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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

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease to pork producers worldwide. Commercially, both live and killed PRRSV vaccines are available to control PRRS, but they are not always successful. Based on the results of mucosal immunization studies in other viral models, a good mucosal vaccine may be an effective way to elicit protective immunity to control PRRS outbreaks. In the present study, mucosal adjuvanticity of *Mycobacterium tuberculosis* whole cell lysate (*Mtb* WCL) was evaluated in pigs administered a modified live PRRS virus vaccine (PRRS-MLV) intranasally. A *Mtb* WCL mediated increase in the frequency of NK cells, $CD8^+$ and $CD4^+$ T cells, and γδ T cells in pig lungs were detected. Importantly, an increased and early generation of PRRSV specific neutralizing antibodies were detected in PRRS-MLV+ *Mtb* WCL compared to pigs inoculated with vaccine alone. In addition, there was an increased secretion of Th1 cytokines (IFNγ and IL-12) that correlated with a reciprocal reduction in the production of immunosuppressive cytokines (IL-10 and TGFβ) as well as T-regulatory cells in pigs vaccinated with PRRS-MLV+ *Mtb* WCL. Further, a complete rescue in arginase levels in the lungs mediated through *Mtb* WCL was observed in pigs inoculated with PRRS-MLV. In conclusion, *Mtb* WCL may be a potent mucosal adjuvant for PRRS-MLV in order to potentiate the anti-PRRSV specific immune responses to control PRRS effectively.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important chronic viral diseases of pigs [1]. The causal organism of this disease is PRRS virus (PRRSV), which belongs to the family *Arteviridae* in the order Nidovirales [2]. The clinical signs of PRRS are reproductive failure, abortion, and high pre-weaning mortality [3]. PRRSV causes immunosuppression resulting in susceptibility of pigs to other poly-microbial infections [4,5]. The virus induces weak, innate immune responses as a result of reduced IFN-α production and dampened natural killer (NK) cell mediated cytotoxicity [5,6], which lead to a weak/delayed adaptive immune response. Although increased PRRSV specific antibodies are generated early post-infection, virus neutralizing (VN) antibodies appear quite late and remain at low levels [7]. A killed PRRSV vaccine is available but it has failed to provide adequate protection. A modified-live PRRS virus vaccine (PRRS-MLV) has been in use to control clinical PRRS in young pigs. Unfortunately, like the PRRSV infection, PRRS-MLV also induces delayed neutralizing antibody and dampened cell-mediated immune (CMI) responses [8–10]. Therefore, it is important to improve the efficacy of PRRSV live vaccines to control PRRS effectively.

Induction of the IFN-γ response by vaccination is important for viral clearance. The pro-inflammatory cytokine IL-6 produced by myeloid cells initiates the virus specific adaptive immune response [11]. Additionally, the CMI response is manipulated by the metabolism of an essential amino acid, L-arginine, whose level in the body is modulated by the enzymes arginase (1 and 2) and nitric oxide synthase [12]. The role of arginase in viral infections of the respiratory tract or in vaccination is limited, however, arginase 2 deficient mice have an increased susceptibility to viral infections [13]. The requirement of arginase to abort the multiplication of herpes simplex virus (HSV) has been reported [14]. In contrast, uncontrolled replication of *Leishmania* was correlated with enhanced arginase activity [15].

Mucosal surfaces cover the largest surface area in the body and almost 80% of the total immune cell population is present at mucosal sites. Nasopharyngeal lymphoid tissues contain the entire

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repertoire of immune cells and are strategically located to orchestrate regional immune functions against airborne infections [16]. Therefore, effective mucosal vaccination is an appropriate strategy to provide protection against various infectious agents. Protective mucosal immunity is mediated by $CD4^+$ T helper (Th) cells, $CD8^+$ cytotoxic lymphocytes (CTLs), and NK cells in HSV genital infections [17]. Similarly, intranasal delivery of an influenza vaccine FluMist (MedImmune, Gaithersburg, MD) provided immunity comparable to that induced by a natural infection [18,19]. An added advantage of mucosal vaccination is that it can induce both mucosal and systemic immune responses [20]. Intranasal immunization of HIV-liposome resulted in an effective virus specific immune response at both mucosal and systemic sites [21].

