 6 were significantly higher than all other treatment groups at all the time points except in group 5 at PC 6 (Fig. 2.8B). Pigs of group 5 and group 4 were also produced significantly increased N protein specific IgG compared to pigs belong to groups 2 and 3 at PC 0 and 15, respectively (Fig. 2.8B).
**PRRSV specific neutralizing antibody titers in the lungs and blood:** PRRSV specific neutralization (VN) titers in the lungs of pigs were measured at PC 15 by indirect immunofluorescence assay. In group 6 pigs the mean VN titer was significantly higher with mean titers of 16 and 27 with 100 and 500 µg/pig vaccine dose, respectively (Fig. 2.9 A&B), compared to other four test groups (with 500 µg/pig dose) and three test groups (with 100 µg/pig dose) (Fig. 2.9 A&B).

In the plasma of group 6 pigs, a significantly higher VN titer compared to other four test groups on the day of booster vaccination, and group 3 on PC 0 and 10 and 15 in 100 µg/pig was observed with either dose of the vaccine (Fig. 2.10 A). The increased VN titer in pigs of group 6 was also remained high at all the other time points compared to group 3 in 500 µg/pig vaccine received animals (Fig. 2.10B). In addition, groups 4 and 5 recorded significantly higher VN titer than group 3 at PC 15 (Fig. 2.10A).

**Replicating infective PRRSV in the lungs and blood:** PRRSV titer is expressed as TCID$_{50}$/gm of lung tissue, we observed replicating infective PRRSV in pig groups 3 to 6 but the viral load was significantly reduced than mock-challenged pigs (group 2) in 100 µg/pig vaccine dose received pigs (Fig. 2.11A). Further, 500 µg/pig dose received group 6 completely cleared the replicating virus from the lungs, and it was significantly reduced compared to groups 2 and 3 (Fig. 2.11B&C).
In the plasma of pigs belong to 100 µg/pig dose category, groups 4, 5 and 6 showed significantly reduced replicating PRRSV titer compared to pig groups 2 and 3 at PC 10 (Fig. 2.12A). In 500 µg/pig dose category, the virus was cleared completely from the circulation from PC 6 to 15 and it was significant compared to groups 2 and 3 at all the tested PCs. Further, PRRSV RNA copy number in the lungs was measured by qRT-PCR (Fig. 2.13 A&B). In the lung homogenates there was a substantial reduction in the viral RNA load in pigs of group 6 (100 µg/pig dose) compared to all other test groups, and it was significantly reduced in groups 4 and 6 in pigs received 500 µg/pig dose compared to mock-challenged pigs (group 2) (Fig. 2.13 A&B).

**Quantification of PRRSV specific IFN-γ secreting cells in the lungs:** The cell-mediated immune response was measured by counting IFN-γ secreting cells (ISCs) frequency in the lung MNCs upon restimulation with total PRRSV protein and individual viral proteins (GP5, nucleocapsid and matrix proteins). Our results have shown higher ISCs in mock challenged pigs (group 2) than other vaccinated pig groups, which received 100 µg/pig vaccine dose (Fig. 2.14A). However, among the vaccinated groups, number of ISCs in group 6 pigs was significantly higher than other vaccinated groups 3, 4, and 5 when lung MNC were restimulated with nucleocapsid or total viral proteins (Fig. 2.14A). While in 500 µg/pig dose category, group 6 recorded significantly higher number of ISCs in lung MNCs than all the other test groups following restimulation with total PRRSV protein. In addition, compared to other vaccinated pig groups (3, 4, and 5) lung MNCs of pigs of group 6 (500 µg/pig dose) restimulated with matrix protein had significantly higher ISCs (Fig. 2.14B).
DISCUSSION

Objective 1: Evaluation of ROS mediated oxidative stress in pigs vaccinated with PRRS-MLV along with M. tb WCL and challenged with homologous or heterologous PRRSV

