RESEARCH REPORT
SWINE HEALTH

Title: Evaluation of adjuvants at the mucosal area for the development of innovative mucosal vaccine against PRRS - **NPB # 08-187**

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Industry Summary:
Porcine Reproductive and Respiratory Syndrome (PRRS) is a chronic and economically important viral disease of pigs. Currently available PRRS virus (PRRSV) vaccines have been administered systemically (intramuscular injection) and they failed to completely protect against reinfections, and absolutely no protection against non-identical PRRS viruses. The aim of this project was to identify potent bacterial based mucosal adjuvants for development of protective anti-PRRSV immunity in pigs. It has been confirmed that effective anti-viral mucosal immunity prevents entry of pathogen in to the body and protects against homologous as well as heterologous infections. But to achieve that goal we need help from suitable adjuvants. Adjuvant is any agent that helps to elicit adequate specific immune responses to vaccine antigens. The PRRSV principally infects lung, therefore we performed all our inoculations by intranasal route (IN) to generate anti-PRRSV specific mucosal immunity. We performed extensive search for suitable adjuvants inoculated IN along with modified live PRRSV vaccine (PRRSV-MLV) (RespPRRS®, Boehringer Ingelheim) in pigs. Out of nine candidate adjuvants that we tested, adjuvanticity of three of them were favorable to generate both anti-PRRSV mucosal (local) and systemic immunity. Further, in our challenge studies pigs were immunized with PRRSV-MLV along with one of the three potent adjuvants (*Mycobacterium tuberculosis* whole cell lysate), and then challenged with homologous or heterologous PRRSV, I/N. Our results detected a significant rescue in the body weight loss, reduced viral load, reduction in lung pathology, reduced fever, supported by favorable anti-PRRSV mucosal and systemic immune responses in mucosally immunized and virulent PRRSV challenged pigs. Thus, we conclude that protective anti-PRRSV mucosal immunity is critical to control PRRSV outbreaks and that could be achieved when conventional PRRSV-MLV administered along with suitable adjuvants, intransally.

Key Words: Mucosal immunity, PRRSV-MLV, adjuvants, intranasal inoculation, innate and adaptive immunity, PRRSV challenge

Scientific Abstract:

PRRS is an economically important chronic endemic viral disease of pigs. Currently practiced control and prevention strategies have been inadequate to reduce economic losses 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. On the other hand, adequate stimulation of the mucosal immune system results in production of both mucosal and systemic protection, so that infectious agents are blocked from entry into the body. But practically, it is difficult to elicit protective mucosal immunity to vaccine antigens due to high alert immune regulatory mechanisms at mucosal surfaces. However, it is possible to overcome that regulatory barrier with the help of potent adjuvants administered along with the vaccine antigens. Based on the immune responses elicited to PRRSV-MLV by the adjuvanticity of nine different bacterial preparations belongs to *Mycobacterium, Streptoccocus,* and *Vibrio* species, three of the preparations: *Mycobacterium tuberculosis* whole cell lysate (*M. tb* WCL); Cholera toxin B subunit; and *Streptoccocus pyogenes* product (Picibanil/OK432) were found to potentiate the PRRSV-MLV (RespPRRS®) specific adaptive immunity. These adjuvants overcame the immune suppression induced by the PRRSV antigens and favored the generation of anti-PRRSV specific adaptive immunity. Subsequently, detailed analysis of on one of the three adjuvants (*M. tb* WCL) with PRRSV-MLV administered IN resulted in upregulated anti-PRRSV specific immune responses. Such as increased PRRSV specific cytotoxic T lymphocytes, NK cells (also rescued its cytotoxicity), and myeloid cells. Also increased the levels of Th1 cytokines (IL-12 and IFNγ), PRRSV specific neutralizing antibody titers, and importantly downregulated the immunosuppressive cytokines (IL-10 and TGFβ) compared to pigs received PRRSV-MLV with no adjuvant. Finally, following virulent heterologous PRRSV challenge in mucosally immunized pigs (PRRSV-MLV with *M. tb* WCL), we found significant rescue in body weight loss, reduced lung inflammation, and significantly less PRRSV load. In addition, favorable anti-PRRSV mucosal and systemic immune responses were detected in mucosally immunized and homologous or heterologous PRRSV challenged pigs. Thus, we conclude that protective anti-PRRSV mucosal immunity is critical to control PRRSV outbreaks, and that could be achieved by intranasal administration of conventional PRRSV-MLV along with a potent adjuvant.

Introduction:

Porcine Reproductive and Respiratory Syndrome (PRRS) is a chronic respiratory disease of pigs of all ages, most severe in young pigs and causes reproductive failure in sows (Benfield, Nelson et al. 1992). A recent study in 17 states of North America representing 94 percent of the U.S. pork producers discovered 50 percent of unvaccinated grower/finisher pigs positive for PRRSV antibodies (APHIS report, January 2009). Overall, the immune responses against PRRSV is ineffective in resolving the infection and potentiates the effects of other swine pathogens (Murtaugh, Xiao et al. 2002). Currently used PRRSV vaccines failed to prevent PRRSV re-infections and also failed to provide cross-protection against non-identical PRRS viruses. Therefore, prevention, management and control of PRRS have been a huge challenge to the swine industry and to PRRSV researchers. We strongly believe that PRRS prevention is possible through generation of protective mucosal immunity with the aid of innovative mucosal vaccines to PRRSV, and this could also provide sustained cross-protection to other strains of the PRRSV. Mucosal immunology is increasingly gaining attention as an area of great potential for the development of vaccines and immunotherapy. Because, mucosa-associated lymphoid tissues contain almost 80% of the total immune cells in the body, and hence induction of mucosal immunity is likely to make an important contribution towards protection against infectious diseases (Singh, Briones et al. 2001). Different vaccine antigens need unique help from adjuvants to elicit protective immune responses, and hence it is important to understand the adjuvanticity of panel of candidate adjuvants to specific vaccine antigens. There are no proven mucosal adjuvants to use in the respiratory mucosal sites of pigs, and they are essential to overcome the immune suppression induced to vaccines at mucosal sites. Especially mediated through immunosuppressive cytokines (IL-10 and TGFβ) and T regulatory cells (Mann, Acevedo et al. 2009). *Our hypothesis was that activation of the mucosal immune system in the respiratory tract of pigs by suitable adjuvants overcomes the initial immunosuppression induced by PRRSV antigens. In addition, it also augments the protective mucosal immunity to PRRSV vaccine, and thus could generate protection against PRRSV outbreaks.*

Objectives:

- **i)** Evaluation of mucosal immune response in the respiratory tract of pigs using innate immune cells specific agents as adjuvants administered by intranasal (IN) route
- **ii)** Administration of RespPRRS® vaccine along with potent adjuvant/s by IN route and evaluate the anti-PRRSV immune response
- **iii)** Perform challenge studies in RespPRRS® with adjuvant vaccinated (IN route) pigs using both homologous and heterologous strain of PRRSV

Materials and Methods:

***Animal groups and inoculations*:** Specific-pathogen-free, 3 - 4 week old Large White x Duroc crossbred weaned pigs (n=154) were obtained from a swine herd of the OARDC, The Ohio State University. Animal studies were performed in the BSL2 animal isolation facility at the FAHRP, OARDC, The Ohio State University, Wooster, OH. Prior to our study pigs were confirmed negative for PRRSV, PRCV, TGEV and porcine circovirus type 2. **All inoculations in this study were carried out by intranasal route (IN).** After a 4 – 6 day acclimation period, pigs from each group were inoculated with 2 ml of different bacterial preparations of *Mycobacterium, Streptoccocus, or Vibrio* species as candidate adjuvants along with or without 2 ml (2 doses) ($1x10^6$ $TCID_{50}$/ml) of PRRSV-MLV (RespPRRS®) (a kind gift from Michael Roof, Bio-R&D, Boehringer Ingelheim Vetmedica Inc.,) (Table 1). Based on available literature the amount of adjuvant inoculated was depending on the type of adjuvant, and it was from 150 μg to 3 mg per pig. All the mycobacterial preparations were received from Colorado University, TB vaccine testing and research material facility (NIH Core Facility). Drs. Larry Schlesinger and Jordi Torreles, Center for Microbial Interface Biology, The Ohio State University, also provided *M. tb* WCL. Other adjuvants: *Streptoccocus pyogenes* product, Picibanil/OK432 (Chugai pharmaceuticals, Japan) with approved MTA; Pamidronate (Sigma, MO, USA); Cholera toxin B subunit (Sigma, MO, USA); and α-Galactosylceramide (TRC chemicals, Canada) were also included in the study. The negative control group was mock inoculated with cell culture medium and diluent used to dissolve the candidate adjuvant (vehicle). To measure the level of adjuvanticity of candidate adjuvants in eliciting anti-PRRSV immunity, pigs were euthanized on indicated days post-inoculation (DPI) (Table 1). For challenge studies, pigs were immunized intranasally with PRRS-MLV and *M. tb* WCL (one of the potent adjuvant chosen from our initial study) followed by challenge with 2 ml of homologous (VR2332, $2.5x10^6$ $TCID_{50}$/ml) or heterologous PRRSV (MN184, $0.5x10^6$ $TCID_{50}$/ml) on DPI-21.

