RESEARCH REPORT
SWINE HEALTH

Title: Development of novel mucosal vaccines for the control of PRRSV outbreaks – **NPB #09-213**

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Industry Summary:

Porcine Reproductive and Respiratory Syndrome (PRRS) is an economically important chronic viral disease of pigs. Available PRRS virus (PRRSV) vaccines are not completely protective to control the disease outbreaks. Conventional PRRSV vaccines administered by intramuscular injection reduced the persistence and duration of viral shedding, but failed to eliminate the wild-type homologous virus. Most importantly, the vaccines failed to prevent re-infections and infection caused by heterologous PRRSV. It is known that successful activation of mucosal immune cells by intranasal immunization provides both local mucosal and systemic immunity to effectively control respiratory viral infections. Aim of this project was to evaluate mucosal adjuvanticity of adjuvants, cholera toxin and OK432 (*Streptoccocus pyogenes* product) to boost modified live PRRSV vaccine (MLV-PRRS) administered intranasally to pigs. Other aim is to evaluate PRRSV killed vaccine delivery system by a nanotechnology based approach to generate protective mucosal immunity to PRRS. Our results suggested that cholera toxin B subunit and OK432 upregulated the anti-PRRSV specific immune response to MLV-PRRS to both homologous and heterologous PRRSV challenge. But unlike the adjuvant *Mycobacterium tuberculosis* whole cell lysate (*M. tb* WCL) these two adjuvants failed to suppress the immunosuppressive responses induced by PRRSV. Our research on development of a killed PRRSV vaccine by entrapping PRRSV killed vaccine in nanoparticles prepared using poly DL-lactide-co-glycolide (PLGA) (nanoparticle-killed-PRRSV vaccine) was successful, and we detected internalization and stimulation of alveolar macrophages by the candidate vaccine in vitro. Our in vivo results suggested that intranasally administered nanoparticle-killed-PRRSV vaccine is capable of inducing protective immune responses, associated with absence of clinical PRRS and significantly reduced viremia and the viral load in the challenged pigs. We conclude that protective anti-PRRSV mucosal immunity is critical to control PRRSV outbreaks and that could be achieved when PRRSV vaccines are administered by intranasal route using suitable potent adjuvants and delivery system.

Scientific Abstract:

Currently practiced control and prevention strategies have been inadequate to reduce PRRS induced economic loss to the pork industry. Stimulating the immune system systemically (i.e. via injection) results mainly in systemic protection, but low mucosal immune responses are generated. Adequate stimulation of the mucosal

immune system results in production of both local mucosal and systemic protection, so that infectious agents are blocked from entry into the body. But it is difficult to elicit protective mucosal immunity without the help of appropriate mucosal adjuvants and vaccine delivery system. Since last few years it has been established that anti-viral mucosal immune responses help in the prevention of viral entry to the body and also protect against both homologous and heterologous viral challenges. In this study, we evaluated the adjuvant effects of cholera toxin B subunit and OK432 to potentiate MLV-PRRS induced specific adaptive immunity. Our results indicated that both these candidate adjuvants upregulated the frequency of various anti-PRRSV specific immune cells, Th1 and Th2 cytokines in immunized and both homologous and heterologous PRRSV challenged pigs. But they failed to dampen the immune suppressive mediators, IL-10, TGFβ and Foxp3+ T-regulatory cells. Our attempt to evaluate killed PRRSV vaccine delivery using nanotechnology-based approach has resulted in satisfactory results. In nanoparticle-killed-PRRSV vaccine inoculated (intranasally) PRRSV homologous (strain VR2332) and heterologous (strain MN184) challenged pigs reduction in viremia with reduced viral load in the lungs was detected compared to both the control unvaccinated and killed PRRSV vaccine immunized virus challenged pigs. Clinically, virulent PRRSV MN184 challenge induced the typical PRRS symptoms in unvaccinated and killed-PRRSV vaccine received pigs but not in nanoparticle-killed-PRRSV vaccine inoculated animals. Microscopically, hematoxylin & eosin stained lung sections of both unvaccinated and killed-PRRSV vaccine inoculated MN184 challenged pigs had severe infiltration of inflammatory cells while the nanoparticle-killed-PRRSV vaccine received pigs had substantially reduced infiltration of inflammatory cells. These results were supported by PRRSV specific immune responses at both mucosal and systemic sites, indicated by increased frequency of various immune cell subpopulation and also cytokines, IFN-α (Innate), IL-12 and IFN-γ (Th1) in the lungs and serum, and also upon restimulation of immune cells. In addition, PRRSV specific increased levels of IgA antibodies and virus neutralizing antibody titers were detected in nanoparticle-killed-PRRSV vaccine received compared to control pig groups. In summary, intranasally administered nanoparticle-killed-PRRSV vaccine is capable of inducing protective immunity to PRRSV, and further studies aimed at few critical modifications to this killed vaccine delivery system may help to take up this strategy to control PRRS in the field.

Introduction:

Porcine reproductive and respiratory syndrome remains as a leading cause of economic burden to the US swine producers in spite of 20 years of research efforts to control the disease. PRRS causes an annual economic loss to the U.S. pork industry of $664 million [1]. According to the Animal and Plant Health Inspection Service report of January 2009, 49.8% of unvaccinated pigs are seropositive to PRRSV in the US. This is based on collective data from 94 percent of pork producers in 17 states, suggesting the widespread prevalence of PRRS in the US. PRRSV causes respiratory and reproductive disease [2-4], with major losses from reproductive failure in sows, including stillbirths, mummifications, weak born piglets and high preweaning mortality [5-7]. The immune responses against PRRSV is ineffective in resolving the infection and potentiates the effects of other swine pathogens [8]. Currently used PRRSV vaccines failed to prevent PRRSV re-infections and induce insufficient cross-protective immunity against heterologous PRRSV. Therefore, prevention, management and control of PRRS remained as challenge to swine producers. We strongly believe that PRRS prevention is possible through generation of protective mucosal immunity with the aid of innovative PRRSV mucosal vaccines, and this could also provide long-lasting cross-protective immunity against genetically variant PRRSV strains. Mucosal immunology is increasingly gaining attention as an area of great potential for the development of vaccines and immunotherapy. Activated innate immune response at mucosal sites play a major role in mucosal immunity against enteric and respiratory infections [9]. Intranasal administration of influenza and parainfluenza-3 vaccines with a potent adjuvant generated enhanced cytotoxic T-lymphocyte and central memory immune response to internal conserved viral proteins resulting in cross-protective immunity against a wide range of heterologous influenza and parainfluenza viruses [10-12]. Recently, a live influenza vaccine administered by intranasal spray (FluMist) containing cold-adapted temperature-sensitive attenuated reassortant viral strains is licensed for use in the US [13].

A killed influenza virus vaccine entrapped in nanoparticles containing *E. coli* heat labile toxin as an adjuvant when administered intranasally to mice, rabbits and pigs elicited protective immunity [14]. Immune responses elicited in pigs by intranasal delivery of nanoparticles based influenza vaccine was significantly better than immunization performed by intramuscular administration of the vaccine [14]. Nanoparticles prepared from PLGA [poly (DL-lactide-co-glycolide)] containing hepatitis B, rotavirus, influenza, or parainfluenza viruses delivered to mucosal sites of mice generated protective immunity [12, 14-16]. Biodegradable biocompatible PLGA nanoparticles are free from any toxicity and they are safe to use in animals and humans, and are also U.S Food and Drug Administration approved [17-19]. The use of nanoparticles as vaccine delivery system allows flexibility in the size, charge and surface properties of the formulations for targeted delivery of the vaccine to mucosal immune cells [20]. ***Our hypothesis was that activation of the mucosal immune system in the respiratory tract of pigs by suitable adjuvants to currently used MLV-PRRS; and administration of killed-PRRSV vaccine using nanoparticles delivery system helps in augmenting cross-protective immunity to control PRRS outbreaks.***

Objectives:

(i) To evaluate the efficacy of MLV-PRRSV vaccine administered intranasally to pigs with adjuvants cholera toxin and OK432 to enhance the anti-PRRSV mucosal immunity.
(ii) To assess the efficacy of killed-PRRSV vaccine entrapped in PLGA microspheres administered intranasally to pigs to augment the anti-PRRSV mucosal immunity.
(iii) To perform challenge studies in immunized pigs using both homologous and heterologous strain of PRRSV and record immune correlates of protection and viral load.