PRRSV replicates in the lungs of infected pigs and the virus-specific immune responses are suppressed and delayed. PRRSV-induced immune-mediated inflammatory responses result in patches of dark brown consolidation in the lungs. Recently, we evaluated the efficacy of PRRSV-MLV co-administered with an adjuvant M. tb WCL intranasally to pigs (Dwivedi et al., 2011a; Dwivedi et al., 2011b). Our results detected enhanced immune correlates of protection associated with significantly reduced lung pathology and protective immunity to a virulent heterologous PRRSV MN184 challenge (Dwivedi et al., 2011a) and to a homologous PRRSV VR2332 challenge. In contrast, unvaccinated PRRSV-challenged pig lungs had significantly increased gross lung consolidation (Dwivedi et al., 2011a). Pathological changes in the lungs of PRRSV infected pigs are due to necrosis of virus-infected and bystander cells mediated by aberrant host immune responses (Chiou et al., 2000; Jung et al., 2009; Sirinarumitr et al., 1998). In the lungs of PRRSV-infected pigs, viral replication, generation of mutagenic viral quasispecies, and the cytokine storm caused by excess secretion of proinflammatory cytokines, result in ROS-mediated oxidative stress and lung pathology (Lee & Kleiboeker, 2007; Sirinarumitr et al., 1998; Xiao et al., 2010).
To measure the ROS levels in the pig immune cells we used a functional assay. The reduction of NBT to formazan by ROS produced by pig immune cells was specific, as the DPI treatment inhibited the mitogen PMA-induced ROS production (Lambert et al., 2008; Li & Trush, 1998), or PRRSV induced ROS (Fig. 2A). Our results are consistent with the quantitatively increased ROS production detected in the lungs of unvaccinated PRRSV challenged pigs (Chiou et al., 2000). Flow cytometric analysis was used to measure the frequency and function of ROS-producing cells (Fattorossi et al., 1990), based on the principle that ROS mediate quenching of the cell surface fluorescent molecule, here FITC tagged to ConA was used as an indicator to measure the ROS function. Quenching of fluorescent molecule is not always directly proportional to the amount of ROS produced, due to difference in the quantity of ROS produced by each type of phagocytic cell. For example, neutrophils produce 2–3 times more ROS than macrophages (Fang, 2004; Nathan & Shiloh, 2000). In flow cytometric analysis, only the frequency of cells producing the ROS is determined, and it is not possible to quantify the ROS produced by each cell, while in a colorimetric assay total or pooled ROS produced by a given number of cells is measured (Elloumi & Holland, 2007; Vowells et al., 1995). Since BAL cells and PBMCs contain varying proportions of different phagocytic cell types, results from the two assays are not exactly comparable, but they are complementary to each other in drawing a meaningful conclusion about ROS production. Overall, flow cytometric analysis determines a qualitative estimation and colorimetric analysis estimates quantitative production of ROS.
Performing both the assays simultaneously on the same set of pig immune cells helped us to interpret both the quantity of ROS and the frequency of immune cells producing ROS. Restimulation of immune cells using killed PRRSV antigens helped us to determine the PRRSV-mediated recall immune response, which we could quantify ex vivo. At certain DPC there was no association between the quantity of ROS and the frequency of ROS-producing cells, suggesting that at times only a very few cells can secrete large amounts of ROS or vice-versa. Overall, PRRSV-challenged unvaccinated pig immune cells secreted enhanced ROS both in terms of quantity and also by the frequency of cells. Compared to the colorimetric assay, the cytometric analysis is more sensitive, objective, and functional, as it determines the frequency of ROS-producing cells (Elloumi & Holland, 2007; Vowells et al., 1995), and this information is critical to estimate the extent and duration of the tissue damage mediated by ROS.

Results of our previously published studies (Dwivedi et al., 2011a; Dwivedi et al., 2011b), and those of the current report, together indicate that mucosal immunization to PRRS induced adequate protective immunity against PRRSV challenge, which may be mediated by optimal levels of ROS production. Optimal production of ROS by immune cells is essential for antimicrobial activity (Acker, 2005; Chvanov et al., 2005; Martin & Edwards, 1993; Quinn & Gauss, 2004), and excess production of ROS causes necrosis of infected cells, as well as apoptosis of bystander cells and phagocytes due to irreversible membrane lipid degradation and DNA fragmentation (Clutton, 1997). Severe lung pathology observed in unvaccinated, virulent PRRSV-challenged pigs (Dwivedi et al., 2011a) might be due to apoptosis of bystander cells, along with necrosis of virus-infected...
cells, consistent with the results of previous studies (Sirinarumitr et al., 1998; Sur et al., 1998). The enhanced lung pathology observed in unvaccinated but virulent PRRSV-challenged pigs might be due to increased expression of the genes involved in excess ROS production (Xiao et al., 2010). In conclusion, intranasal immunization of PRRS-MLV with a potent mucosal adjuvant, M. tb WCL induces protective immunity, and importantly, limited the PRRSV-induced pulmonary pathology mediated by ROS.

**Objective 2: Evaluation of immune correlates of protection against PRRSV in M. tb WCL adjuvanted PLGA nanoparticle-based PRRSV inactivated vaccine immunized and virus challenged pigs.**