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 in a week. The gross pathology in lung and other organs were recorded during necropsy and scored as described previously (Jung, Alekseev et al. 2007). We collected blood on DPI 0, 4, 7, 14, 21, 30, and 60, and on days post-challenge (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 (Jung, Renukaradhya et al. 2009) and stored at -20^0C until used in assays. Collected blood, lung tissue, bronchoalveolar lavage (BAL) fluid, tracheobronchial lymph nodes (TBLN) and isolated MNC as previously described (7, 30, 32). Lung-mononuclear cells (lung-MNC or LMNC) were isolated from lungs as previously described (7) with few modifications. Briefly, lung was perfused with heparinized saline, minced and then treated with collagenase type II and DNase I. Subsequently cell pellet was subjected to Percoll density gradient centrifugation and the interface rich in lung-MNC were collected for our study.

Table 1. Experimental design to study the mucosal immune response in the respiratory tract of pigs inoculated with mucosal adjuvant/s and RespPRRS® vaccine, IN

	Groups – inoculations	Euthanized on DPI/DPC	Total pigs
A	A. Mock + Vehicle	3 DPI	3
	B. *M. tb* - Mycolic acid methyl esters (MAME) (300 μg/pig)	3 DPI	3
	C. *M. tb* - Whole cell lysate (WCL) (3 mg/pig)	3 DPI	3
	D. *M. tb* - Phosphatidylinositol mannosides (PIM) 1&2 (150 μg/pig)	3 DPI	3
	E. *M. tb* - PIM 6 (100 μg/pig)	3 DPI	3
	F. *M. tb* - Cell wall fraction (300 μg/pig)	3 DPI	3
	G. *M. tb* - Total lipids (1.5 mg/pig)	3 DPI	3
	H. *M. tb* – Lipoarabinomannon (LAM) (150 μg/pig)	3 DPI	3
B	A. Mock + Vehicle	7 and 15 DPI	6
	B. RespPRRS® + Vehicle	7 and 15 DPI	6
	C. RespPRRS® + *M. tb* WCL (3 mg/pig)	7 and 15 DPI	6
	D. RespPRRS® + *M. tb* α-PIM 1, 2 & 6 (250 μg/pig)	7 and 15 DPI	6
	E. RespPRRS® + *M. tb* LAM (150 μg/pig)	7 and 15 DPI	6
	F. RespPRRS® + α-Galctosylceramide (500 μg/pig)	7 and 15 DPI	6
	G. RespPRRS® + OK432 (500 μg/pig)	7 and 15 DPI	6
	H. RespPRRS® + Pamidronate (30 mg/pig)	7 and 15 DPI	6
	I. RespPRRS® + Choleratoxin B subunit (100 μg/pig)	7 and 15 DPI	6
C	A. Mock + Vehicle	7, 14, 30 and 60	12
	B. RespPRRS® + *M. tb* WCL (1 mg/pig)	7, 14, 30 and 60	12
	C. RespPRRS® + *M. tb* WCL (3 mg/pig)	7, 14, 30 and 60	12
D	A. Mock + Vehicle		4
	B. Mock (3 mg/pig) + challenge with PRRSV strain (VR2332)	14, 30, and 60	9
	C. Mock (3 mg/pig) + challenge PRRSV strain (MN-184)	14, 30, and 60	9
	D. RespPRRS®+ *M. tb* WCL (3 mg/pig) + challenge with homologous PRRSV strain (VR-2332)	14, 30, and 60	9
	E. RespPRRS®+ *M. tb* WCL (3 mg/pig) + challenge with heterologous PRRSV strain (MN-184)	14, 30, and 60	9

(DPI - Days Post-Inoculation; DPC - Days Post-Challenge)

Experimental procedures:

1. ***Analysis of lung pathology:*** The gross pathology in lung and other organs were recorded during the necropsy and scored as described previously (Jung, Alekseev et al. 2007).

2. ***Phenotypic analysis of immune cells:*** To determine the phenotype of immune cells and their activation status, PBMC, lung-MNC, BAL cells, TBLN-MNC were immunostained with antibodies specific to pig cell surface markers: NK ($CD3^-CD4^-CD8^+$); NKT ($CD3^+$PBS57 loaded CD1d tetramer$^+$); γδ T cells ($CD8a^+TcRN4^+$); T-helper cells ($CD3^+CD4^+CD8^-$); cytotoxic T lymphocytes ($CD3^+CD4^-CD8^+$); T-helper/memory phenotype ($CD3^+CD4^+CD8^+$); T-regulatory cells ($CD4^+CD25^+Foxp3^+$); dendritic cells (DCs) rich fraction ($CD172^+CD11c^+$MHC class II$^+$); and granulocytes/macrophages/monocytes ($CD172^+$). Subsequently, the cells were subjected to flow cytometric analyses using our 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 of 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 with respective candidate adjuvant, killed PRRSV homologous (VR2332) or heterologous (MN184) antigens (100 μg/ml), or recombinant PRRSV proteins (N, M3', M5') (gift from Dr. Michael Murtaugh) for 48 h. All the cells cultured without any restimulation were included as control. Culture supernatants harvested was analyzed for different cytokines by ELISA.

   ELISPOT assay: PBMC and LMNC were restimulated with killed VR2332 or MN184 antigens in anti-pig IFNγ capture antibody pre-coated ELISPOT plates. Subsequently, treated with anti-pig IFNγ detection antibody and the IFNγ secreting cells were analyzed after staining with soluble substrate using an ELISPOT reader (Zhang, Alekseev et al. 2008).

4. ***Analysis of cytokine response:*** Serum collected at different DPI and DPC, lung homogenates prepared on the day of necropsy, and *in vitro* restimulated primed immune cells culture supernatants 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 by us previously (Azevedo, Yuan et al. 2006; Jung, Renukaradhya et al. 2009).

5. ***PRRSV specific neutralizing antibody response:*** Serum collected was tested for anti-PRRSV specific neutralizing antibody titers by standard immunofluorescence neutralization assay (Benfield, Nelson et al. 1992; Christopher-Hennings, Holler et al. 2001).

6. **Anti-PRRSV Isotype antibody assay:** PBMC and lung-MNC were restimulated with killed VR2332 antigens coated ELISA plate for 24 hr at 37^0C. 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 (Zhang, Tian et al. 2007).

7. ***Quantification of viral load*:** For determination of viral load, all the serum samples were analyzed for PRRSV titers using MARC145 cells by standard immunofluorescence assay (Benfield, Nelson et al. 1992; Christopher-Hennings, Nelson et al. 1998; Christopher-Hennings, Holler et al. 2001).