To increase the efficacy of mucosal immunization, a potent adjuvant or delivery system is needed to overcome the immune tolerant mechanisms at mucosal sites [22,23]. Mucosal administration of a live attenuated vaccine with a suitable adjuvant induces long lasting protection in various studies performed in bovine herpes virus-1, influenza, and parainfluenza-3 virus [24–26]. Killed *Mycobacterium tuberculosis* is an excellent candidate adjuvant used in the preparation of Freund's complete adjuvant [27], but its use in humans and in food animals is contraindicated due to a severe granulomatous inflammatory reaction induced at the injection site. This adverse effect results from toxic cell wall components of *Mtb* (such as mycolic acids, arabinogalactan, wax D) [28,29]. However, adjuvanticity of various purified components of *Mtb* have been evaluated individually with satisfactory results [30,31]. In particular, certain individual components and a total fraction of whole cell lysate (WCL) of *Mtb* free from its toxic cell wall constituents have been demonstrated to possess superior adjuvanticity in rodents, guinea pigs, and rabbits [32–35]. However, the knowledge related to mucosal adjuvanticity of *Mtb* WCL to protect against viral infections of the respiratory tract is limited.

The purpose of this study was to enhance the efficacy of PRRS-MLV with the help of *Mtb* WCL by inducing protective mucosal immunity. Initially, we performed studies in pigs to choose a suitable bacterial candidate to use as a mucosal adjuvant by inoculating nine different bacterial preparations belonging to *Mycobacterium*, *Vibrio*, and *Streptococcus* species intranasally; the detailed results of which will be presented elsewhere. From that study, we selected *Mtb* WCL for its potent adjuvant properties such as its ability to augment PRRSV specific Th1 cytokines and immune cells, and suppress immunosuppressive responses. In this study, immune responses of pigs administered PRRS-MLV+/− *Mtb* WCL intranasally were evaluated at both mucosal and systemic sites.

2. Materials and methods

2.1. Cells, PRRSV, and adjuvant

Stable mycoplasma-free MARC-145 cells which support the growth of PRRSV [36] were used to prepare PRRSV antigens and to perform immunological assays. MARC-145 cells were maintained in DMEM (Lonza, Riverside, CA) with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) at 37 °C with 5% CO_2. PRRS-MLV (Ingelvac® PRRS) was provided by Dr. Mike Roof (Bio-R&D, Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO). Wild-type PRRSV VR2332 was provided by Eric Nelson (South Dakota State University). *M. tuberculosis* whole cell lysate (*Mtb* WCL) was provided by Drs. Dobos and Belisle (NIH/NIAID funded contract "TB Vaccine Testing and Research Materials"; Colorado State University, Fort Collins, CO). Purified and biotin labeled cytokine specific anti-porcine antibodies, IL-6, IL-12, and IL-10 (R&D Systems, Minneapolis, MN), IFN-γ (BD Pharmingen, San Diego, CA), TGF-β (Invitrogen, Camarillo, CA) were purchased from commercial sources and used to perform the sandwich ELISA.

2.2. Pigs and inoculations

Conventional large White-Duroc crossbred weaned specific-pathogen-free piglets were transported to the animal facilities of the Food Animal Health Research Program at the Ohio Agricultural Research and Development Center, Wooster, OH. The swine herd was confirmed seronegative for antibodies by ELISA to PRRSV, porcine respiratory corona virus, transmissible gastroenteritis virus, and porcine circo virus 2. Piglets were bled on arrival and tested to confirm the absence of PRRSV antibodies. Pigs were allowed to acclimate for one week before initiation of our study. For the duration of our study, all animals received food and water *ad libitum*. All inoculations such as adjuvant (*Mtb* WCL, 3 mg/pig) and vaccine (2×10^6 $TCID_{50}$ per pig) were administered intranasally. All pigs were maintained, samples collected, and were euthanized as per the protocol approved by the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC), The Ohio State University, Ohio.

Twenty-two pigs were randomly allocated to one of three groups: group 1, mock pigs ($n = 4$) received normal saline (2 ml); groups 2 and 3 (each group $n = 9$) were vaccinated with PRRS-MLV+/− *Mtb* WCL. Both vaccine and adjuvant each in 2 ml volume were administered at the same time into each nostril. Three pigs each from groups 2 and 3 were euthanized on 15, 30, and 60 post-immunization day (PID). Mock inoculated pigs ($n = 4$) were euthanized separately prior to euthanasia of any vaccinated pigs. In another study (data shown in Fig. 1), nine pigs were allocated into three groups ($n = 3$ per group), and inoculated intranasally with PRRS-MLV+/− *Mtb* WCL as described above, and pigs were euthanized at PID 7. Mock inoculated pigs ($n = 3$) were euthanized separately prior to euthanasia of any vaccinated pigs.