Materials and Methods:

***Animal groups and inoculations*:** Specific-pathogen-free, 3 - 4 week old Large White x Duroc crossbred weaned pigs (n=129) were obtained from a swine herd of the OARDC, The Ohio State University. Animal studies were performed in our BSL2 animal isolation facility at the FAHRP, OARDC, The Ohio State University, Wooster, Ohio. Prior to our study pigs were confirmed negative for PRRSV, PRCV, TGEV and porcine circovirus type 2. After 4 – 6 day acclimation period, pigs from each group were inoculated with indicated adjuvant and 2 doses ($2x10^6$ pfu/pig) of MLV-PRRS (Boehringer Ingelheim Vetmedica, Inc., kind gift from Michael Roof, Bio-R&D), or killed PRRSV antigen entrapped in PLGA nanoparticles and challenged with either PRRSV strains homologous (VR2332, $2x10^6$ $TCID_{50}$ per pig in 2 ml) or heterologous (MN184, $0.5x10^6$ $TCID_{50}$ per pig in 2 ml) post-immunization day (DPI) 21 (Table 1). **All inoculations in this study were carried out once by intranasal route.** As adjuvants, *Streptoccocus pyogenes* product OK432 (Chugai pharmaceuticals, Japan) and cholera toxin B subunit (Sigma, MO, USA) were used as adjuvant in this study. As a negative control pigs mock inoculated with cell culture medium and the vehicle used to dissolve the candidate adjuvant were included. To measure the level of adjuvanticity of candidate adjuvants in eliciting anti-PRRSV immunity, pigs were euthanized on indicated DPI or day-post challenge (DPC) (Table 1).

Clinical signs, blood and tissue sampling: Body weights were recorded on day 0 and at weekly intervals until the end of our study. Pigs were observed daily for clinical signs and body temperature was recorded twice a week. The gross pathology in the lungs and lymph nodes were recorded during necropsy and scored as described previously [21]. We collected blood on DPI 0, 4, 7, 14 and 21, and on DPC 0, 4, 7, 14, 21, 28, 35, 42, 48, and 60. The lung homogenates were prepared from all the pigs on the day of necropsy [22] and stored at -20^0C until used in assays. Samples of blood, lung tissue, bronchoalveolar lavage (BAL) fluid, tracheobronchial lymph nodes (TBLN) were collected and isolated mononuclear cells (MNC) as described [23, 24].

Table 1. Experimental design to study mucosal immune response in the respiratory tract of pigs inoculated with a mucosal adjuvant and PRRSV-MLV (RespPRRS®) vaccine or killed PRRSV vaccine antigens. (DPI - Days Post-Inoculation; DPC - Days Post-Challenge)

Experimental groups - administered intranasally (IN)	Euthanasia	Pig #s	Total
Evaluate immune correlates of protection			
A. Mock	30 DPI	3 pigs / group	9
B. RespPRRS®	30 DPI		9
C. RespPRRS® +cholera toxin (10, 100 and 200μg/pig)	30 DPI		9
D. RespPRRS® +OK432 (50, 250 and 500μg/pig)	30 DPI		9
E. Killed PRRSV (VR2332) antigen (100, 500 and 1000 μg/pig with microspheres)	30 DPI		9
Evaluate immune correlates of protection, viral load and clinical parameters	DPC	4 pigs / group	
1. Mock	30, 60 and 90		12
2. RespPRRS® with cholera toxin and challenged on PVD 21 with homologous PRRSV strain (VR2332)	30, 60 and 90		12
3. RespPRRS® with cholera toxin and challenged on PVD 21 with heterologous PRRSV strain (MN184)	30, 60 and 90		12
4. RespPRRS® with OK432 and challenged on PVD 21 with homologous PRRSV strain (VR2332)	30, 60 and 90		12
5. RespPRRS® with OK-432 and challenged on PVD 21 with heterologous PRRSV strain (MN184)	30, 60 and 90		12
6. Killed PRRSV (VR2332) in microspheres and challenged on PVD 21 with homologous PRRSV strain (VR2332)	30, 60 and 90		12
7. Killed PRRSV (VR2332) in microspheres and challenged on PVD 21 with heterologous PRRSV strain (MN184)	30, 60 and 90		12

Experimental procedures:

1. *Analysis of lung pathology:* The gross pathology in the lungs and other organs were recorded during the necropsy and scored as described previously [21].

2. *Phenotypic analysis of immune cells:* To determine the phenotype of immune cells and their activation status, PBMC, lung-MNC (LMNC), BAL cells, TBLN-MNC were immunostained with antibodies specific to pig cell surface markers: NK ($CD3^-CD4^-CD8\ \alpha^+$); γδ T cells ($CD8\alpha^+TcRN4^+$); T-helper cells ($CD3^+CD4^+CD8^-$); cytotoxic T lymphocytes ($CD3^+CD4^-CD8^+$); T-helper/memory cells ($CD3^+CD4^+CD8^+$); T-regulatory cells ($CD4^+CD25^+Foxp3^+$); dendritic cells (DCs) rich fraction ($CD172^+CD11c^+$MHC class II^+); and granulocytes/macrophages/monocytes ($CD172^+$). Subsequently cells were subjected to flow cytometric analyses using BD FACS AriaII (BD Biosciences) flow cytometer. We acquired 50,000 events for each combination of staining and the results were analyzed using FlowJo software (Tree Star, Inc. OR, USA).

3. *Recall cytokine response by immune cells:* We determined the functional response of *in vivo* primed immune cells in pigs which received mock or PRRS-MLV +/- adjuvant followed by mild or virulent PRRSV challenge. PBMC, LMNC, BAL and TBLN-MNC were subjected to *in vitro* restimulation in the absence or presence of killed VR2332 or MN184 antigens (100 μg/ml) for 48 hrs, and the harvested culture supernatants was analyzed for different cytokines.

4. *Analysis of cytokine response:* Serum collected at different DPI and DPC, lung homogenates prepared on the day of necropsy, and *in vitro* restimulated culture supernatant were analyzed for innate (IFNα), pro-inflammatory (IL-6), Th1 (IFN-γ and IL-12), Th2 (IL-4), T-regulatory (IL-10 and TGFβ) cytokines by ELISA as described previously [23, 24].

5. *PRRSV specific neutralizing antibody response:* Serum samples were tested for PRRSV specific neutralizing antibody titers by standard immunofluorescence assay [25, 26].

6. *Anti-PRRSV Isotype antibody assay:* PRRSV specific antibodies present in the lung homogenate and serum were analyzed by ELISA using killed VR2332 antigens coated 96-well plates. Isotype of the anti-PRRSV specific antibody secreted and bound to antigen coated plate was analyzed using anti-pig IgA and IgG secondary antibodies conjugated with HRP by ELISA [27].

7. *Quantification of viral load*: For determination of viral load, serum samples and lung homogenates were analyzed for PRRSV titers using MARC145 cells by standard immunofluorescence assay [25, 26, 28]. Also lung sections were subjected to immunohistochemistry [28, 29].

8. *Pig NK cytotoxic assay:* A non-radioactive colorimetric assay was used to determine the innate NK cell-mediated cytotoxicity in vaccinated PRRSV challenged pigs as described by us previously [30].

Results:

Objective 1: To evaluate the efficacy of MLV-PRRSV vaccine administered intranasally to pigs with adjuvant cholera toxin and OK-432 to enhance the anti-PRRSV mucosal immunity.