8. ***Pig NK cytotoxic assay:*** To determine the innate NK cell-mediated cytotoxicity in PRRSV infected pigs, we standardized a non-radioactive colorimetric assay (Jung, Renukaradhya et al. 2009). Briefly, pig PBMC were used as source of NK cells (effectors) against K-562 (human myeloblastoid cell line) or Yac-1 (mouse T lymphoma cell line) (target cells) maintained in RPMI supplemented with FBS. Effectors were plated at 10-fold dilutions with fixed number of targets to get different E:T ratios. The seeded plate was incubated for 24 hr at 37^0C in a CO_2 incubator, and then supernatant was harvested and measured the amount of lactate dehydrogenase (LDH) present in the supernatant, which is directly proportional to NK specific lysis of target cells. The percent NK specific killing will be calculated using the formula: $[(OD_{E+T} - OD_E - OD_{T\text{-}spon})/OD_{T\text{-}total}-OD_{T\text{-}AM})] \times 100$. Where: E = effectors; T = targets; AM = assay medium; T-total = targets with 0.5% NP40; T-AM = targets with assay medium; T-spon = T-AM minus OD of AM control wells.

Results:

As per Objective 1, our initial goal was to identify suitable mucosal adjuvants to potentiate the anti-PRRSV specific mucosal immunity to PRRSV-MLV from a panel of bacterial based candidate adjuvants (Table 1A&B). Pig NK cell cytotoxic activity in response to all the six *Mycobacterium* based candidate adjuvants was assayed

in pig PBMC. Our results indicated that pig NK cell specific cytotoxicity was substantially enhanced in response to five of the candidate adjuvants except to PIM 6. This suggests that pig NK cells could be activated using *Mycobacterium* based candidate adjuvants *in vivo*. In addition, substantially more of pro-inflammatory (IL-6) and Th1 (IL-12 and IFNγ) cytokines were detected in serum in response to LAM, PIMs, and WCL compared to vehicle and other adjuvant inoculated pigs. The knowledge related to cytokine responses of *in vivo* primed immune cells of lung draining TBLN to adjuvants is critical. Our results indicated that TBLN-MNC in response to WCL and upon restimulation with WCL *in vitro* secreted more of IL-4, IL-6, IL-12, and IFNγ compared to vehicle and other candidate adjuvant inoculated pigs. Cell wall fraction (CWE) of *M. tb* also stimulated TBLN-MNC to secrete more of IL-4 and IL-12, but due to secretion of higher amounts of immunosuppressive cytokines (IL-10 and TGF-β) CWE was excluded as a potential candidate mucosal adjuvant. To evaluate the phenotype of different mucosal immune cells in TBLN, lung, and blood of candidate adjuvants received pigs we performed flow cytometric analysis. Increased frequency of NK cells (%) was detected in PBMC and LMNC of pigs inoculated with PIM 1&2 and WCL compared to pigs inoculated with other adjuvants or vehicle. Increased frequency of T-helper cells was also detected in pigs inoculated with WCL in PBMC and lung-MNC compared to pigs inoculated with other adjuvants or vehicle. LAM increased the frequency of NK and T-helper cells in TBLN. One to 2 fold increase in the frequency of swine DCs was detected in response to WCL, LAM and PIM 1&2 in lung, blood and TBLN.

In summary, WCL, PIMs and LAM were identified to possess adequate mucosal adjuvanticity in the pig respiratory tract, and thus could potentially enhance the adaptive arm of the immune system to PRRSV vaccine antigens.

In our Objective 2, based on results from Objective 1 we chose *Mycobacterium* candidate adjuvants WCL, PIMs, and LAM to determine their specific adjuvanticity to PRRSV-MLV. In addition, we also included well known other candidate adjuvants: γδ T cells stimulant, Pamidronate; humoral immune response inducer, Cholera toxin B subunit; a potent NK and DCs stimulator, OK-432; and a strong NKT cell activator, α-Galactosylceramide (Table 1B). To evaluate the adjuvanticity of these candidate adjuvants to PRRSV-MLV, we analyzed the Th1, Th2, pro-inflammatory, and immunosuppressive cytokines in serum and also their secretion by mucosal and systemic immune cells. Normally, PRRSV infection enhances the immunosuppressive cytokines (IL-10 and TGFβ) in both lung and blood of infected pigs resulting in delayed and dampened protective Th1 immune responses. Even following IN inoculation of PRRSV-MLV alone, we detected a trend in the upregulation of IL-10 and TGFβ (Fig. 1A, B&C), along with reduced Th1 cytokines secretion (Fig. 1E&F). This suggests that inhibition of IL-10 and TGFβ secretion in pigs to PRRSV-MLV is critical to enhance the protective anti-PRRSV adaptive immune responses.

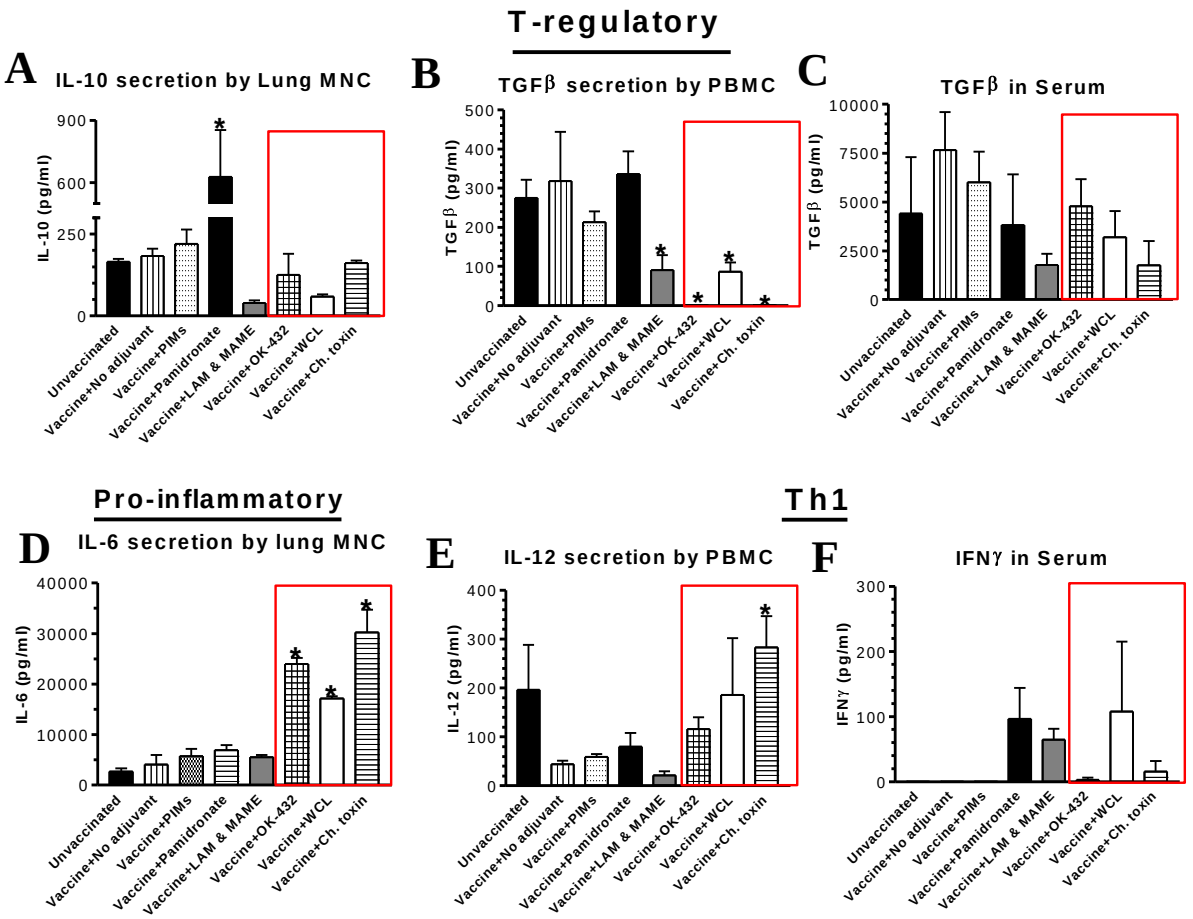


Fig. 1. Cytokine responses in pigs to PRRSV-MLV administered intransally (IN) with various candidate mucosal adjuvants. Pigs were inoculated IN (3 pigs/group) with PRRSV-MLV with or without indicated candidate adjuvants and the pigs were euthanized on post-immunization day (PID) 7. Concentrations of cytokines in serum and secreted by lung-MNC and PBMC upon *in vitro* restimulation (recall memory response) for 48 h with killed PRRSV vaccine strain (VR2332) antigens was analyzed by ELISA. Each bar represents the average cytokine concentration of 3 pigs +/- SEM. Symbol '*' indicates statistically significant difference ($P<0.05$) between pig groups administered with PRRSV-MLV with vs without indicated adjuvant analyzed by nonparametric Kruskal-Wallis test.