2.3. Collection of blood, preparation of lung homogenates, and isolation of immune cells

Three to 5 ml of blood was collected on PID 0, 4, 7, 14, 21, 28, 31, 35, 42, 49, 56, and 60. Serum was separated from the blood, aliquoted, and stored at −20 °C. Lung homogenates were prepared as described previously [5]. For isolation of peripheral blood mononuclear cells (PBMC), blood was collected in acid citrate dextrose solution. For isolation of bronchoalveolar lavage fluid (BAL) cells, lung mononuclear cells (lung MNC) and MNC from tracheobronchial lymph nodes (TBLN) draining the lungs, the procedure was followed as described previously [5,37,38].

2.4. PRRSV specific isotype antibody analysis in serum

To analyze PRRSV isotype specific IgA and IgG antibodies in serum, ELISA plates were coated with pre-titrated crude killed PRRSV (VR2332) antigens (10 μg/ml) in carbonate–bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. Plates were washed in PBS-Tween-20 and then treated with blocking buffer (1% BSA in PBS) for 2 h at RT. Test serum was added and incubated at RT for 2 h. Plates were washed and the bound PRRSV isotype specific antibody was detected using anti-pig IgA and anti-pig IgG secondary antibodies conjugated with HRP (KPL, Gaithersburg, MD). Plates were developed using a chromogen ABTS and the OD was read at 405 nm. To eliminate the background activity, a control plate with bicarbonate buffer (pH 9.6) and without PRRSV antigens was blocked, treated with the test serum, and developed separately. The OD values obtained from control wells were subtracted from the respective experimental wells to obtain the corrected values.