In our earlier study (NPB grant #8-187) we have demonstrated that cholera toxin B subunit (100 μg/pig) and OK432 (500 μg/pig) potentiate the anti-PRRSV specific adaptive immunity when co-administered intranasally with MLV-PRRS compared to MLV-PRRS alone. In this project we wanted to determine the mucosal adjuvanticity of these two potent candidate adjuvants in immunized PRRSV challenged pigs. To accomplish this objective pigs were grouped as described (Table 1) and inoculated intranasally with mock (diluent used to dissolve the candidate adjuvant), MLV-PRRS with OK432 (50 and 500 μg/pig) or cholera toxin B subunit (100 and 250 μg/pig) and then challenged on DPI 21 using either

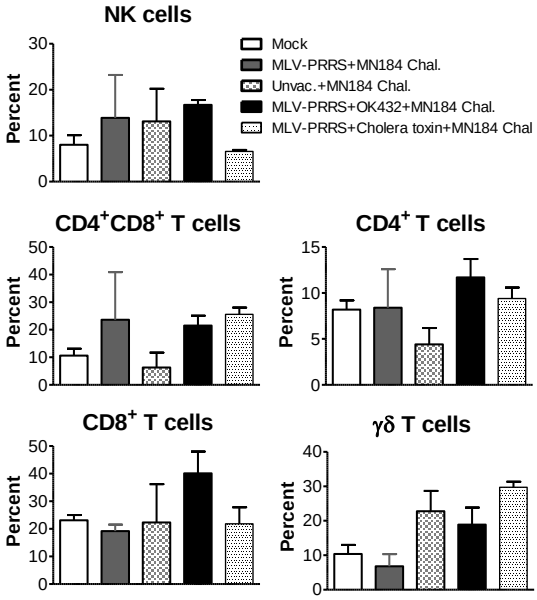


Fig. 1. Analysis of the frequency of immune cells in PBMCs of pigs administered with MLV-PRRS intranasally with candidate adjuvants. Pigs were administered with the combination of MLV-PRRS and indicated adjuvants and challenged with PRRSV strain MN184 on DPI 21 and euthanized on DPC 21. PBMCs were immunostained using fluorophore-conjugated antibodies to identify the frequency of NK cells ($CD3^-CD4^-CD8\alpha^+$), CTLs ($CD3^+CD4^-CD8\alpha^+$), Th/memory cells ($CD3^+CD4^+CD8\alpha^+$), Th cells ($CD3^+CD4^+CD8\alpha^-$), and γδ T cells ($TcR1N4^+CD8\alpha^+$) by flow cytometry. Each bar represents the average cell frequency of 3 pigs +/- SEM. Asterisk indicates statistically significant difference between MLV-PRRS+OK432 and MLV-PRRS inoculated pig groups.

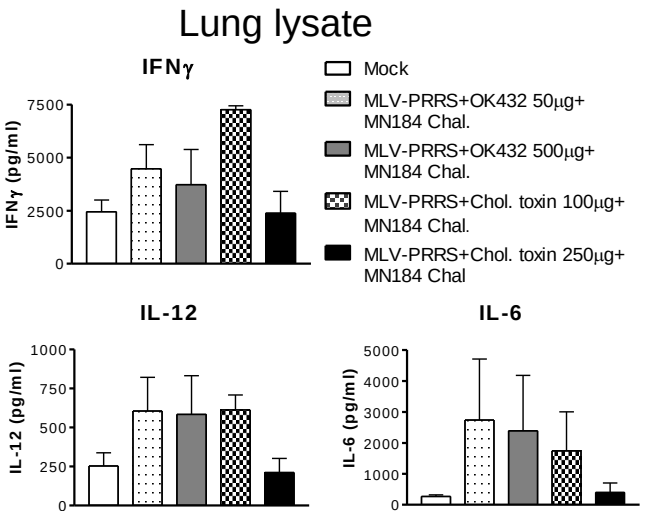


Fig. 2. Analysis of the lung cytokines in pigs administered with MLV-PRRS intranasally with candidate adjuvants. Pigs were administered with the combination of MLV-PRRS and indicated adjuvants at two concentrations and challenged with PRRSV strain MN184 on DPI 21 and euthanized on DPC 21. Lung lysates prepared from all the pigs were analyzed for indicated cytokines by ELISA. Each bar represents the average cytokine concentration of 3 pigs +/- SEM.

homologous (VR2332) or virulent heterologous (MN184) PRRSV strains. Three pigs in each group were euthanized on DPC 30. Clinically, only unimmunized virulent PRRSV MN184 challenged pigs had mild fever with reduced food intake during first two weeks. Analysis of various immune cells in the blood of mucosally immunized PRRSV strain MN184 challenged pigs suggested that the candidate adjuvant OK432 substantially upregulated the frequency of NK cells, $CD4^+$, $CD8^+$ T cells compared to mock and virus challenged pigs which were unvaccinated or MLV-PRRS alone administered (Fig. 1). While the cholera toxin did not upregulate the frequency of analyzed immune cells (Fig. 1). A similar trend in the frequency of immune cells in pigs vaccinated and challenged with PRRSV strain VR2332 was observed (data not shown).

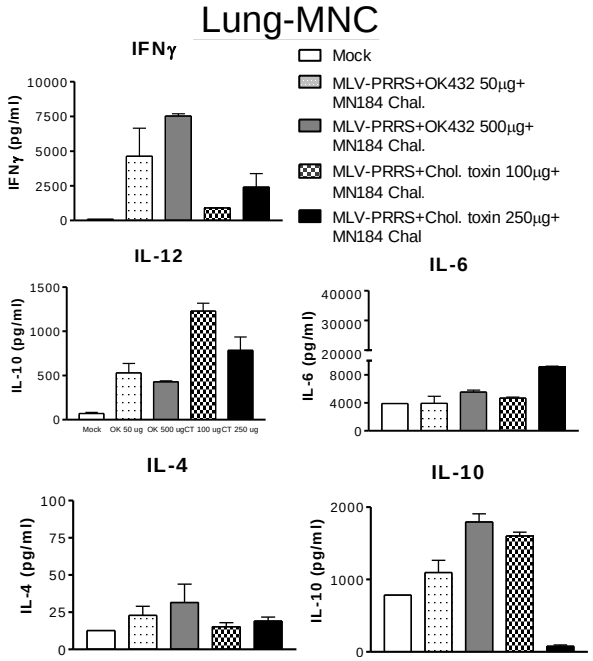


Fig. 3. Analysis of the recall cytokine response by lung-MNC of pigs administered with MLV-PRRS intranasally with candidate adjuvants. Pigs were administered with the combination of MLV-PRRS and indicated adjuvants and challenged with PRRSV strain MN184 on DPI 21 and euthanized on DPC 21. Lung-MNC isolated from all the pigs were restimulated in the presence of killed MN184 antigens and the supernatant harvested from cultures after 48 hr were analyzed for indicated cytokines by ELISA. Each bar represents the average cytokine concentration of 3 pigs +/- SEM.

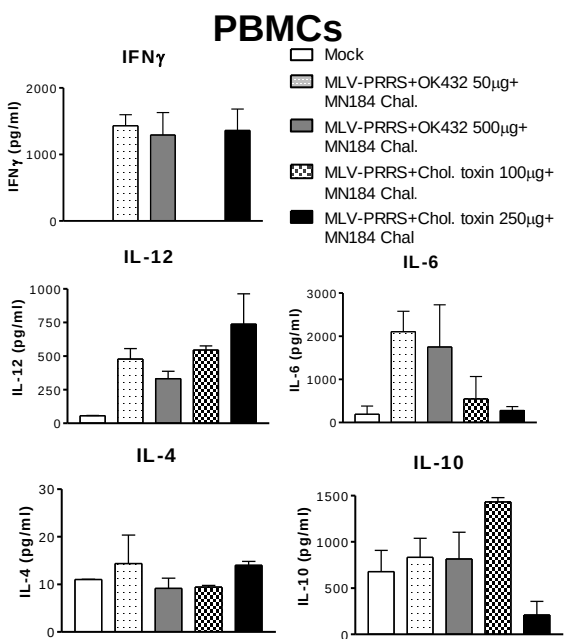


Fig. 4. Analysis of the recall cytokine response by PBMCs of pigs administered with MLV-PRRS intranasally with candidate adjuvants. Pigs were administered with the combination of MLV-PRRS and indicated adjuvants and challenged with PRRSV strain MN184 on DPI 21 and euthanized on DPC 21. PBMCs isolated from all the pigs were restimulated in the presence of killed MN184 antigens and the supernatant harvested from cultures after 48 hr were analyzed for the indicated cytokines by ELISA. Each bar represents the average cytokine concentration of 3 pigs +/- SEM.