Due to adjuvanticity of candidate adjuvants LAM+MAME, OK-432, *M. tb* WCL, and Cholera toxin, reduced levels of immunosuppressive cytokine (TGF-β) was detected in *in vitro* restimulated PBMC and LMNC to PRRS-MLV in pigs (Fig. 1A&B), and also in serum (Fig. 1C). Increased levels of Th1 cytokine, IL-12 was secreted by lung-MNC and PBMC of pigs vaccinated with OK-432, *M. tb* WCL, and Cholera toxin, and also higher concentration of IFNγ was detected in serum of pigs which received pamidronate, LAM+MAME and WCL along with PRRS-MLV compared to control and other candidate adjuvant inoculated pigs (Fig. 1E). In serum, a higher level of IFNγ was detected when *M. tb* WCL was used as adjuvant (Fig. 1F). An increased level of pro-inflammatory cytokine, IL-6 was secreted by LMNC of pigs vaccinated with OK-432, WCL, or Cholera toxin compared to control and other candidate adjuvant inoculated pigs (Fig. 1D). However, candidate adjuvants pamidronate and PIMs were responsible for higher amounts of immunosuppressive and lower amounts of Th1 and pro-inflammatory cytokines in lung and blood (Fig. 1); therefore, we considered them as poor adjuvants to elicit protective anti-PRRSV immune responses in pigs.

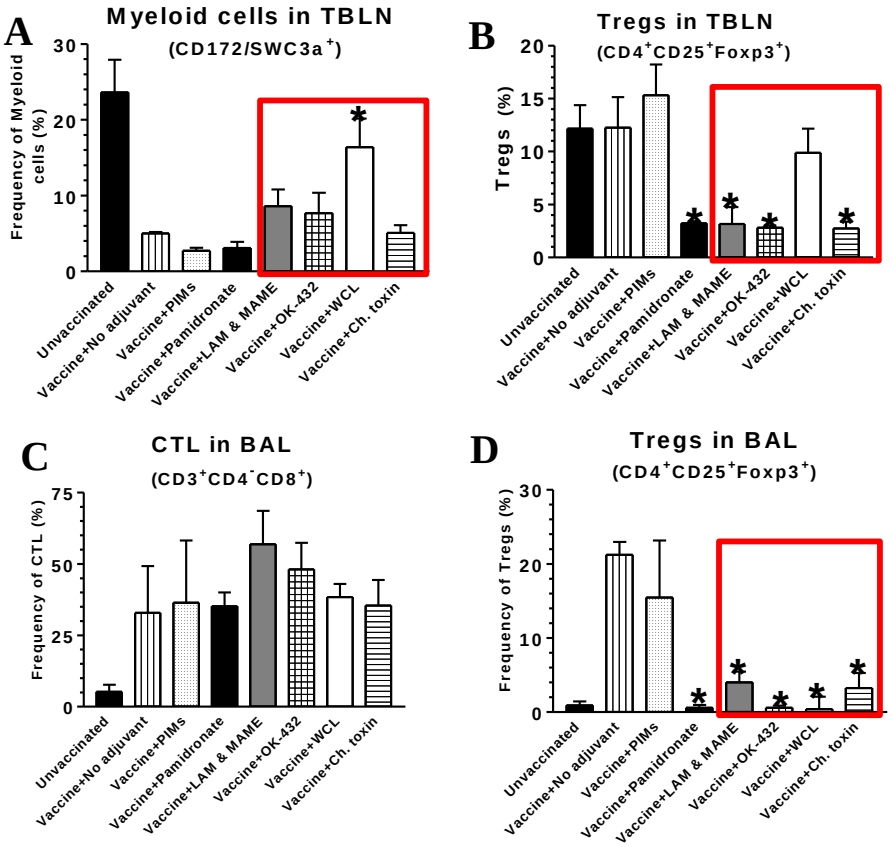


Fig. 2. Analysis of frequency of mucosal immune cells in pigs administered with PRRSV-MLV IN with candidate adjuvants. Pigs were inoculated IN (3 pigs/group) with PRRSV-MLV with or without indicated candidate adjuvants and the pigs were euthanized on days-post immunization (DPI)-7. TBLN-MNC and BAL cells were immunostained using fluorophore-conjugated antibodies to identify the frequency of myeloid (SWC3a/CD172a$^+$), CTL (CD3$^+$CD4$^-$CD8α^+), or T-regulatory (CD4$^+$CD25$^+$Foxp3$^+$) cells by flow cytometry. Each bar represents the average cell frequency of 3 pigs +/- SEM. Symbol '*' indicates statistically significant difference ($P<0.05$) between pig groups administered with PRRSV-MLV with vs without indicated adjuvant analyzed by nonparametric Kruskal-Wallis test.

The phenotype of different lymphoid (T-helper, CTL, T-regulatory and NK cells) and myeloid cells present in MNC harvested from TBLN, lung, BAL fluid and blood was analyzed by flow cytometry. The lung draining TBLN is the important lymphoid organ where specific adaptive immune responses are initiated, which is mediated by interaction of antigen presenting cells (APC) with naïve lymphocytes. Pigs received only PRRSV-MLV had significantly lower frequency of myeloid cells and their frequency was rescued to near normal levels in pigs received PRRSV-MLV with adjuvants (Fig. 2A). A significant rescue in total myeloid cells, in particular, swine DCs rich population in TBLN-MNC of pigs which received PRRSV-MLV along with *M. tb* WCL (Fig. 2A) was detected. The anti-PRRSV specific CTL responses are critical and we detected relatively higher frequencies of CTL in BAL cells of pigs received PRRSV-MLV with LAM+MAME and OK-432 compared to control and other candidate adjuvant inoculated pigs (Fig. 2C). The T-regulatory cells (CD4$^+$CD25$^+$Foxp3$^+$) (Tregs) are known immunosuppressive lymphocyte subpopulation, as they secrete IL-10 and also mediate other regulatory functions responsible for dampening the host adaptive immune responses (Kaser, Gerner et al. 2008). A significant reduction in porcine Tregs in TBLN-MNC and BAL cells of pigs which received PRRSV-MLV with pamidronate, LAM+MAME, OK-432, WCL, and Cholera toxin compared to unvaccinated control and other candidate adjuvant inoculated pigs (Fig. 2 B&D) was detected. Low levels of immunosuppressive cytokines were detected in corresponding pig groups which had lesser frequencies of Tregs (Fig. 1&2). We also measured the levels of PRRSV specific neutralizing antibody responses in serum of pigs' vaccinated IN with PRRSV-MLV +/- adjuvant at different time points. Only mucosal vaccination with adjuvant (*M. tb* WCL) induced higher levels of anti-PRRSV specific neutralizing antibody titers (Fig. 3).

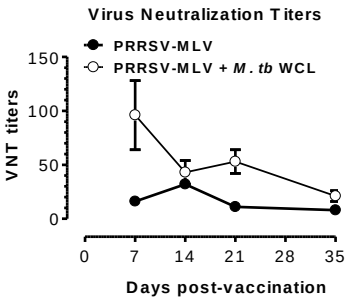


Fig. 3. Only PRRSV-MLV with adjuvant received pigs elicited enhanced anti-PRRSV specific neutralizing antibodies. Serum collected from PRRSV-MLV +/- *M. tb* WCL, IN (6 pigs/group) inoculated pigs at different indicated DPI were analyzed for PRRSV specific neutralizing antibody titers by a standard virus neutralization assay.

NK cell is the important innate immune player in clearance of virus infected cells from the body. About 5-10% of pig lymphocytes are NK cells and upon activation they perform important functions like, innate cytotoxicity, secrete Th1 cytokine (e.g. IFNγ) and activates DCs. A greater than 60% drop in NK cell mediated cytotoxicity in PRRSV (SD23983) infected pigs as early as one week post-infection, and subsequently reduced to more than 90% by two weeks, and then remained low up to four weeks post-infection (Fig. 4A). Even NK cell cytotoxic function of PRRSV-MLV inoculated pigs in absence of adjuvant was reduced by approximately 40 and 90% by DPI-7 and -60, respectively (Fig. 4C and data not shown).