Potent mucosal vaccines against pathogens which predominantly cause disease at mucosal sites have been proven efficacious and confirmed for mucosal delivery of vaccines against influenza, parainfluenza, respiratory syncytial virus, rotavirus, and HIV/SIV (Guillonneau et al., 2009; Imaoka et al., 1998; Kamijuku et al., 2008; Ogra, 1984; Schmidt et al., 2002). The anti-PRRSV immunity induced by currently used PRRSV vaccines (administered by parenteral route) have failed to protect pigs against emerging heterologous PRRSV strains and reinfections (Labarque et al., 2004; Mengeling et al., 2003). PRRS is a primary respiratory tract disease and therefore to effectively control PRRS a protective mucosal vaccine may be warranted. Analyses of immune correlates both in terms of humoral and cell-mediated response together may help to quantify the protective immunity.
Though live PRRSV vaccines are widely in use in the field, they failed to protect pigs against reinfections and against genetically/antigenically variant strains (Botner et al., 1997; Martelli et al., 2009). Also the killed vaccines are limited by their immunogenicity to elicit protective immune response even against a genetically closely related viral strains (Kimman et al., 2009). A major issue associated with live vaccines is the reversion of vaccine virus strain to virulent infectious mutants, leading to transmission of virus to unimmunized herds (Madsen et al., 1998; Nielsen et al., 2002). Since inactivated vaccines are safe, reinforcement of the killed vaccines is needed. In this direction, there have been several studies reported which have examined various approaches to develop a protective killed vaccine against PRRS. Intranasal delivery of a killed PRRSV vaccine along with an adjuvant CpG ODN (TLR-9 ligand) augmented both systemic and mucosal anti-PRRSV specific antibody response, enhanced the antigen specific T cell proliferation and secretion of cytokines (IFN-γ and IL-6) (Zhang et al., 2007). Killed vaccine comprising of UV or BEI inactivated PRRSV with Freund's incomplete adjuvant was able to elicit a virus specific immune response and also partial protection to a homologous viral challenge (Vanhee et al., 2009). There are several advantages associated with development of potent inactivated PRRSV vaccine: (1) inactivation procedures such as UV inactivation or treatment with chemicals such as BEI rescues IFN-α downregulation caused by the PRRSV; (2) virus neutralizing epitopes present on GP5 protein of the inactivated PRRSV are preserved; (3) inactivation processes abrogate the development of regulatory subsets of T cells (Tregs), because induction of Tregs is one of the major immune evasion strategies by PRRSV (Darwich et al., 2010; Kim et al., 2011; Wills et al., 1997).
Particulate bioadhesive delivery systems prolong the mucosal residence time of the vaccine and also selectively target the Ags to mucosal M-cell which in turn delivered to local APCs. This pathway of vaccine delivery is likely to be the most effective means to induce effective mucosal immune response (Clark et al., 2001; Kuolee & Chen, 2008). Poly(esters) are biodegradable synthetic (homo or hetero) polymers, for example PLGA is a copolymer of lactic and glycolic acids [poly (lactideco- glycolide) or PLGA], and it is a US Food and Drug Administration (FDA) and European Medicines Agency (EMEA) approved polymer. PLGA has been widely employed for the preparation of nanoparticles for vaccine and drug delivery (Duncan, 2005; McNeil, 2005). These copolymers undergo hydrolysis of their ester linkages in the presence of water and the time required for the degradation depends on the ratio of monomers used in the preparation. Higher the content of glycolide units results in a lower is the time required for its degradation (Shive & Anderson, 1997). Moreover, lactic acid component of the polymer is responsible for hydrophobicity of the polymer, when the lactide proportion in the polymer is lower (as in 50:50 PLGA) degradability is faster. On the other hand, when the proportion is higher (as in 85:15 PLGA) degradability is longer (Jain, 2000; Jalil & Nixon, 1990). Thus, PLGA degrades in the body producing its original monomers of lactic and glycolic acids which are the byproducts of various metabolic pathways (Lu et al., 2009). PLGA nanoparticles are designed to be taken up across the epithelium after intranasal administration (Cahill et al., 1995). The immune enhancing effect of antigens encapsulated in PLGA was first demonstrated over two decades ago, wherein it was shown that microparticles mediated delivery of antigens increased both B- and T-cell responses to entrapped vaccine antigens (Eldridge et al., 1991; Nixon et al., 1996). In the present study, PLGA particles with a
75:25 ratio of lactic acid and glycolic acid were used. Protein Ags entrapped in PLGA are released slowly in vitro and outside the cells, and the average complete protein release time is 4-7 weeks (Mukherjee et al., 2008; Thomas et al., 2011), which is ideal for sustained activation of mucosal immune cells. While the nanoparticle entrapped vaccine Ags phagocytosed by APCs are readily released inside the cell and are capable of inducing an enhanced cell-mediated immune response (Eldridge et al., 1991; Nixon et al., 1996).

Size and surface characteristics of nanoparticles play important role in opsonization processes and clearance kinetics (Moghimi & Hunter, 2001; Moghimi & Szebeni, 2003). In our study, the range of nanoparticles entrapped either with adjuvant or vaccine was between 200 and 600 nm. Considering the role of size on the in vivo kinetics of nanoparticles, particles of 200 – 600 nm size range are suitable for efficient uptake by M-cells and APCs. This is because earlier reports have shown that larger sized particles (more than 5-10 µm) were able to serve as antigen depots and are efficient in eliciting local immune response, and NPs with ≤ 100 nm are readily pass through the intercellular space of vascular endothelium (Heegaard et al., 2011; Kim et al., 2002; Zho & Neutra, 2002). We did not detect any adverse reactions upon intranasal administration of nanoparticle entrapped product in vaccinated pigs. Another strategy that could have augmented their uptake by APCs is by increasing the surface hydrophilic nature of the particles by coating them with surfactant, Polaxamer 188 (Araujo et al., 1999; Rajapaksa et al., 2010). In addition, sucrose and magnesium hydroxide were added to the emulsion during primary water-in oil phase of nanoparticle preparation. Sucrose prevents
degradation of protein structure to withstand harsh conditions such as sonication and solvent evaporation processes, while magnesium hydroxide neutralizes the acidic environment generated by hydrolysis of PLGA which may destabilize encapsulated protein antigens inside APCs (Gupta et al., 2007; Gupta et al., 2006; Shenderova et al., 1999).