With regards to levels of lung cytokines present in the lung homogenates cholera toxin (100 μg/pig) upregulated the secretion of IFNγ and IL-12; and OK432 upregulated the secretion of IFNγ, IL-12 and IL-6 in MLV-PRRS co-administered pigs following challenge using PRRSV MN184 compared to mock pigs (Fig. 2). Interestingly, lower dose of both the adjuvants also induced a satisfactory anti-PRRSV immune response. Analysis of recall cytokine response by lung MNC and PBMCs indicated that Th1 (IFNγ and IL-12) cytokines were secreted at substantially higher levels following MN184 challenge by the adjuvanticity of both the adjuvants to MLV-PRRS (Fig. 3 and 4). Also in pigs immunized with these two adjuvants and then challenged with PRRSV VR2332 strain an increased secretion of the Th1 cytokines, albeit at lower levels was detected (data not shown).

Potentiated PRRSV specific immune response was detected in restimulated PBMCs of pigs isolated from MLV-PRRS co-administered with the adjuvant OK432 and challenged using MN184, indicated by substantially higher levels of IFNγ, IL-12 and IL-6 secretion (Fig. 4). Combination of cholera toxin with MLV-PRRS upregulated the IFNγ secretion in PBMCs of pigs challenged with VR2332 and MN184, while only IL-12 secretion was increased in MN184 challenged animals (Fig. 4 and data not shown). Overall, both the doses of candidate adjuvants induced comparable levels various anti-PRRSV specific immune cells, and Th1 and Th2

cytokines in mucosally immunized and either PRRSV strain challenged pigs compared to control animal immune cells. In addition, the production of immunosuppressive cytokine (IL-10) was also detected at higher levels by the adjuvanticity of these two candidate adjuvants to MLV-PRRS (Fig. 3 and 4).

Objective 2: To assess the efficacy of killed-PRRSV vaccine entrapped PLGA microspheres administered intranasally to pigs to augment anti-PRRSV mucosal immunity.

There are several field reports concerning the reversion of attenuated vaccine strains to virulence, and therefore there is a need of a protective killed PRRSV vaccine to use in pregnant sows and boars to prevent the rapid dissemination of reverted PRRSV to offspring. Our aim was to develop and analyze a nanoparticle-based killed PRRSV vaccine, we prepared PLGA [poly(d, l-lactic-co-glycolic acid)] microspheres (nanoparticles) entrapped killed PRRSV vaccine following a procedure described previously [14, 15]. Briefly, PRRSV (VR2332) was grown in MARC 145 cells and the clarified virus from the infected cell culture supernatant was subjected to ultracentrifugation at 25,000 rpm for 2 hr and the viral pellet was sonicated and entrapped in PLGA nanoparticles. The killed PRRSV antigens encapsulation efficiency in the nanoparticles was approximately 50%. Encapsulated nanoparticles were 100 - 500 nm in size as determined using scanning electron microscopy (Fig. 5i a). Engulfment of nanoparticles by pig alveolar macrophages (Mϕs) (Fig. 5i b and c) was indicated by the presence of PRRSV-nucleocapsid antigens inside the macrophages analyzed by confocal microscopy (Fig. 5i d).

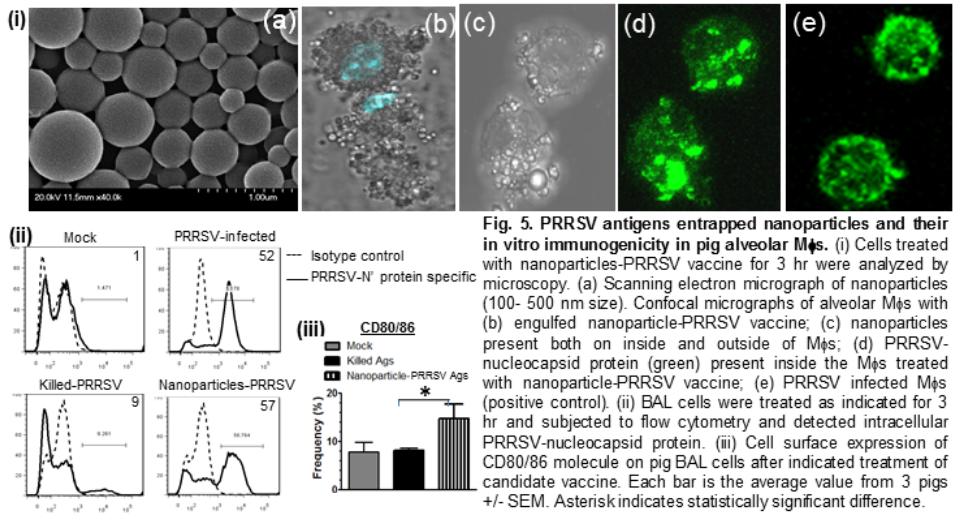


Fig. 5. PRRSV antigens entrapped nanoparticles and their in vitro immunogenicity in pig alveolar Mϕs. (i) Cells treated with nanoparticles-PRRSV vaccine for 3 hr were analyzed by microscopy. (a) Scanning electron micrograph of nanoparticles (100- 500 nm size). Confocal micrographs of alveolar Mϕs with (b) engulfed nanoparticle-PRRSV vaccine; (c) nanoparticles present both on inside and outside of Mϕs; (d) PRRSV-nucleocapsid protein (green) present inside the Mϕs treated with nanoparticle-PRRSV vaccine; (e) PRRSV infected Mϕs (positive control). (ii) BAL cells were treated as indicated for 3 hr and subjected to flow cytometry and detected intracellular PRRSV-nucleocapsid protein. (iii) Cell surface expression of CD80/86 molecule on pig BAL cells after indicated treatment of candidate vaccine. Each bar is the average value from 3 pigs +/- SEM. Asterisk indicates statistically significant difference.

Further, internalized nanoparticle-killed-PRRSV vaccine antigen in the Mϕs (Fig. 5i d) was detected inside endosomal compartments determined by a multi-color confocal microscopy (data not shown). Indicating that nanoparticles entrapped PRRSV antigens were targeted to antigen presenting cells (APCs), and the PRRSV antigens were also translocated to appropriate compartments inside the APCs. This will possibly help in the generation of effective anti-PRRSV specific adaptive immunity when these nanoparticles based inactivated PRRSV vaccine is administered intranasally to pigs.

Quantitative assessment of nanoparticle-killed-PRRSV vaccine in BAL cells in vitro: Different concentration of PRRSV vaccine antigens entrapped in nanoparticles or an equivalent amount of killed antigens were incubated with BAL cells for 3 hrs at 37^0C. PRRSV antigens were detected in significantly higher frequency of BAL cells than control killed antigens treated cells (Fig 5ii). Control BAL cells infected with PRRSV for 9 hr at 37^0 C is shown (Fig. 5ii). Further, there was a significant increase in the expression of an activation marker CD80/86 on the surface of Mϕs treated with nanoparticle-killed-PRRSV vaccine compared to control PRRSV- antigens treated cells (Fig. 5iii).

Objective 3: To perform challenge studies in immunized pigs using both homologous and heterologous strain of PRRSV and record immune correlates of protection and viral load.

We performed in vivo PRRSV challenge studies in pigs unvaccinated, vaccinated using nanoparticle-killed-PRRSV vaccine or killed PRRSV vaccine followed by PRRSV viral challenge using homologous (VR2332) or virulent heterologous (MN184) PRRSV strains.

Heterologous PRRSV MN184 challenge study:

Mucosal immunization with nanoparticle-killed-PRRSV vaccine reduced the lung pathology to a virulent heterologous PRRSV challenge. Pigs were either unimmunized or immunized with nanoparticle-killed-PRRSV vaccine or control killed PRRSV vaccine and then challenged on DPC 21 using a virulent heterologous PRRSV strain MN184. Pigs which were either unvaccinated or received killed vaccine and then challenged using PRRSV strain MN184 had fever with reduced feed intake during first two-week post-challenge, but nanoparticle-killed-PRRSV vaccine received virus challenged pigs were apparently healthy.