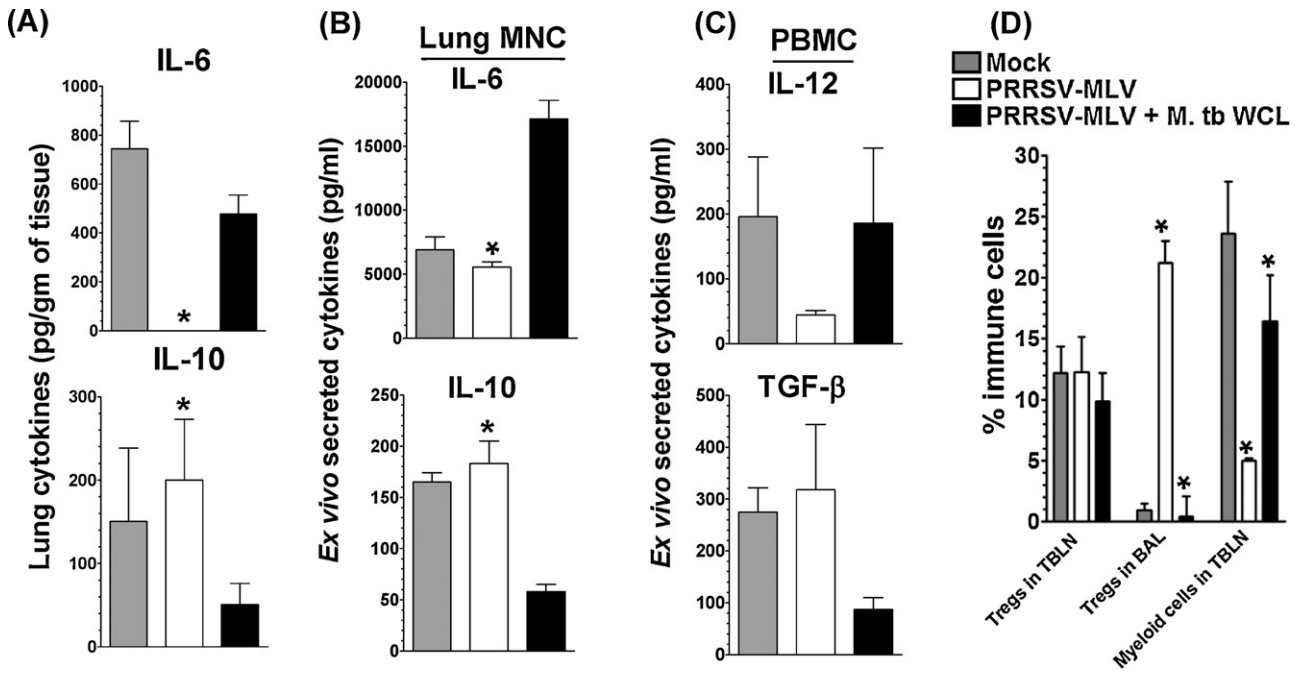

Fig. 1. Mucosal vaccination of pigs using adjuvant *Mtb* WCL potentiated the PRRSV specific immune responses. Pigs were inoculated with PRRS-MLV+/− *Mtb* WCL, intranasally, and euthanized at PID 7. (A) Lung homogenate prepared from lung samples, and the harvested supernatant from cultures of (B) lung-MNC and (C) PBMC restimulated in the presence of killed PRRSV VR2332 antigens were analyzed for indicated cytokines by ELISA. (D) TBLN MNC and BAL cells were immunostained to analyze the frequency of Tregs and total myeloid cells ($CD172^+$) by flow cytometry. Each bar represents the average cytokines or percent immune cells from three pigs ± SEM. Asterisk denotes statistically significant difference between pigs inoculated with PRRS-MLV+/− *Mtb* WCL.

2.5. Analyses of PRRSV neutralizing antibody titers

A standard indirect immunofluorescence assay (IFA) was followed for determining the virus neutralizing antibody titers [39,40]. Briefly, serum was heat treated for complement inactivation, diluted two fold in DMEM, and incubated with an equal volume of PRRSV VR2332 containing 500 $TCID_{50}$ per well for 2 h at 37 °C. One hundred microliters of that suspension was transferred into a 96-well microtiter plate containing a confluent monolayer of MARC-145 cells, and the plate was incubated for 24 h at 37 °C in a CO_2 incubator. Cytopathic effects were examined following fixation with acetone water and the addition of anti-PRRSV nucleocapsid mAb (clone SDOW17) and Alexa-488 conjugated anti-mouse IgG(H+L) secondary antibody. These cytopathic effects were observed under a fluorescent microscope after mounting with glycerol-PBS (6:4 ratio).

2.6. Pig NK cell cytotoxic assay

NK assay to determine the pig NK-cell mediated cytotoxicity was performed as described previously [5]. Briefly, PBMC were used as the source of NK cells (effectors) against K-562 (human myeloblastoid cell line) or Yac-1 (mouse T lymphoma cell line) target cells. Effectors and targets were incubated at different E:T ratios and the amount of released lactate dehydrogenase (LDH) was measured by a colorimetric assay. Released LDH is directly proportional to the NK specific lysis of target cells.

2.7. Analysis of PRRSV specific recall/memory immune response

Five million lung MNC were subjected to *in vitro* restimulation in a 24-well tissue culture plate in the presence of killed crude PRRSV VR2332 antigens (50 μg/ml) in enriched RPMI-1640 [5] for 48 h at 37 °C. Harvested culture supernatant was analyzed for cytokines by ELISA. Cells cultured in the absence of any antigens were included as a control, and the amount of cytokines secreted by these cells was subtracted from the respective restimulated experimental well values.

2.8. Analysis of cytokine response

Lung homogenates and cell culture supernatants were analyzed for cytokines IFN-γ, IL-12, IL-6,IL-10, and TGFβ by ELISA as described previously [5,11].

2.9. Flow-cytometric study of different immune cell populations

Flow cytometric analysis was performed to determine the phenotype and the frequency of different immune cells by a multicolor immunoassay as described previously [5,37,41]. Immunostained cells were acquired using a FACS AriaII flow cytometer (BD Biosciences). The analysis was performed using a FlowJo software (Tree Star, Inc., OR, USA) to enumerate different immune cell populations based on the cell surface marker expression as follows: NK cells ($CD3^-CD4^-CD8\alpha^+$) [42]; T-helper cells ($CD3^+CD4^+CD8^-$); cytotoxic T lymphocytes (CTLs) ($CD3^+CD4^-CD8^+$); γδ T cells ($CD8\alpha^+TcR1N4^+$); T-regulatory cells ($CD4^+CD25^+FOXP3^+$); myeloid cells ($CD172^+$). Frequencies of individual lymphoid and myeloid cell subsets were analyzed from a total 50,000 to 100,000 events.