Several studies have shown that mucosal administration of nanoparticle (NP) entrapped vaccines induce better and long-lasting immune response by efficient uptake of antigen by Peyer’s patch microfold cells (M-cells) of nasal associated lymphoid tissues (NALT) and gut associated lymphoid tissues (GALT) (Clark et al., 2001; Illum et al., 2001). Further, among the mucosal routes for vaccine delivery, intranasal delivery of NP vaccine resulted in higher and longer IgG and IgA antibody response compared to rectal, oral, and intramuscular routes of immunization (Manocha et al., 2005). Other physicochemical property associated with strong influence on the mucoadhesiveness and bioavailability of nanoparticles is the surface charge of polymers. Generally, positively charged (cationic) particles (e.g., PLGA) have advantage over their negatively charged (anionic) counterparts, because they are better positioned to interact with negatively charged mucin as well as negatively charged cell membranes of M-cells and are most effectively taken up by macrophages (Jaganathan & Vyas, 2006; Joseph et al., 2006; Josephson et al., 1999).

The NPs alone are better delivery systems than immunopotentiating adjuvants. To further augment adjuvant effects to entrapped vaccine antigens co-administration with an
appropriate immunopotentiator was found advantageous in eliciting robust protective immune responses (Brayden, 2001; Lavelle & O'Hagan, 2006; O'Hagan & Valiante, 2003). Since we have already established the adjuvanticity of \( M. \text{tb} \) WCL to PRRS-MLV (Binjawadagi et al., 2011; Dwivedi et al., 2011a; Dwivedi et al., 2011b), we co-administered NP entrapped/unentrapped vaccine with entrapped/unentrapped \( M. \text{tb} \) WCL to evaluate the differential immune correlates of protection in vaccinated and heterologous PRRSV challenged pigs.

In the current study, we have demonstrated that \( M. \text{tb} \) WCL adjuvanted NP entrapped killed PRRSV vaccine augmented cross-protective immunity against PRRSV challenge. This was characterized by following immune correlates: (1) better clearance of the challenged infective virus both at the lungs and circulation; (2) significantly reduced PRRSV RNA load in the lungs; (3) generation of significantly increased levels of PRRSV neutralizing titers at both mucosal sites and circulation; (4) significantly increased PRRSV specific IgG and IgA antibody response detected to total viral protein and also against individual structural proteins of the virus at both mucosal and systemic sites; and finally (5) increased IFN-\( \gamma \) response (ISCs) in the lungs. All these immune parameters suggested the generation of adequate protective immunity to a killed PRRSV vaccine by our immunization strategy.

It is important to emphasize that all mucosal sites are inter-connected by a common mucosal immune system and administration of protective antigens at one primary site will stimulate proliferation and migration of antigen-specific lymphocytes, providing
both systemic and local mucosal immunity (Bowersock & Martin, 1999; McDermott & Bienenstock, 1979; Mestecky, 1987). Antibodies against PRRSV can be detected as early as 5 days post-infection and by the end of second week all the animals become seroconverted (Diaz et al., 2005; Meier et al., 2003). Generally, following PRRSV infection early antibodies were detected against nucleocapsid protein, and antibodies against M protein and GP5 develop later (Nelson et al., 1994). These early antibodies are shown to be devoid of neutralizing capacity (Yoon et al., 1994) and these could be responsible for antibody dependent enhancement (ADE) of viral replication (Yoon et al., 1996; Yoon et al., 1997). In contrast, we observed significantly higher levels of antibody titers against PRRSV nucleocapsid protein and total viral proteins, but varying levels of response against GP5 and matrix proteins. Importantly, the increased antibody titers were comparably proportional to PRRSV neutralizing titers in nanoparticle-based vaccine received pigs. This suggests that high levels of viral protein specific antibodies observed in pigs received NP- PRRSV K-Ag plus \( M. \ tb \) WCL may not be contributing towards ADE, rather helped in induction of strong humoral and cell-mediated immunity as represented by increased number of IFN-\( \gamma \) response in the lungs, VN titers and better virus clearance both at mucosal sites and in circulation.