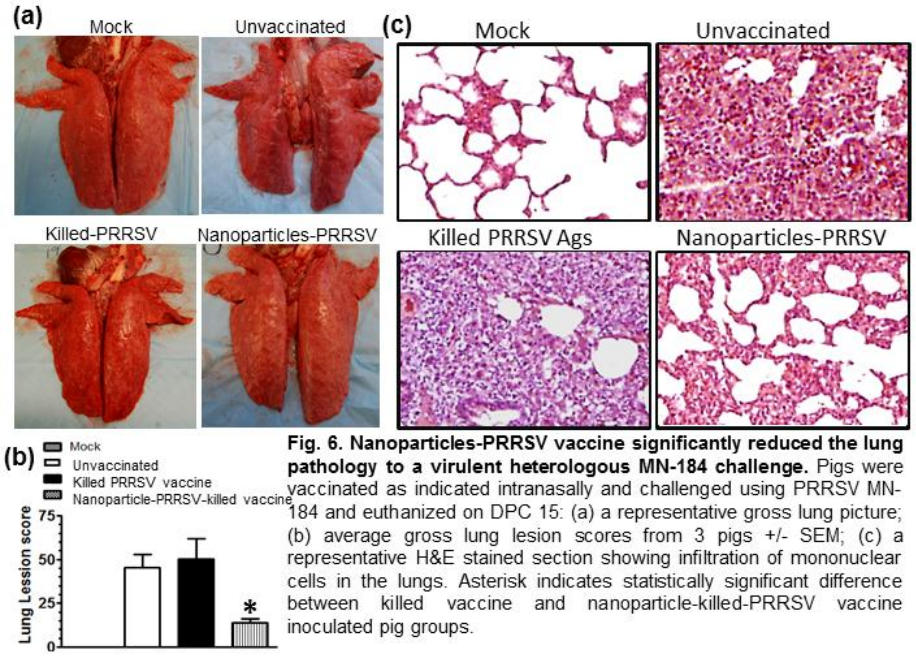


Fig. 6. Nanoparticles-PRRSV vaccine significantly reduced the lung pathology to a virulent heterologous MN-184 challenge. Pigs were vaccinated as indicated intranasally and challenged using PRRSV MN-184 and euthanized on DPC 15: (a) a representative gross lung picture; (b) average gross lung lesion scores from 3 pigs +/- SEM; (c) a representative H&E stained section showing infiltration of mononuclear cells in the lungs. Asterisk indicates statistically significant difference between killed vaccine and nanoparticle-killed-PRRSV vaccine inoculated pig groups.

During necropsy severe gross lung lesions were detected in unvaccinated killed PRRSV vaccine immunized virus challenged pigs, and the gross lung lesion scores were significantly reduced at DPC 15 in nanoparticle-killed-PRRSV vaccine received pigs (Fig. 6 a and b). In addition, microscopically significant reduction in infiltration of lymphocytes was detected (Fig. 6c). Clinical observations were correlated with reduced PRRSV antigen positive cells by immunohistochemistry in the lungs (Fig. 7 a&b) and the lung viral titer at DPC 15 (Fig. 7c). In blood the viral load was reduced at DPC 8 and completely cleared by DPC 15 (Fig. 7d) in nanoparticle-killed-PRRSV vaccine received compared to both the unvaccinated and killed PRRSV vaccine administered pigs.

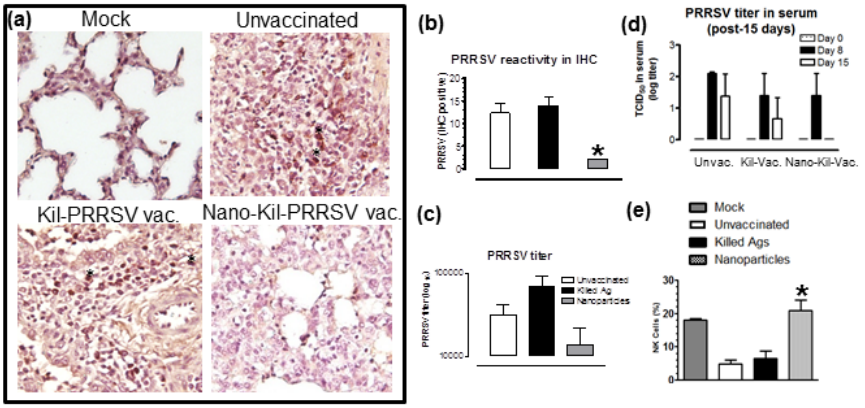


Fig. 7. Decreased PRRSV load and titer in nanoparticle-killed-PRRSV immunized heterologous virus challenged pigs. Pigs were vaccinated intranasally as indicated and challenged using a virulent heterologous PRRSV strain MN184: (a) immunohistochemistry (IHC) picture of a representative pig lung showing PRRSV antigens (asterisk); (b) average number of PRRSV antigen positive cells in IHC stained slides counted from 10 different fields; (c) PRRSV titer in the lungs (log_{10} values); (d) PRRSV titer in the serum (log_{10} values); (e) the frequency of NK cell in the pig lungs was determined by flow cytometry. Each bar is an average value from 3 pigs +/- SEM. Asterisk indicates statistically significant difference between killed vaccine and nanoparticle-killed-PRRSV vaccine inoculated pig groups.

Rescue in NK cell cytotoxic function in microsphere immunized pigs: NK cell population and its cytotoxic function were downregulated in the lungs of unimmunized and killed PRRSV vaccine received MN184 challenged pigs, while their numbers were significantly rescued to physiologically normal levels in nanoparticle-killed-PRRSV vaccine received compared to killed vaccine administered pigs. In addition, the NK cell killing function was also rescued (although not statistically significant) in nanoparticle-killed-PRRSV vaccine received compared to both the unvaccinated and killed PRRSV vaccine administered pigs (Fig. 7e and data not shown).

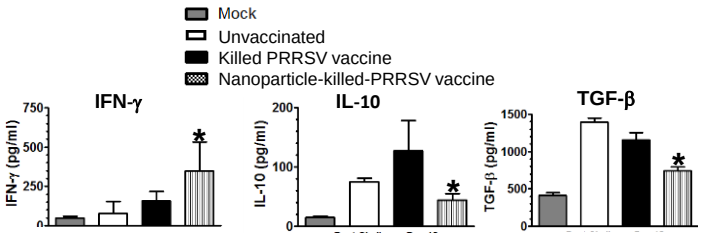
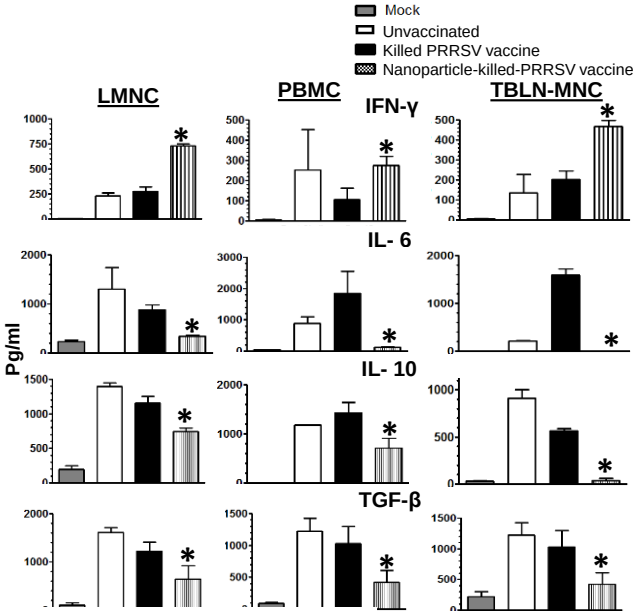
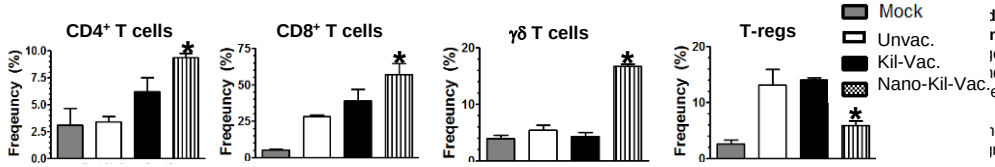


Fig. 8. Secretion of enhanced Th1 and decreased immunosuppressive cytokines in nanoparticle-killed-PRRSV vaccine inoculated pigs. Pigs were vaccinated as indicated intranasally and challenged using a virulent heterologous PRRSV MN184. Lung homogenates prepared from pigs euthanized on DPC 15 were analyzed for indicated cytokines by ELISA. Each bar indicates the average cytokine from 3 pigs +/- SEM. Asterisk indicates statistically significant difference between killed vaccine and nanoparticle-killed-PRRSV vaccine inoculated pig groups.