2.10. Analysis of arginase levels in pig lungs

A colorimetric assay was used to measure arginase levels in the pig lungs, and the protocol was standardized based on previously described methods [43]. BAL cells were lysed using lysis buffer containing 0.5% TritonX-100 and protease inhibitor cocktail for 30 min on ice. Cell debris and nuclei were clarified by centrifugation and the clear supernatant was aliquoted. Each aliquot was treated with 10 mM $MnCl_2$ and heated for 10 min at 56 °C. Another identical aliquot not treated with $MnCl_2$ was kept at RT. L-Arginine 0.5 M (pH 9.7) was added to all the tubes and incubated for 25 min at 37 °C. The reaction was stopped using a mixture of 96% H_2SO_4,

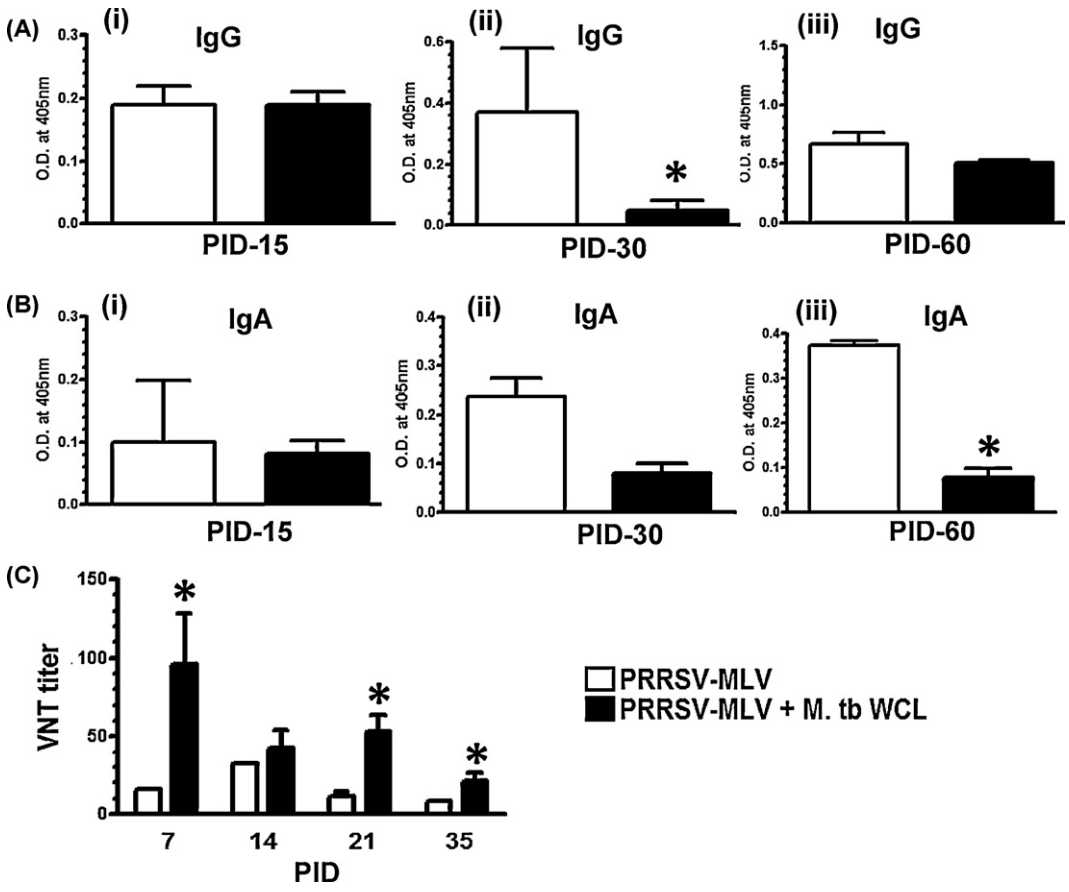

Fig. 2. Enhanced PRRSV specific neutralizing antibody titers in mucosally vaccinated pigs mediated by *Mtb* WCL. Pigs were inoculated with PRRS-MLV+/− *Mtb* WCL, intranasally, and euthanized at PID 15, 30, and 60. Serum samples collected at indicated PID was analyzed for anti-PRRSV isotype specific (A) IgG and (B) IgA antibodies by ELISA. (C) Serum samples collected at indicated PID were analyzed for anti-PRRSV specific neutralizing antibody titers by a standard immunofluorescence assay. Each bar represents the average OD from three pigs ± SEM, or average virus neutralizing antibody titers from six pigs ± SEM. Asterisk denotes statistically significant difference between pigs inoculated with PRRS-MLV+/− *Mtb* WCL.