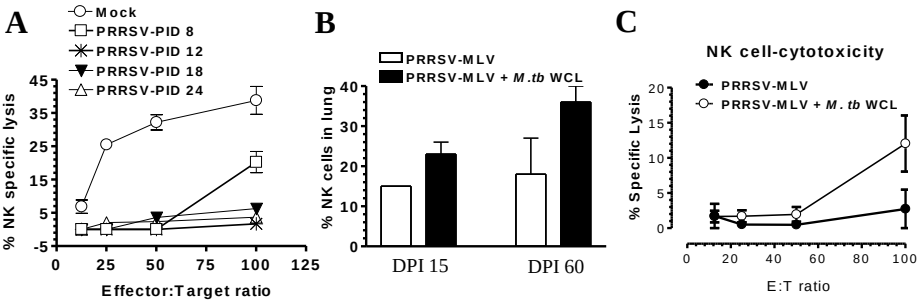


Fig. 4. PRRSV inhibits NK cell-mediated cytotoxicity and PRRSV-MLV inoculated only with the adjuvant rescued both NK cells frequency and their cytotoxic function. (A) PBMC of mock or PRRSV (SD23983) infected pigs on indicated post-infection days (PID) (effectors) were cultured with K-562 (targets) for 24 hr and the lactate dehydrogenase (LDH) released in to the supernatant was measured using the LDH substrate at OD490nm. Pigs were immunized with PRRSV-MLV +/- *M. tb* WCL, (B) the frequency of lung NK cells were analyzed by flow cytometry and (C) PBMC isolated from mucosally immunized pigs +/- *M. tb* WCL at DPI-60 were assayed for NK cell-mediated cytotoxicity as mentioned above.

This confirms the ability of PRRSV including its vaccine strain (PRRS-MLV) mediated suppression of the host innate NK cell-mediated cytotoxic function. However, increased frequencies of NK cells in pigs vaccinated using PRRSV-MLV along with LAM+MAME, WCL, OK-432, and Cholera toxin compared to control and other candidate adjuvant inoculated pigs was detected at DPI-30 (data not shown). In PRRSV-MLV with *M. tb* WCL inoculated pigs rescue in both the frequency and its cytotoxic functions were also detected at DPI-60 (Fig. 4B&C). All these results suggested that PRRSV-MLV when administered alone failed to elicit adequate anti-PRRSV immune responses, but when inoculated with potent adjuvant induced the favorable anti-PRRSV specific immune responses.

Overall, evaluation of adjuvanticity at mucosal surfaces in the pig respiratory tract to PRRSV-MLV resulted in identification of three potential candidate adjuvants, *M. tb* WCL, OK-432, and Cholera toxin B subunit. These adjuvants have the ability to overcome the immunosuppression induced by PRRSV-MLV and promoted specific adaptive immune responses. As indicated by less of immunosuppressive cytokines and Tregs in lung and TBLN. In addition, these three adjuvants favored the production of pro-inflammatory and Th1 cytokines, critical for the generation of protective anti-PRRSV immunity.

A

Body temperature

PRRSV Challenge	Vaccinated		Unvaccinated	
	Mean	SEM	Mean	SEM
VR2332	104.70	0.10	**104.85**	**0.06**
MN184	104.64	0.08	**104.94**	**0.11**

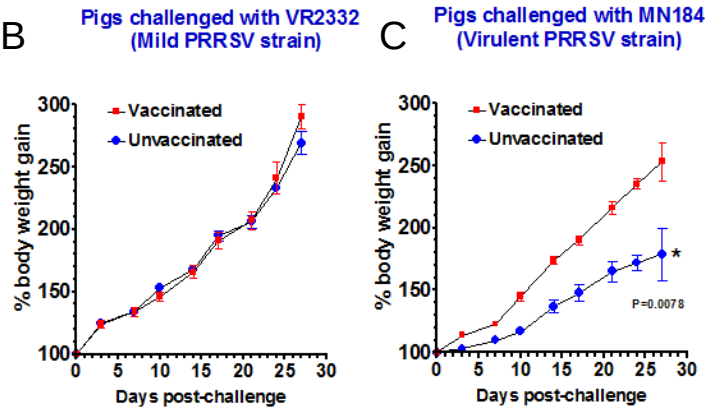


Fig. 5. Mucosal vaccination of pigs significantly rescued the body weight loss to virulent PRRSV challenge and had reduced fever. Pigs were unvaccinated or vaccinated using PRRSV-MLV along with *M. tb* WCL intranasally, and on DPI-21 challenged with a (B) homologous PRRSV (strain VR2332) or (C) heterologous PRRSV (strain MN184). Percent body weight gain was calculated by keeping day 0 post-challenge weight as 100%. Statistically significant rescue in body weight gain in vaccinated and virulent PRRSV challenged pigs compared to control was observed. (A) Mean body temperature of pigs recorded on every third day from DPC-0 to 30 +/- SEM (n=6 - 9 pigs/group). Symbol '*' denote statistically significant difference *(P<0.05)* between unvaccinated vs vaccinated pig groups analyzed by two-tailed unpaired t test.

In our Objective 3,

based on results from Objective 2 we chose one of the three potent adjuvants (*M. tb* WCL) and pigs were inoculated IN with PRRSV-MLV, and then performed challenge studies using PRRSV homologous (VR2332) or heterologous (MN184) strains. All the animals were analyzed clinically, immunologically, and also determined the PRRSV load.

Clinically, mucosal PRRSV-MLV vaccination with M. tb WCL provided heterologous anti-PRRSV immunity and significant protection

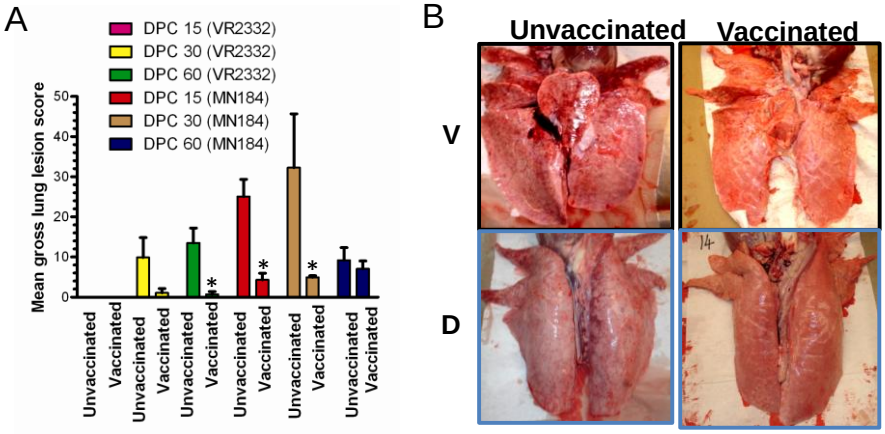


Fig. 6. Mucosal vaccination of pigs significantly reduced the lung pathology to virulent heterologous PRRSV challenge. Pigs were unvaccinated or vaccinated using PRRSV-MLV along with *M. tb* WCL IN and on DPI-21 challenged with a virulent PRRSV (strain MN184). (A) Gross lung lesion scores of both homologous (VR2332) and heterologous (MN184) PRRSV challenged pigs are shown. (B) A representative pig lung at DPC-30 with severe lung consolidation in unvaccinated challenged pig compared to a control is shown. V - ventral view and D - dorsal view. Each bar represents the average lung lesion scores from 3 pigs +/- SEM. Symbol '*' denote statistically significant difference *(P<0.05)* between unvaccinated vs vaccinated pig groups analyzed by two-tailed unpaired t' test.