<u>Increase in Th1 and reduction in immunosuppressive cytokine response in the lungs of nanoparticle-killed-PRRSV vaccine received pigs:</u> In the lungs significantly increased secretion of IFN-γ and significantly lower immunosuppressive cytokines IL-10 and TGF- β were detected in nanoparticle-killed-PRRSV vaccine received compared to killed PRRSV vaccine administered PRRSV challenged pigs (Fig. 8). In the culture supernatant of MNC isolated from the lungs, blood and TBLN restimulated using killed MN184 antigens secreted significantly increased level of amounts of IFN-γ and significantly lower compared to cytokines IL-10, TGF-β and IL-6 in nanoparticle-killed-PRRSV vaccine received killed PRRSV vaccine administered pigs (Fig. 9).



<u>Nanoparticle-killed PRRSV vaccine regulated the lymphoid and immune cell population:</u> In the lungs of nanoparticle-PRRSV vaccine received MN184 challenged significantly increased frequencies of $CD4^+$ T T cells and γδ T cells were detected. In population of important immune regulatory regulatory cells was at significantly lower compared to killed PRRSV vaccine received challenged pigs (Fig. 10).



decreased immunosuppressive ne inoculated pigs. Pigs were ed using a virulent heterologous d TBLN-MNC isolated from pigs ed MN184 antigens and cytokines for indicated cytokines by ELISA. nce between killed vaccine and roups.

Fig. 10. Enhanced frequency of $CD4^+$, $CD8^+$ and γδ T cells and reduced immunosuppressive T-regs in nanoparticle-killed-PRRSV vaccine inoculated pigs. Pigs were vaccinated as indicated intranasally and challenged using a virulent heterologous PRRSV MN184. Lung MNC of pigs euthanized on DPC 15 were immunostained and analyzed by flow cytometry: (a) $CD3^+CD4^+CD8\alpha^-$ cells; (b) $CD3^+CD4^-CD8\alpha^+$ cells; (c) γδ T cells ($TcR1N4^+CD8\alpha^+$); (d) T-regs ($CD4^+CD25^+Foxp3^+$). Each bar indicates the average percent of cytokine from 3 pigs +/- SEM. Asterisk indicates statistically significant difference between Kil-Vac and Nano-Kil-Vac inoculated pig groups. Unvac.: Unvaccinated; Kil-Vac: Killed PRRSV vaccine: Nano-Kil-Vac: Nanoparticles entrapped killed PRRSV vaccine.

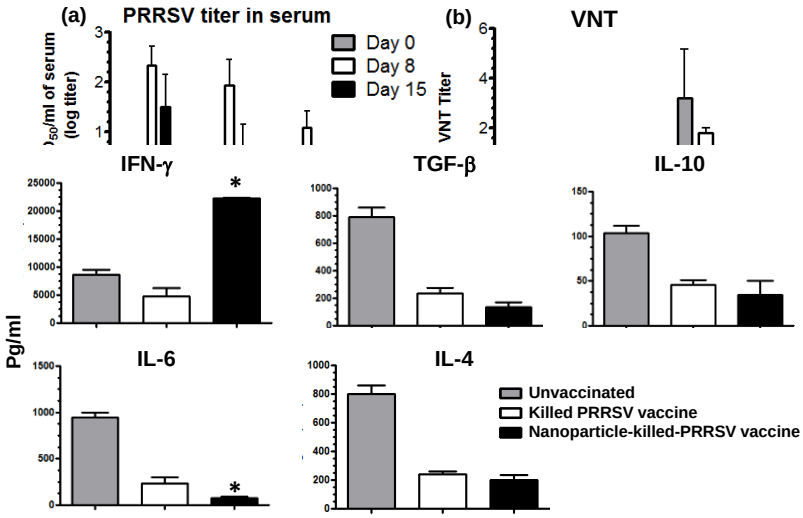


Fig. 12. Increased IFN-γ and reduced immunosuppressive cytokine secretion in the lungs of nanoparticle-killed-PRRSV vaccine inoculated homologous PRRSV challenged pigs. Pigs were vaccinated as indicated intranasally and challenged using a homologous PRRSV strain VR2332 and euthanized at DPC 15. Lung homogenates were analyzed for indicated cytokines by ELISA. Each bar indicates the average cytokine amount from 3 pigs +/- SEM. Asterisk indicates statistically significant difference between killed vaccine and nanoparticle-killed-PRRSV vaccine inoculated pig groups.

Homologous PRRSV VR2332 challenge study:

<u>Mucosal immunization with nanoparticle-killed PRRSV vaccine induced the protective immune response to a homologous viral challenge.</u> Pigs were unimmunized or immunized exactly as described above but challenged using a homologous PRRSV strain VR2332. As expected since VR2332 is a mild PRRSV vaccine strain we did not observe any typical clinical PRRS symptoms in any virus challenged pig groups. However, we detected substantial differences in the viral load and immune responses. The viremia in unvaccinated and killed vaccine

immunized VR2332 challenged pigs was comparable (Fig. 11a), but in nanoparticle-killed PRRSV vaccine immunized pigs substantially reduced viremia was detected at DPC 8 and the viremia was completely cleared by DPC 15 (Fig. 11a). This response was associated with a detectable virus neutralizing antibody titers in nanoparticle-killed-PRRSV vaccine received VR2332 challenged pigs (Fig. 11b). The total amount of PRRSV specific IgA antibodies were significantly higher in the serum at DPC 15 (Fig. 11c), and in contrast specific IgG antibody levels were significantly lower in nanoparticle-killed-PRRSV vaccine received compared to both the unvaccinated and killed PRRSV vaccine administered pigs (Fig. 11d). While in the lungs total secretory IgA antibody levels were comparable in both the vaccinated pig groups (Fig. 11e).

Reciprocal regulation of Th1 and immunosuppressive cytokines in nanoparticle-killed-PRRSV vaccine received pigs: In the lung homogenate significantly higher levels of innate IFN-γ and significantly lower level of immunosuppressive cytokines, IL-10 and TGF-β were detected in nanoparticle-killed-PRRSV vaccine received compared to killed PRRSV vaccine received VR2332 challenged pigs (Fig. 12). But the IL-4 levels were comparable in both the vaccinated pig groups (Fig. 12). The lung MNC (LMNC) isolated from the lungs, blood and TBLN restimulated using killed VR2332 antigens secreted different cytokines at variable amounts. We detected significantly higher level IFN-γ and IL-12 by LMNC, PBMC and TBLN-MNC in nanoparticle-killed-PRRSV vaccine received compared to killed PRRSV vaccine received virus challenged pigs (Fig. 13).