Virus-specific neutralizing antibodies play an important role in the clearance of PRRSV viremia (Batista et al., 2004; Bautista & Molitor, 1999; Lopez & Osorio, 2004; Lowe et al., 2005). They block interaction of porcine sialoadhesin (PoSn) receptor on the susceptible cells with GP5 of PRRSV, thus limiting the internalization of the virus (Vanderheijden et al., 2003). Moreover, the mean VN titer in nanoparticle-based
vaccinated pigs was higher than the proposed protective titer of 8 in blood, and was close to sterility titer of 32 at the site of infection (lungs) at 2 weeks post-challenge (Lopez & Osorio, 2004; Osorio et al., 2002). The protective and sterilizing PRRSV neutralizing titers were proposed based on an earlier study involving passive transfer of serum with known virus neutralizing titers into pregnant sows. In vaccinated animals, apart from virus neutralizing antibodies other factors such as components of innate and cell-mediated immune responses also contribute to viral clearance. Therefore, vaccinated pigs with lower VN titers could also resist the replication of PRRSV in pigs. Further investigations are needed to correlate the precise VN titers and viral clearance in vaccinated pigs. In addition to significantly reduced viral load in the blood, we observed correspondingly reduced infective PRRSV and viral RNA copies in the lungs of pigs of respective treatment groups. Normally, PRRSV-specific NA appear around the 3rd or 4th week post-infection (Diaz et al., 2005; Takikawa et al., 1996) and also titer varies significantly with the strain of PRRSV, but NP-PRRSV K-Ag + M. tb WCL vaccinated pigs could induce generation of high levels of PRRSV-specific NA at an early time point and that an increased VN titer was consistently detected from the day of challenge. Improved titers of PRRSV-specific NA combined with proportionally reduced viral load both at the lungs and circulation in the pigs administered with the NP-based adjuvanted vaccine has raised the possibility of future practical application of our study to the pork industry.

Virus specific cell-mediated immune response (CMI) is represented by upregulated Th1 based response that is specialized in clearing virus-infected cells, and the key cytokine
responsible for such effect is IFN-\(\gamma\). The IFN-\(\gamma\) is produced by NK cells, \(\gamma\delta\) T cells, CD4\(^+\) T cells, CD8\(^+\) T cells, and CD4\(^+\)CD8\(^+\) memory/T-helper lymphocytes (Costers et al., 2009). Associated with the reduced lung lesions in mucosally immunized PRRSV challenged pigs, higher frequency of IFN-\(\gamma\) secreting cells (ISCs) in the lungs was detected in pigs immunized with NP- PRRSV K-Ag + \(M. \, tb\) WCL vaccine. Although number of ISCs specific to individual viral proteins (GP5, matrix and nucleocapsid proteins) were less than mock-challenged pigs, but significantly higher ISCs were detected in lung MNCs of NP-PRRSV K-Ag + \(M. \, tb\) WCL vaccinated pigs restimulated with total PRRSV proteins and matrix protein, and it is consistent with an earlier report (Bautista & Molitor, 1997).

Unvaccinated virus challenged pigs showed more number of ISCs compared to any of the test pigs except the group 6 which received NP-PRRSV K-Ag (500 \(\mu g/pig\)) + \(M. \, tb\) WCL. A possible reason could be the non-structural PRRSV proteins (NSPs) present in the killed vaccine would have suppressed the host IFN-\(\gamma\) response. PRRSV NSPs have been found to be suppressors of host type I IFN production (Beura et al., 2010; Chen et al., 2010), and also the presence of low levels of replicating challenged virus failed to elicit the IFN-\(\gamma\) response. In contrast, higher number of ISCs recorded in group 6 appears to be mediated by the additive adjuvanticity of nanoparticle based delivery system and immunopotentiator \(M. \, tb\) WCL. Other major reason for improved CMI response could be that, PLGA entrapped agents were cross-presented to Th1 cells in the context of MHC class I molecules of CD8\(^-\) DCs and macrophages \textit{in vivo}, otherwise predominantly Th2 biased weak humoral immune response is elicited in killed vaccine administered pigs.
(Schliehe et al., 2011). Thus, our results indicate that mucosal immunization of pigs using NP entrapped PRRSV K-Ag adjuvanted with unentrapped \textit{M. tb} WCL boosted both innate and adaptive, mucosal and systemic immune responses critical for anti-PPRSV immunity (Manocha et al., 2005).

Since adjuvants are necessary for enhancement of innate, adaptive, and long-lasting immunological memory (Heegaard et al., 2011), immediate availability of the adjuvant at the mucosal surfaces is critical for rapidly initiating the required protective immunity. Such a response is possible even at mucosal sites due to presence of abundant DCs, macrophages, mast cells, neutrophils and lymphocytes (Brandtzaeg, 2009). Our results supported an earlier report, that initial innate stimulation following pathogen invasion or by a strong adjuvant is likely to induce activation of Th1 and IgG secreting cells which migrate to peripheral environments and contribute to a strong systemic immune response (Dory et al., 2005). In our study, necessity of immediate adjuvant effect was indicated by poor protective immune responses and incomplete virus clearance in pigs co-administered with entrapped PRRSV K-Ag and \textit{M. tb} WCL in nanoparticles.