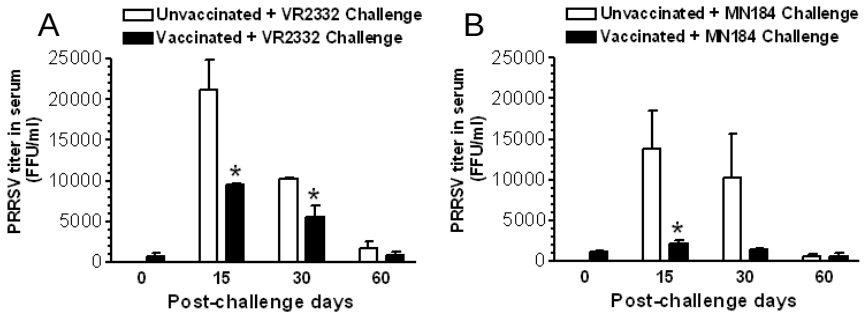
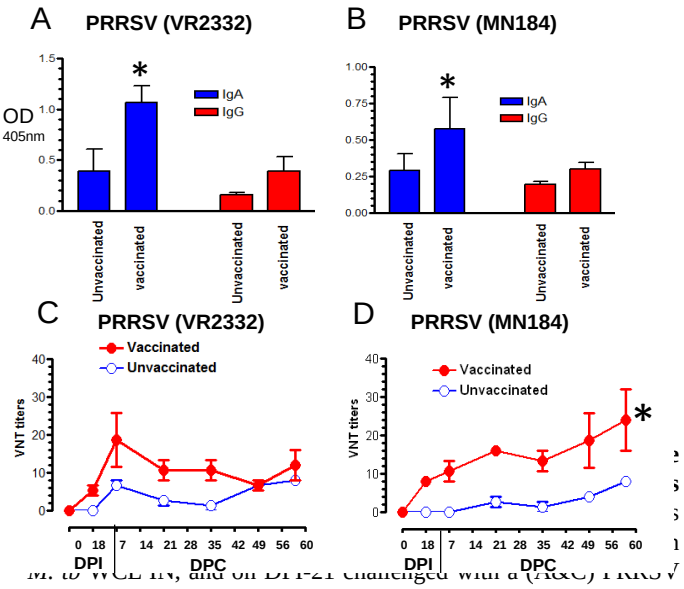


Fig. 7. Mucosal vaccination of pigs significantly decreased the PRRSV load. Pigs were unvaccinated or vaccinated using PRRSV-MLV along with *M. tb* WCL IN, and serum samples collected on indicated DPC were analyzed for PRRSV titers. (A) Pigs challenged with mild PRRSV (VR2332) and (B) pigs challenged with virulent PRRSV (MN184). Each bar represents the average viral titer from 3 pigs +/- SEM. Symbol '*' denote statistically significant difference *(P<0.05)* between unvaccinated vs vaccinated pig groups analyzed by two-tailed unpaired t test.

Clinically, unvaccinated and virulent PRRSV (MN184) challenged pigs had mild cough and reduced food intake during DPC-1 to -15, while mucosally immunized challenged pigs remained healthy with no apparent clinical PRRS disease. In addition, the virulent PRRSV challenged pigs had fever and the mean body temperature of nine pigs until DPC-30 was 0.3^0F higher than vaccinated and challenged pigs (Fig. 5A). Rescue in the body weight loss in mucosally immunized pigs to mild vaccine strain of PRRSV (VR2322) challenge was modest (Fig. 5B). However, a significant rescue in the body weight loss to a virulent heterologous PRRSV challenge was observed (Fig. 5C).

The gross lung examination in homologous mild PRRSV challenged pigs revealed no appreciable macroscopic lesions at DPC-15 (data not shown). But more lung lesions were observed at DPC-30 and -60, and the mean lung lesion scores were even significantly higher in unvaccinated VR2332 challenged pigs compared to vaccinated challenged animals at DPC-60 (Fig. 6A). However, as expected unvaccinated and virulent PRRSV challenged pigs had severe macroscopic lung lesions on both ventral and dorsal surfaces of the lungs compared to mucosally immunized and challenged pigs (Fig. 6B). The lung lesion scores were significantly higher at both DPC-15 and -30 in those virulent PRRSV

challenged pigs (Fig. 6A). Consistent with the lung lesions, in mucosally immunized and mild or virulent PRRSV challenged pigs significantly reduced PRRSV load was detected in serum at DPC-15 and -30 (Fig. 7 A&B).



M. tb WCL IN, and on DPI-21 challenged with a (A&C) PRRSV (VR2332) or (B&D) PRRSV (MN184). (A&B) Total anti-PRRSV isotype specific antibodies secreted by lung cells at DPC-60 upon restimulation with killed VR2332 antigens was analyzed by swine isotype antibody specific ELISA. (B&D) PRRSV specific neutralizing antibodies in serum of pigs collected at different indicated DPI and DPC were analyzed by virus neutralization test (VNT). Each bar or data point represents the average OD or VNT from 3 pigs +/- SEM. Symbol '*' denote statistically significant difference *(P<0.05)* between unvaccinated vs vaccinated pig groups analyzed by nonparametric Kruskal-Wallis test. .

Immunological responses in pigs supported the observed protective anti-PRRSV immunity generated to PRRSV-MLV inoculated with M. tb WCL

(i) **Analysis of anti-PRRSV specific neutralizing antibodies:** Pathogen specific mucosal secretory IgA antibody response is critical to prevent the entry of pathogens into the body. Pigs mucosally vaccinated and challenged with mild or virulent PRRSV had significantly higher concentrations of anti-PRRSV specific secretory IgA antibodies secreted by lung-MNC upon *in vitro* restimulation with killed PRRSV (VR1332) antigens (Fig. 8 A&B). Even substantially higher levels of anti-PRRSV specific IgG antibodies were also secreted by lung-MNC (Fig. 8 A&B). Further, we assessed the PRRSV specific neutralization antibody titeres in the serum of pigs collected at various DPC. Both mild and virulent PRRSV challenged unvaccinated pigs had very low PRRSV specific neutralizing antibody titers (Fig. 8 C&D). A modest increase in PRRSV specific neutralization titers was detected in mucosally immunized mild PRRSV challenged pigs at DPC-7 to 35, however, significantly higher titers were detected in mucosally immunized pigs challenged with virulent PRRSV from DPC-7 to 60, until the end of our study (Fig. 8 C&D).

(ii) Recall memory IFNγ secreting cell response in blood and lung: We performed ELISPOT assay to determine the IFNγ secreting cell response in PBMC and lung-MNC of both vaccinated or unvaccinated and PRRSV challenged pigs. Cells were restimulated *in vitro* using killed PRRSV antigens belongs to vaccine strain (VR2332) or heterologous virulent strain (MN184). Both the killed PRRSV antigens restimulated the *in vivo* primed cells to secrete IFNγ at comparable levels (Fig. 9). The mucosally immunized VR2332 challenged pigs PBMC at DPC-15 had lower frequencies of IFNγ secreting cell spots, and at DPC-30 in both blood and lung higher IFNγ secreting cells were detected (Fig. 9A). Supporting the clinical responses, in mucosally immunized virulent PRRSV challenged pigs, significantly higher levels of recall memory IFNγ secreting cells as early as DPC-15 and substantially higher at DPC-30 compared to unvaccinated challenged pigs was detected (Fig. 9B). Most importantly, in those pigs even lung-MNC had substantially higher frequencies of IFNγ secreting cells compared to control unvaccinated pigs at DPC-30 (Fig. 9B).