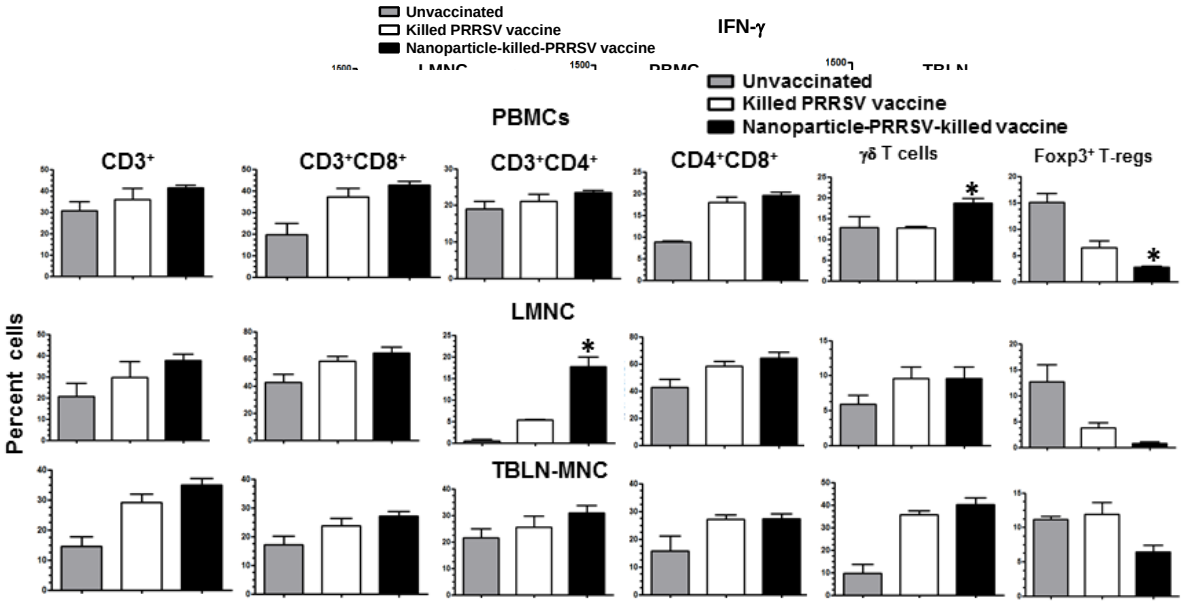


Fig. 14. Marginally increased frequency of T lymphocyte subsets in blood, lungs and TBLN of nanoparticle-killed-PRRSV vaccine inoculated homologous PRRSV challenged pigs. Pigs were vaccinated as indicated intranasally and challenged using a homologous PRRSV strain VR2332 and euthanized at DPC 15. PBMCs, LMNC and TBLN-MNC were immunostained and analyzed for indicated lymphocyte subsets by flow cytometry. Each bar indicates the average percent of cells from 3 pigs +/- SEM. Asterisk indicates statistically significant difference between killed vaccine and nanoparticle-killed-PRRSV vaccine inoculated pig groups.

Nanoparticle-killed-PRRSV vaccine did not modulate a majority of lymphoid cell subset to VR232 challenge: In the lungs, blood and TBLN of nanoparticle-killed-PRRSV vaccine received VR2332 challenged pigs the frequency of $CD3^+$ (total lymphocytes), $CD8^+$ and CD4CD8 double positive T cells were comparable to killed PRRSV vaccine received pigs (Fig. 14). Only the frequency of $CD4^+$ T cells in the LMNC (but not in PBMC and TBLN) and γδ T cells in PBMC (but not in LMNC and TBLN) were significantly higher in nanoparticle-killed-PRRSV vaccine received compared to killed PRRSV vaccine administered VR2332 challenged pigs (Fig. 14). In addition, fewer numbers of Foxp3+ T-regulatory cells were detected in blood, lungs and TBLN of nanoparticle-killed-PRRSV vaccine received compared to killed PRRSV vaccine received pigs, and the reduction in their frequency was even statistically significant in the PBMCs (Fig. 14).

Discussion:

Cholera toxin is a potent mucosal adjuvant when administered orally. The cholera toxin B subunit binds to monosialoganglioside (GM1) molecule and helps in its uptake by mucosal immune cells. Adjuvanticity of cholera toxin in pigs was demonstrated [31], but its mucosal adjuvanticity when administered intranasally is not known. OK432 (Chugai Pharmaceuticals, Japan) is a penicillin G treated, lyophilized, low virulent Su strain of group A *Streptococcus pyogenes* of human origin [32]. Human and porcine NK cell activity is augmented both *in vivo* and *in vitro* by OK432 [32] (Dwivedi and Renukaradhya et al., unpublished). OK432 induces DCs maturation by upregulating the expression of co-stimulatory molecules and promoting the production of inflammatory cytokines (8, 12). Consistent with the previous observations in pigs immunized intranasally with MLV-PRRS co-administered with either cholera toxin B subunit or OK432 and then challenged with PRRSV a boosted virus specific adaptive immune responses were detected. However, unlike the adjuvant *M. tuberculosis* whole cell lysate [23, 24] these two candidate adjuvants failed to dampen PRRSV induced immunosuppressive responses. In fact, the production of IL-10 was higher in pigs received cholera toxin B subunit and OK432 co-administered with MLV-PRRS. We have previously demonstrated that even MLV-PRRS alone administered intranasally upregulate the IL-10 secretion in the lungs and blood of pigs [23, 24]. Thus, these two candidate adjuvants may not be suitable for augmenting PRRSV mucosal immunity to MLV-PRRS.

Nanotechnology has become one of the important research endeavors of the 21st century. Nanoparticles offer the advantage of increasing the potency of the killed vaccines as they protect the vaccine antigens from protease mediated degradation and helps in the pulse-release of the vaccine; in addition, they have adjuvant effects [33]. The inherent ability of APCs to passively phagocytize particulate matter makes the nanoparticles mediated delivery of PRRSV killed vaccine an attractive tool, but it needs detailed investigation. Already there are several studies on application of nanotechnology, for example killed influenza virus vaccine entrapped in nanoparticles containing *E. coli* heat labile toxin administered intranasally to mice, rabbits, and pigs induced protective immunity [14]. Immune response elicited in pigs by intranasal delivery was significantly higher than intramuscular immunization [14]. PLGA nanoparticle based hepatitis B, rotavirus, influenza, and parainfluenza virus vaccines delivered to mucosal sites in mice induced protective immune response [12, 14-16]. Biodegradable and biocompatible PLGA nanoparticles are free from any toxicity in animals and humans, and are approved by U.S Food and Drug Administration [17-19].

We have demonstrated that PLGA nanoparticles containing PRRSV killed vaccine antigens could be prepared, and they are readily phagocytized by alveolar Mϕs, and also they are localized in endosomes. Further, internalized nanoparticle-PRRSV vaccine in Mϕs upregulated the expression of co-stimulatory molecule CD80/86, which is essential for processing and presentation of PRRSV antigens to induce adaptive immunity. Clinically, pigs immunized intranasally using nanoparticle-killed-PRRSV vaccine and challenged using virulent heterologous PRRSV did not suffer from PRRS disease, while the killed vaccine and the unvaccinated challenged pigs had fever and reduced feed intake during first two-week post-viral challenge. This was associated with significant cross-protective immune response indicated by reduced gross and microscopic lung lesions associated with reduced viremia and viral load in the lungs compared to control killed PRRSV vaccine immunized virus challenged pigs.

Reduced clinical findings and lung pathology to a heterologous PRRSV MN184 challenge in nanoparticle-PRRSV vaccine received pigs was supported by immune correlates such as: (i) a significant rescue in the NK cell frequency in the lungs in addition to a partial rescue in the NK-cell mediated cytotoxicity; (ii) potentiated virus targeted adaptive immune response suggested by significantly increased IFN-γ and reduced IL-10 and TGF-β in the lungs; (iii) phenotypic analysis of immune cells demonstrated a significant increase in $CD4^+$, $CD8^+$ T cells and γδ T cells; with a reciprocal reduction in $Foxp3^+$ T-regulatory cells in the lungs. In conclusion,

we have demonstrated an enhanced innate and adaptive immune response capable of providing cross-protective immunity to PRRSV in pigs immunized intranasally with PLGA nanoparticle-killed-PRRSV vaccine.

As expected, although homologous PRRSV strain VR2332 did not cause clinical disease both in nanoparticle-killed-PRRSV vaccine and control challenged pigs; the viremia and virus neutralizing antibody titers and cell-mediated immune responses suggested a protective immune response to a homologous viral challenge by nanotechnology based PRRSV vaccine. These findings were supported by enhanced virus specific adaptive immune response indicated by significantly increased IFN-γ and IL-12 in the lungs, blood and TBLN; associated with reduced IL-10 and TGF-β in the lungs. In addition, a substantial reduction in the frequency of immunosuppressive T-regulatory cells was detected in nanoparticle-killed-PRRSV vaccine received VR2332 challenged pigs.