Previous inactivated vaccines against PRRSV have been associated with limited success in eliciting protective immunity even against homologous challenge virus, and failed completely to protect against heterologous PRRSV strains. Moderate success was reported in a few vaccine trials when adjuvanted killed vaccine was used, which was supported by moderately increased PRRSV NA titers, associated with partial virus clearance (Kim et al., 2011; Scortti et al., 2007; Wills et al., 1997). However, in our
study we observed clearance of replicating virus both in the lungs and in circulation, suggesting the importance of sustained bioavailability of vaccine antigens and the timely need of a potent adjuvant (i.e., immunopotentiating) effect combine to elicit protective immune responses in pigs against PRRSV.

In summary, intranasal vaccination of pigs with PLGA nanoparticle entrapped inactivated PRRSV with *M. tb* WCL has the potential to induce a protective immune response against PRRSV resulting in the effective clearance of the virus both from the site of infection and systemically. Thus, better cross-protection against virulent heterologous PRRSV is possible by effective mucosal immunization with an adjuvanted inactivated nanoparticle-based vaccine. Further, investigations are required with respect to fractional analysis of different components of *M. tb* WCL for their contribution towards the adjuvanticity, and focus on the cloning and expression of major immune potentiating fractions for commercial production and possible human use of this potent mucosal adjuvant. Our future research will be aimed at: (1) mucosal vaccination trials using adjuvanted killed PRRSV vaccine in the real field situation to control PRRS outbreaks in endemic areas; (2) to pursue this study in pregnant sows for experimental evaluation of protection against vertical transfer of PRRSV infection to piglets; and (3) comparison of this vaccination strategy with mucosally/parenterally administered attenuated live PRRSV vaccines.
### TABLES

#### (A)

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<tr>
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<th>BAL-MNC</th>
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<tr>
<td>Vac + VR2332</td>
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<td>NBT+PMA</td>
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<td>0.484±0.03</td>
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Table 2.1. Colorimetric analysis of ROS produced by phagocytes present in BAL-MNC and PBMC of pigs restimulated with killed PRRSV Ags. Each number is an average OD value from three pigs +/- SEM. Higher the OD<sub>650nm</sub> value higher the production of ROS. Asterisks indicate statistically significant difference (P<0.05) between vaccinated and unvaccinated pig groups. Vac or Unvac+ VR2332 or MN184 – pigs vaccinated or unvaccinated with PRRSV-MLV+ M. tb WCL and challenged using PRRSV VR2332 or MN184.
Table 2.2: Flow cytometric analysis of the frequency of ROS producing cells present in BAL-MNC and PBMC of pigs restimulated with killed PRRSV (VR2332 or MN184) Ags. Each number is an average frequency of fluorescent positive cells in percentages of three pigs +/- SEM. Higher the value lesser the production of ROS. Asterisks indicate statistically significant difference (P<0.05) between vaccinated and unvaccinated pig groups. Vac or Unvac+ VR2332 or MN184 – pigs unvaccinated or vaccinated with PRRSV-MLV+ M. tb WCL and challenged using PRRSV VR2332 or MN184.