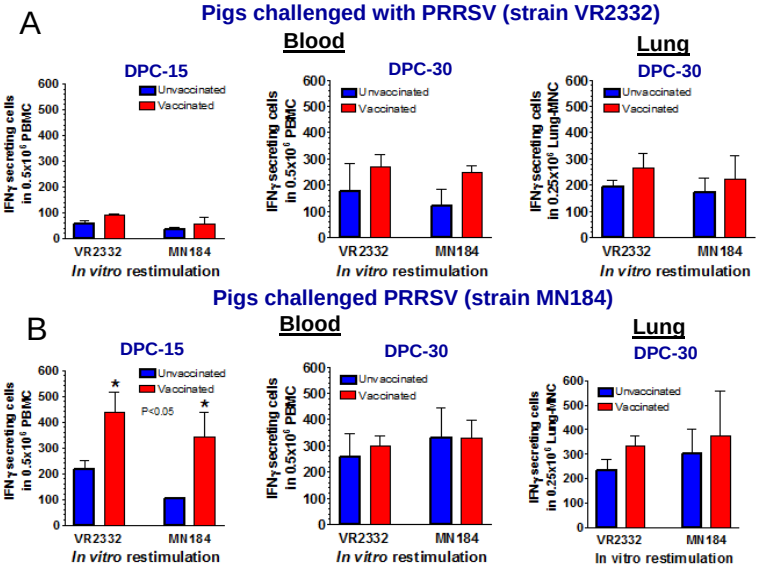


Fig. 9. Mucosally vaccinated pigs had elevated IFNγ secreting cells in blood and lung. Pigs were unvaccinated or vaccinated (PRRSV-MLV + *M. tb* WCL) and then challenged with (A) mild PRRSV (VR2332) or (B) virulent PRRSV (MN184) on DPI-21, and pigs were euthanized on DPC-15 and -30. PBMC and lung-MNC were restimulated with killed VR2332 or MN184 antigens, respectively, and the IFNγ secreting cells were analyzed by ELISPOT. Each bar represents the average number of IFNγ secreting cell spots from 3 pigs +/- SEM. Symbol '*' denote statistically significant difference (*P<0.05)* between unvaccinated vs vaccinated pig groups analyzed by two-tailed unpaired t test.

(iii) **Other cytokine responses in PRRSV challenged pigs:** In mucosally immunized and virulent PRRSV (MN184) challenged pigs recall memory Th1 (IL-12) cytokine response was detected at significantly higher levels following *in vitro* restimulation with PRRSV nucleocapsid protein (Fig. 10A). Immunosuppressive cytokines (IL-10 and TGFβ) in serum at various DPC indicated that mucosal vaccination with *M. tb* WCL significantly reduced the secretion of IL-10 at DPC-14 and 21 compared to unvaccinated challenged pigs and substantially lower at other DPC tested (Fig. 10B). This correlated with higher frequency of IFNγ secreting cells detected at that DPC in the same pig group (Fig. 10B & 9B). Even serum TGFβ levels were also produced substantially low in mucosally vaccinated virulent PRRSV challenged pigs compared to control animals at all DPC (Fig. 10C).

(iv) Lymphocyte subpopulations and myeloid cells in PRRSV challenged pigs: Evaluation of frequency of various immune cells at both mucosal area (BAL, lung-MNC, and TBLN-MNC) and in systemic circulation (PBMC) is critical to determine the innate and adaptive immune responses in pigs mucosally vaccinated and challenged with homologous or heterologous PRRSV. In mucosally

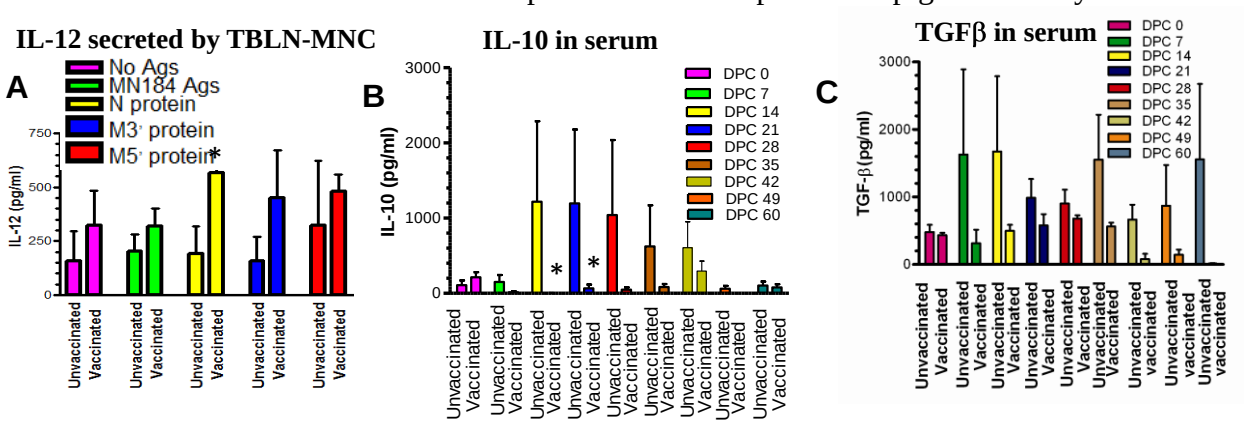


Fig. 10. Mucosally vaccinated pigs challenged using virulent heterologous PRRSV had more of Th1 (IL-12) and less of T-regulatory (IL-10 and TGFβ) cytokines. Pigs were unvaccinated or vaccinated (PRRSV-MLV + *M. tb* WCL) and then challenged with PRRSV MN184 strain and euthanized on DPI-60. TBLN-MNC were restimulated with killed MN184 antigens or recombinant PRRSV proteins (N, M3' or M5') for 48 hr and culture supernatants were analyzed for (A) IL-12. Serum samples collected on indicated DPC were analyzed for (B) IL-10 and (C) TGFβ by ELISA. Each bar represents the average cytokine levels from 3 pigs +/- SEM. Symbol '*' denote statistically significant difference (*P<0.05*) between unvaccinated vs vaccinated pigs analyzed by two-tailed unpaired t test.

							cinated	
							TBLN	PBMC
							28.4 ± 7.4	ND
							ND	**23.6 ± 17.3**
							ND	ND
							20.1 ± 0.6	**11.1 ± 2.7**
Dendritic cells	ND	5.2 ± 1.7	ND	0.007±0.1	ND	7.9 ± 0.8	2.3 ± 2.2	ND
Myloid Cells	30.2 ± 2.3	ND	ND	13.6 ± 1.5	37.6 ± 5.3	ND	ND	14.3 ± 2.3
γδ T cells	3.3 ± 0.5	12.9 ± 1.8	16.39 ± 3.9	ND	**5.3 ± 1.4**	**17.2 ± 3.8**	21.2 ± 5.0	ND

Table 2. Mucosally vaccinated pigs challenged using mild strain of PRRSV had increased frequencies of anti-PRRSV specific immune cells in different tissues. Pigs were unvaccinated or vaccinated (PRRSV-MLV + *M. tb* WCL) and then challenged with PRRSV vaccine strain (VR2332) and euthanized on (A) DPC-15 (B) DPC-60. The Lung-MNC (LMNC), BAL cells, TBLN-MNC, and PBMC were immunostained for indicated lymphocyte subpopulations, and dendritic cells, and then subjected to flow cytometry. ND: No difference between respective pig groups.

immunized VR2332 challenged pigs, cytotoxic T cells (CTLs), memory/Th cells, γδ T cells, and DCs were significantly higher in BAL fluid and moderately higher in lung-MNC at DPC-15 compared to unvaccinated VR2332 challenged pigs (Table 2A). In mucosally immunized and VR2332 challenged pigs at DPC-30 one to two fold increase in T-helper cells and total myeloid cells were detected in lung and PBMC (data not shown). At DPC-60 significantly higher frequencies of total Th cells, memory/Th cells, and γδ T cells were detected in lung-MNC and PBMC of vaccinated VR2332 challenged pigs (Table 2B). Importantly, immunosuppressive Tregs were significantly lower at DPC-15 in BAL of immunized VR2332 challenged pigs, although little higher Tregs were detected at DPC-60 in lung (Table 2A).