In summary, our study has demonstrated that PLGA nanoparticle mediated delivery of killed PRRSV vaccine has a great promise in reducing PRRSV load to both homologous and heterologous PRRSV challenge. Further, critical modifications to this candidate vaccine such as boosting the vaccine with an additional dose, including a potent mucosal adjuvant and including a parenteral dose together with an intranasal dose may help in inducing increased breadth of cross-protective immunity. Thus, some more investigation on the nanoparticle-killed-PRRSV vaccine delivery system may help to introduce this innovative vaccine delivery technology to the field.

References:

[1] Holtkamp D, Kliebenstein J. PRRS Costs Industry $664 Million Annually. Pork Checkoff Study, http://wwwporkorg/News/1265/PRRSCostsIndustry664Millionaspx 2011.

[2] Christopher-Hennings J, Nelson EA, Hines RJ, Nelson JK, Swenson SL, Zimmerman JJ, et al. Persistence of porcine reproductive and respiratory syndrome virus in serum and semen of adult boars. J Vet Diagn Invest 1995 Oct;7(4):456-64.

[3] Collins JE, Benfield DA, Christianson WT, Harris L, Hennings JC, Shaw DP, et al. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J Vet Diagn Invest 1992 Apr;4(2):117-26.

[4] Wensvoort G, Terpstra C, Pol JM, ter Laak EA, Bloemraad M, de Kluyver EP, et al. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet Q 1991 Jul;13(3):121-30.

[5] Mengeling WL, Lager KM, Vorwald AC. Clinical consequences of exposing pregnant gilts to strains of porcine reproductive and respiratory syndrome (PRRS) virus isolated from field cases of "atypical" PRRS. Am J Vet Res 1998 Dec;59(12):1540-4.

[6] Rowland RR. The stealthy nature of PRRSV infection: the dangers posed by that ever-changing mystery swine disease. Vet J 2007 Nov;174(3):451.

[7] Meulenberg JJ. PRRSV, the virus. Vet Res 2000 Jan-Feb;31(1):11-21.

[8] Murtaugh MP, Xiao Z, Zuckermann F. Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. Viral Immunol 2002;15(4):533-47.

[9] Mann JF, Acevedo R, Campo JD, Perez O, Ferro VA. Delivery systems: a vaccine strategy for overcoming mucosal tolerance? Expert Rev Vaccines 2009 Jan;8(1):103-12.

[10] Kamijuku H, Nagata Y, Jiang X, Ichinohe T, Tashiro T, Mori K, et al. Mechanism of NKT cell activation by intranasal coadministration of alpha-galactosylceramide, which can induce cross-protection against influenza viruses. Mucosal Immunol 2008 May;1(3):208-18.

[11] Guillonneau C, Mintern JD, Hubert FX, Hurt AC, Besra GS, Porcelli S, et al. Combined NKT cell activation and influenza virus vaccination boosts memory CTL generation and protective immunity. Proc Natl Acad Sci U S A 2009 Mar 3;106(9):3330-5.

[12] Shephard MJ, Todd D, Adair BM, Po AL, Mackie DP, Scott EM. Immunogenicity of bovine parainfluenza type 3 virus proteins encapsulated in nanoparticle vaccines, following intranasal administration to mice. Res Vet Sci 2003 Apr;74(2):187-90.

[13] Belshe R, Lee MS, Walker RE, Stoddard J, Mendelman PM. Safety, immunogenicity and efficacy of intranasal, live attenuated influenza vaccine. Expert Rev Vaccines 2004 Dec;3(6):643-54.

[14] Singh M, Briones M, O'Hagan DT. A novel bioadhesive intranasal delivery system for inactivated influenza vaccines. J Control Release 2001 Feb 23;70(3):267-76.

[15] Thomas C, Gupta V, Ahsan F. Influence of surface charge of PLGA particles of recombinant hepatitis B surface antigen in enhancing systemic and mucosal immune responses. Int J Pharm 2009 Jun 12.

[16] Nayak B, Panda AK, Ray P, Ray AR. Formulation, characterization and evaluation of rotavirus encapsulated PLA and PLGA particles for oral vaccination. J Microencapsul 2009 Mar;26(2):154-65.

[17] Semete B, Booysen L, Lemmer Y, Kalombo L, Katata L, Verschoor J, et al. In vivo evaluation of the biodistribution and safety of PLGA nanoparticles as drug delivery systems. Nanomedicine 2010 Oct;6(5):662-71.

[18] Duncan R. Nanomedicine gets clinical. Materials Today 2005;8:16-7.

[19] McNeil SE. Nanotechnology for the biologist. J Leukoc Biol 2005 Sep;78(3):585-94.

[20] Lawson LB, Norton EB, Clements JD. Defending the mucosa: adjuvant and carrier formulations for mucosal immunity. Curr Opin Immunol 2011 Jun;23(3):414-20.

[21] Jung K, Alekseev KP, Zhang X, Cheon DS, Vlasova AN, Saif LJ. Altered pathogenesis of porcine respiratory coronavirus in pigs due to immunosuppressive effects of dexamethasone: implications for corticosteroid use in treatment of severe acute respiratory syndrome coronavirus. J Virol 2007 Dec;81(24):13681-93.

[22] Jung K, Renukaradhya GJ, Alekseev KP, Fang Y, Tang Y, Saif LJ. Porcine reproductive and respiratory syndrome virus modifies innate immunity and alters disease outcome in pigs subsequently infected with porcine respiratory coronavirus: implications for respiratory viral co-infections. J Gen Virol 2009 Nov;90(Pt 11):2713-23.
[23] Dwivedi V, Manickam C, Patterson R, Dodson K, Murtaugh M, Torrelles JB, et al. Cross-protective immunity to porcine reproductive and respiratory syndrome virus by intranasal delivery of a live virus vaccine with a potent adjuvant. Vaccine 2011 May 23;29(23):4058-66.
[24] Dwivedi V, Manickam C, Patterson R, Dodson K, Weeman M, Renukaradhya GJ. Intranasal delivery of whole cell lysate of Mycobacterium tuberculosis induces protective immune responses to a modified live porcine reproductive and respiratory syndrome virus vaccine in pigs. Vaccine 2011 May 23;29(23):4067-76.
[25] Benfield DA, Nelson E, Collins JE, Harris L, Goyal SM, Robison D, et al. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J Vet Diagn Invest 1992 Apr;4(2):127-33.
[26] Christopher-Hennings J, Holler LD, Benfield DA, Nelson EA. Detection and duration of porcine reproductive and respiratory syndrome virus in semen, serum, peripheral blood mononuclear cells, and tissues from Yorkshire, Hampshire, and Landrace boars. J Vet Diagn Invest 2001 Mar;13(2):133-42.
[27] Zhang L, Tian X, Zhou F. Intranasal administration of CpG oligonucleotides induces mucosal and systemic Type 1 immune responses and adjuvant activity to porcine reproductive and respiratory syndrome killed virus vaccine in piglets in vivo. Int Immunopharmacol 2007 Dec 15;7(13):1732-40.
[28] Christopher-Hennings J, Nelson EA, Nelson JK, Rossow KD, Shivers JL, Yaeger MJ, et al. Identification of porcine reproductive and respiratory syndrome virus in semen and tissues from vasectomized and nonvasectomized boars. Vet Pathol 1998 Jul;35(4):260-7.
[29] Rowland RR. The interaction between PRRSV and the late gestation pig fetus. Virus Res 2010 Dec;154(1-2):114-22.
[30] Renukaradhya GJ, Alekseev K, Jung K, Fang Y, Saif LJ. Porcine reproductive and respiratory syndrome virus-induced immunosuppression exacerbates the inflammatory response to porcine respiratory coronavirus in pigs. Viral Immunol 2010 Oct;23(5):457-66.
[31] Foss DL, Murtaugh MP. Mucosal immunogenicity and adjuvanticity of cholera toxin in swine. Vaccine 1999 Feb 26;17(7-8):788-801.
[32] Bonavida B, Katz J, Hoshino T. Mechanism of NK activation by OK-432 (Streptococcus pyogenes). I. Spontaneous release of NKCF and augmentation of NKCF production following stimulation with NK target cells. Cell Immunol 1986 Oct 1;102(1):126-35.
[33] Gupta RK, Chang AC, Siber GR. Biodegradable polymer microspheres as vaccine adjuvants and delivery systems. Dev Biol Stand 1998;92:63-78.