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<tr>
<td>Vac + VR2332</td>
<td>91.9±1.9</td>
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<td>ConA-FITC+PMA+NBT 93.1±2.7</td>
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<td>ConA-FITC+PMA+NBT+DPI 95.47±1.3</td>
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<td>Vac+ MN184</td>
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<td>96.1±1.3</td>
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<tr>
<td>Unvac+MN184</td>
<td>80.7±8.2</td>
<td>81.0±4.6</td>
<td>88.2±3.2</td>
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<td>89.5±1.2</td>
<td>86.5±0.6</td>
<td>ConA-FITC+PMA+NBT 91.7±4.1</td>
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<tr>
<td>Unvac+VR2332</td>
<td>88.5±3.6</td>
<td>92.7±6.0</td>
<td>80.3±3.8</td>
<td>ConA-FITC+PMA+NBT+DPI 92.4±1.6</td>
</tr>
<tr>
<td>Vac+ MN184</td>
<td>86.8±2.4</td>
<td>93.0±1.2</td>
<td>90.6±0.3*</td>
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<tr>
<td>Unvac+MN184</td>
<td>87.5±2.0</td>
<td>89.2±0.7</td>
<td>81.2±1.1*</td>
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Figure 2.1. Enhanced ROS production in unvaccinated PRRSV challenged pigs detected by a colorimetric assay. Groups of three pigs were unvaccinated or vaccinated (PRRS-MLV + M. tb WCL) intranasally, and challenged using either PRRSV VR2332 (A and B), or MN184 (C and D). Pigs were euthanized on the indicated days post-challenge (DPC). Bronchoalveolar lavage fluid (BAL) cells (A and C), and peripheral blood mononuclear cells (PBMCs) (B and D), were restimulated using crude killed PRRSV Ags and then treated with NBT. Each bar represents the difference in OD value obtained by subtracting the mean OD (n = 3) of vaccinated from unvaccinated and PRRSV challenged pigs at the respective DPC. OD values greater than zero indicate excess ROS production in unvaccinated compared to vaccinated pigs. Control bar represents the difference in the OD of mock pig immune cells obtained by subtracting the OD of PMA+ DPI from PMA-treated cells, signifying that stimulation of cells by PMA and inhibition by DPI, respectively, are ROS-specific. Asterisks indicate statistically significant differences between vaccinated and unvaccinated pigs.
Figure 2.1
Figure 2.2: Quantification of the frequency of PRRSV-induced ROS-producing cells by flow cytometry. Standardization of an assay to detect ROS-producing pig immune cell population: (A)(i) ROS production by mock pig BAL cells. Cells were either labeled or unlabeled with ConA-FITC and treated as indicated. A reduction in the frequency of fluorescence-positive cells in DPI untreated cells indicates ROS-mediated activity. (A)(ii) PRRSV-induced ROS production by pig BAL cells. Cells from pigs inoculated with PRRS-MLV + M. tb WCL and challenged using PRRSV MN184 were restimulated using killed PRRSV Ags, labeled with ConA-FITC, and treated with or without NBT. The cell surface immunofluorescence was analyzed by flow cytometry. A reduced frequency of NBT-treated cells indicates quenching of FITC by PRRSV-induced ROS. (B–E) Analysis of vaccine-mediated ROS production. Groups of pigs (n = 3) were unvaccinated or vaccinated (PRRS-MLV + M. tb WCL) intranasally, and challenged using either PRRSV VR2332 (B and C), or MN184 (D and E), and the pigs were euthanized at the indicated days post-challenge (DPC). BAL cells (B and D) and PBMCs (C and E) were restimulated using killed PRRSV Ags, and then labeled with ConA-FITC. The cells were subjected to flow cytometry, and the frequency of fluorescence-positive cells was recorded. Each bar represents the difference in frequency values obtained by subtracting the mean frequency of unvaccinated from that of the vaccinated pigs at the indicated DPC. A frequency greater than zero indicates more ROS production in unvaccinated compared to vaccinated pigs. The control bar represents the difference in the frequency of mock pig immune cells obtained by subtracting PMA- from PMA+ DPI- treated cells, signifying that stimulation of cells by PMA and inhibition by DPI, respectively, are ROS-specific. Asterisks indicate statistically significant differences between the vaccinated and unvaccinated pig groups.
Figure 3: A representative graph showing a positive control Con A-FITC labeled cells treated with NBT and PMA (150ng/ml) showed significant reduction in number of fluorescent cells compared to DPI treated cells, indicating inhibited ROS production by DPI and in turn increased number of fluorescent cells (A). Reduction in number of fluorescent cells when Con A-FITC labeled and stimulated with killed PRRSV antigen and NBT treated compared to killed PRRSV antigen stimulated but NBT untreated labeled cells, indicating increased ROS production (B).

Figure 2.2
Figure 2.3: Estimation of PRRSV specific IgG antibody production in the lungs. BAL fluid samples collected from pigs of all the indicated groups were subjected to estimation of IgG production against PRRSV envelope protein GP5, matrix protein, and total PRRSV viral protein by ELISA. Each bar indicates the mean ± SEM at OD$_{405}$ nm value of three pigs of the indicated group. Lowercase letters indicate statistically significant (P ≤ 0.05) differences between two indicated groups of pigs as described in materials and methods.

A → 100 µg/pig vaccine dose category, B → 500 µg/pig vaccine dose category
Figure 2.4: Estimation of PRRSV specific IgA antibody production in the lungs. BAL fluid samples collected from pigs of all the indicated groups were subjected to estimation of IgG production against PRRSV envelope protein GP5, matrix protein, and total PRRSV viral protein by ELISA. Each bar indicates the mean ± SEM at OD405 nm value of three pigs of the indicated group. Lowercase letters indicate statistically significant (P ≤ 0.05) differences between two indicated groups of pigs as described in materials and methods. A → 100 µg/pig vaccine dose category, B → 500 µg/pig vaccine dose category.
Figure 2.5: Estimation of PRRSV specific total IgG antibody titer in blood. Plasma samples collected from pigs on the days of vaccinations and indicated post-PRRSV MN184 challenge day (PC) were 10-fold diluted to determine virus specific IgG titer against total PRRS viral protein by ELISA. Each symbol indicates the mean ± SEM at OD$_{405}$ nm value of three pigs of the indicated group. Lowercase letters indicate statistically significant (P ≤ 0.05) differences between two indicated groups of pigs as described in materials and methods.

A → 100 µg/pig vaccine dose category, B → 500 µg/pig vaccine dose category
Figure 2.6: Estimation of PRRSV GP5 protein specific IgG antibody titer in blood. Plasma samples collected from pigs on the days of vaccination and indicated post-PRRSV MN184 challenge day (PC) were 10-fold diluted to determine viral surface protein GP5 specific IgG titer by ELISA. Each symbol indicates the mean ± SEM at \( \text{OD}_{405} \) nm value of three pigs of the indicated group. Lowercase letters indicate statistically significant (\( P \leq 0.05 \)) differences between two groups of pigs as described in materials and methods.