In mucosally vaccinated and virulent PRRSV (MN184) challenged pigs at DPC-15 significantly higher frequencies of total lymphocytes, DCs, and NK cells were detected in lung and TBLN (Table 3A). Importantly, immunosuppressive Tregs were significantly lower at DPC-15 in those immunized pigs compared to unvaccinated and virulent PRRSV challenged animals (Table 3A). At DPC-30, pigs mucosally vaccinated and challenged with MN184 had increased frequencies of CTL and NK cells compared to unvaccinated and respective PRRSV challenged pigs (data not shown). A trend in increase in favorable immune cells continued even at DPC-60 with

A

Phenotype of immune cells	Unvaccinated				Vaccinated			
	BAL	**LMNC**	**TBLN**	**PBMC**	**BAL**	**LMNC**	**TBLN**	**PBMC**
Total T cells	12.4 ± 3.4	9.2 ± 1.54	17.8 ±0.9	38.9± 2.3	**25 ± 0.7**	11 ± 3.0	**27.7 ± 10.9**	30.6± 15.2
Th cells	13± 2.0	5.9 ± 0.47	18± 0.5	12.8± 1.4	14.8± 5.4	**7.3 ± 2.6**	**38.7± 9.8**	8.7± 4.4
Dendritic cells	1.6± 0.06	10.2± 6.1	7.5 ± 1.1	0. 56 ± 0.26	**3.2± 0.8**	8.5± 8.2	**28.2 ± 3.3**	**6.4 ± 0.25**
Tregs	35 ± 3.3	3.8 ± .8	10.7± 1.2	1.5 ± .01	**15.1 ± 4.6**	**2.6 ± 1**	**3.0 ± 2**	1.6 ± 0.47
NK Cells	22.5± 5.3	5.0± 1.5	3.88± .52	33.7± 6.7	**31.7± 1.5**	**8.2± 3.4**	**5.0± 0.41**	12± 6.2

B

Cell Phenotype	Unvaccinated				Vaccinated			
	BAL	**LMNC**	**TBLN**	**PBMC**	**BAL**	**LMNC**	**TBLN**	**PBMC**
Lymphocytes	6.9 ± 1.6	4.1 ± 1.1	30.5 ± 6.4	37.4 ± 6.5	**10. 1 ± 2.9**	4.2 ± 2.3	34.54 ± 3.7	29.8 ± 9.7
Memory / Th cells	0.01 ± .01	2.4 ± 1..8	29 ± 2.4	9.7± 3.4	0.01 ± .01	**6.24 ± 1.78**	17.7 ± 5	1.9 ± 1.8
Tregs	0.4 . ± 4	3.5 ± .2	34.6 ± 11.6	4.8 ± 3.9	0 ± 0	8.7 ± 2.7	33.7 ± 12.3	**2.7 ± .2**
γδ cells	8.5± 2.3	2.3 ± .5	33.5 ± 3	25.5 ± 1.5	5.9 ± 2.9	2.7 ± .6	24.8 ± 7.7	14.6 ± 9.2
Dendritic cells	0.10 ± .05	6.9 ± 2.4	13.0± 2.1	3.6± 1.6	0.04± .01	5.4 ± 1.4	9.8 ± 2.	2.7± .7
Myloid Cells	87.4 ± 3.8	30.9 ± 3.6	16.7 ± 6.7	11 ± .4	91.3 ± 1.5	24.2 ± 2.5	**25.1± 2.9**	12.3 ± 3.0

Table 3. Mucosally vaccinated pigs challenged using virulent strain of PRRSV had increased frequencies of anti-PRRSV specific immune cells in different tissues. Pigs were unvaccinated or vaccinated (PRRSV-MLV + *M. tb* WCL) and then challenged with PRRSV strain (MN184) and euthanized on (A) DPC-15 (B) DPC-60. The Lung-MNC (LMNC), BAL cells, TBLN-MNC, and PBMC were immunostained for indicated lymphocyte subpopulations, and dendritic cells, and then subjected to flow cytometry. ND: No difference between respective pig groups.

significantly higher frequencies of total lymphocytes, memory/Th cells, total myeloid cells in immunized virulent PRRSV challenged pigs compared to control animals (Table 3B).

I. Discussion:

The mucosal adjuvanticity of various bacterial preparations tested in the respiratory tract of pigs with or without PRRSV-MLV suggested the requirement of an adjuvant which could overcome the immune suppression

induced by the PRRSV, and also could potentiate the anti-PRRSV specific adaptive immunity. Based on the various anti-PRRSV specific innate and adaptive immune responses that we detected at both mucosal and systemic sites in pigs inoculated with or without adjuvant to PRRSV-MLV, we conclude that it is possible to elicit broadly protective anti-PRRSV specific immunity.

A previous study using PRRSV-MLV (with no adjuvant) inoculated by intranasal route and challenged with non-identical PRRSV had reduced shedding of both vaccine and challenge virus in boar semen (Christopher-Hennings 1998). One more study on intranasal delivery of killed PRRSV vaccine along with CpGODN elicited strong anti-PRRSV specific secretory IgA antibody response, but the lack of PRRSV challenge studies in immunized pigs failed to correlate the observed immune response to protection (Zhang, Tian et al. 2007). In our study, we confirmed the advantages of bacterial based potent mucosal adjuvants in mucosal delivery of PRRSV-MLV in eliciting protective anti-PRRSV immunity, and even the immune responses generated were correlated well in protection against viral challenge. In the current study, we used 4 - 5 week old piglets and hence did not address the PRRSV shedding in semen. But we detected significantly reduced PRRSV in the serum of mucosally vaccinated and both homologous and heterologous PRRSV challenged pigs.

The PRRSV infection induces B cell lymphoplasia leading to massive total antibody response, but unfortunately with poor anti-PRRSV specific neutralizing antibody titers. The PRRSV is responsible for suppressed innate NK cell function, delayed or dampened IFNα, IFNγ, IL-6 and IL-12 production. Most importantly, the PRRSV initiates production of long lasting immunosuppressive cells (Tregs) and cytokines (IL-10 and TGFβ). Even the vaccine (PRRSV-MLV) virus is not excluded from induction of all these undesirable responses in mucosally immunized pigs. Based on studies by others in experimental animals, and currently used commercial mucosal vaccines in humans (Influenza Flu-mist, Polio oral-drops, Rotaviral oral vaccines etc.,), generation of protective mucosal immunity is critical to viruses whose site of infection is predominantly at mucosal sites. Our study demonstrated that effective mucosal vaccination overcomes the viral proteins induced immunosuppression and potentiates the virus-specific adaptive immune responses. Our approach was routed in a similar direction to combat the PRRSV-MLV induced undesirable effects and to elicit protective mucosal and systemic anti-PRRSV immunity. The use of an appropriate adjuvant (such as *M. tb* WCL) along with PRRSV-MLV administered by intranasal route overcame the immunosuppression, and also potentiated the innate and anti-PRRSV specific adaptive immunity.

There are many reports confirmed how immune dysfunctions caused by PRRSV favors the secondary microbial infections (bacterial, viral, fungal etc.,), resulting in exacerbation of respiratory and reproductive disease in pigs. In turn, responsible for the huge economic losses to the pork industry, much higher than approximately estimated $600 million annual losses to the US economy. Even though we did not appreciate apparent clinical disease in PRRSV vaccine strain (VR2332) challenged pigs, we did detect favorable anti-PRRSV immune responses only in pigs mucosally vaccinated and then challenged compared to unvaccinated challenged pigs. But in real field situation due to immune dysfunctions induced by even mild PRRSV strains and possible exposure to secondary microbial infections, milder PRRSV strains also induce clinical disease with reduced body weight gain. However, virulent PRRSV (such as strain MN184) alone is capable of inducing severe clinical disease, and in real field situation with exposure to other pathogens, pigs will suffer even more with significant reduction in body weight gain. Consistent with that situation even in our study, we did find severe PRRSV disease in pigs unvaccinated and MN184 challenged. But our mucosal vaccination approach significantly rescued the pigs from respiratory disease to virulent PRRSV challenge and in turn reduced the body weight loss. Associated favorable immunological responses and reduced viral load in serum indicated that broadly protective anti-PRRSV specific immunity is possible by eliciting an effective mucosal immunity against PRRSV in pigs.

In summary, our studies confirmed that protection against both identical and non-identical PRRSV is possible by eliciting effective anti-PRRSV mucosal immunity with the help of PRRSV-MLV administered

intransally along with potent adjuvants to pigs. Local mucosal protection is critical for the PRRSV which infects principally lungs and also induce strong innate immunosuppression. Potent mucosal adjuvants can overcome both immunosuppression induced by PRRSV proteins and also potentiates the PRRSV specific adaptive immune responses. Our future research will be aimed at large scale mucosal vaccination against PRRSV in real field situation. Further, extend our research to circumvent the PRRSV persistence in pig tonsils by mucosal vaccination approach to effectively control PRRS outbreaks.

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