A \( \rightarrow \) 100 \( \mu \)g/pig vaccine dose category, B \( \rightarrow \) 500 \( \mu \)g/pig vaccine dose category.
Figure 2.7: Estimation of PRRSV matrix protein specific IgG antibody titer in blood. Plasma samples collected from pigs on the days of vaccination and indicated post-PRRSV MN184 challenge day (PC) were 10-fold diluted to determine matrix protein specific IgG titer by ELISA. Each symbol indicates the mean ± SEM at OD$_{405}$ nm value of three pigs of the indicated group. Lowercase letters indicate statistically significant (P ≤ 0.05) differences between two groups of pigs as described in materials and methods.

A $\rightarrow$ 100 µg/pig vaccine dose category, B $\rightarrow$ 500 µg/pig vaccine dose category
Figure 2.8: Estimation of PRRSV nucleocapsid protein specific IgG antibody titer in blood. Plasma samples collected from pigs on the days of vaccination and indicated post-PRRSV MN184 challenge day (PC) were 10-fold diluted to determine nucleocapsid protein specific IgG titer by ELISA. Each symbol indicates the mean ± SEM at OD\textsubscript{405} nm value of three pigs of the indicated group. Lowercase letters indicate statistically significant (P ≤ 0.05) differences between two indicated groups of pigs as described in materials and methods.

A → 100 µg/pig vaccine dose category, B → 500 µg/pig vaccine dose category
Figure 2.9: Estimation of PRRSV specific neutralization titers in the lungs. Lung homogenates of pigs were subjected to estimate PRRSV neutralization titer (VNT) by indirect immunofluorescence assay. Each bar indicates the mean VNT ± SEM of three pigs of the indicated group. Lowercase letters indicate statistically significant (P ≤ 0.05) differences between two indicated groups of pigs as described in materials and methods. A → 100 µg/pig vaccine dose category, B → 500 µg/pig vaccine dose category.
Figure 2.10: Estimation of PRRSV specific neutralization titers in blood. Plasma samples collected from pigs on the days of vaccination and indicated post-PRRSV MN184 challenge day (PC) were two-fold diluted to estimate PRRS virus neutralization titer (VNT) by indirect immunofluorescence assay. Each symbol indicates the mean VNT ± SEM of three pigs of the indicated group. Lowercase letters indicate statistically significant (P ≤ 0.05) differences between two indicated groups of pigs as described in materials and methods.

A → 100 µg/pig vaccine dose category, B → 500 µg/pig vaccine dose category
Figure 2.11: Determination of infective PRRSV load in the lungs of pigs by indirect immunofluorescence assay. Lung homogenates of pigs were analyzed to determine the virus titer, represented as TCID$_{50}$/gm of lung tissue. Each bar indicates the mean viral titer ± SEM of three pigs of the indicated group. Lowercase letters indicate statistically significant (P ≤ 0.05) differences between two indicated groups of pigs as described in materials and methods.

A $\rightarrow$ 100 µg/pig vaccine dose category, B $\rightarrow$ 500 µg/pig vaccine dose category, C $\rightarrow$

Each picture (C to H) is a representative lung homogenate at indicated dilution from three pigs of the indicated group under 500 µg/pig vaccine dose category.
Figure 2.12: Determination of infective PRRSV load in the blood of pigs by indirect immunofluorescence assay. Plasma samples collected from pigs on the indicated days post-PRRSV MN184 challenge (PC) were analyzed to determine the viral titer, represented as TCID$_{50}$/ml of plasma. Each symbol indicates the mean VNT ± SEM of three pigs of the indicated group. Lowercase letters indicate statistically significant (P ≤ 0.05) differences between two indicated groups of pigs as described in materials and methods.

A → 100 µg/pig vaccine dose category, B → 500 µg/pig vaccine dose category
Figure 2.13: Determination of PRRSV RNA copy number in the lungs of pigs by quantitative realtime-PCR (qRT-PCR). Lung homogenates of pigs were analyzed to quantify the viral RNA copies. Each bar indicates the mean viral RNA copy number ± SEM of three pigs of the indicated group. Lowercase letters indicate statistically significant (P ≤ 0.05) differences between two indicated groups of pigs as described in materials and methods.

A → 100 µg/pig vaccine dose category, B → 500 µg/pig vaccine dose category
Figure 2.14: Estimation of the frequency of IFN-γ secreting cells (ISCs) in the lungs of pigs. Lung MNCs isolated from individual pigs were restimulated with one of the indicated viral proteins (GP5, Nucleocapsid, or Matrix protein) or total PRRSV MN184 protein in an ELISPOT assay and ISCs spots were quantified. Each bar indicates the mean of ISCs spots per million LMNCs ± SEM of three pigs of the indicated group. Lowercase letters indicate statistically significant (P ≤ 0.05) differences between two indicated groups of pigs as described in materials and methods.

A → 100 µg/pig vaccine dose category, B → 500 µg/pig vaccine dose category


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tonsils of experimentally infected pigs depends on the level of CD8\textsuperscript{high} T cells. *Viral Immunol* 16, 395-406.


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