85% H_3PO_4, and H_2O mixed in the ratio of 1:3:7. The enzyme activity was measured after adding 4% α-isonitrosopropiophenone and heat treating for 30 min at 90–95 °C. Standard concentration of urea (0.10 M) was diluted ten fold and subjected to similar treatment as described above. Optical density was measured at 540 nm and the values were converted to units based on the OD values of the urea standards. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea per min.

2.11. Statistical analysis

All of the data were expressed as the mean of three or six pigs ± SEM. Statistical analyses were performed using nonparametric Wilcoxon *t*-test when functionality was compared between two study groups, and paired *t*-test with repeated measures when functionality was compared among different PID (SAS software, SAS Institute Inc., Cary, NC). Statistical significance was assessed as $P < 0.05$.

3. Results

3.1. Intranasal delivery of PRRS-MLV along with Mtb WCL elicited anti-PRRSV specific immune responses

Initially, at PID 7, PRRSV specific *Mtb* WCL-mediated adjuvant effect was analyzed in pigs. Lung homogenates from PRRS-MLV inoculated pigs had significantly higher amounts of the immunosuppressive cytokine IL-10 and lower levels of the pro-inflammatory cytokine IL-6 (Fig. 1A). Also, lung-MNC of PRRS-MLV inoculated pigs secreted significantly higher amounts of IL-10 and lower amounts of IL-6 compared to pigs that received PRRS-MLV+ *Mtb* WCL (Fig. 1B). As an indicator of a systemic immune response, PBMC from pigs receiving PRRS-MLV secreted more of an another immunosuppressive cytokine TGF-β and less of Th1 response inducing cytokine IL-12 compared to pigs received PRRS-MLV+ *Mtb* WCL (Fig. 1C).

An increased immunosuppressive cytokine response was associated with a significant increase in the frequency of T-regulatory cells (Tregs) in the lungs of pigs receiving PRRS-MLV compared to pigs inoculated with PRRS-MLV+ *Mtb* WCL (Fig. 1D). In addition, the frequency of myeloid cells ($CD172^+$) in TBLN of PRRS-MLV inoculated pigs was significantly less (Fig. 1D); particularly the dendritic cells (DCs) rich fraction ($CD172^+CD11c^+$SLA class II^+) (data not shown) when compared to pigs vaccinated with PRRS-MLV+ *Mtb* WCL. To note, *Mtb* WCL did not induce any adverse effects in the pig respiratory tract. We also performed an *in vivo* dose kinetics study of *Mtb* WCL in pigs vaccinated intranasally with PRRS-MLV and detected an enhanced anti-PRRSV specific immune response when *Mtb* WCL was used at 3 mg per pig (data not shown).

3.2. Enhanced generation of PRRSV specific neutralizing antibodies was mediated by Mtb WCL

Serum samples were analyzed for PRRSV specific total IgG and IgA antibodies; and surprisingly, a significantly reduced secre-

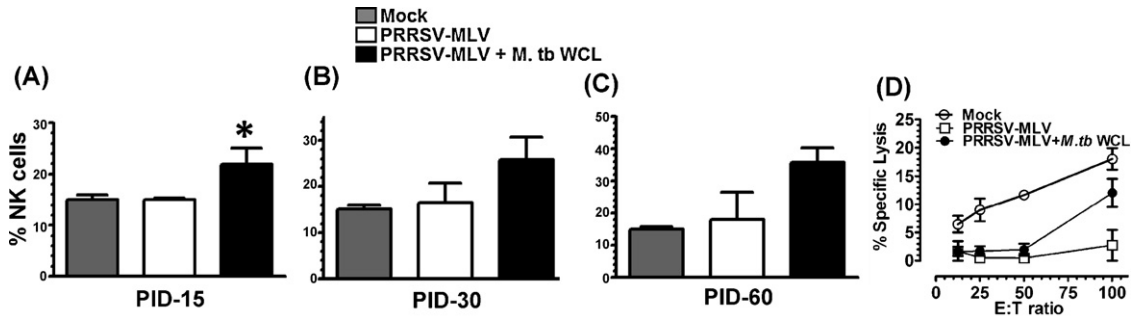

Fig. 3. Upregulation of NK cell frequency and function by *Mtb* WCL to PRRS-MLV in pigs. Pigs were inoculated with PRRS-MLV+/− *Mtb* WCL, intranasally, and euthanized at PID 15, 30 and 60. (A–C) Lung immune cells were immunostained to analyze the frequency of NK cells by flow cytometry. (D) A standard colorimetric NK cell cytotoxicity assay was used to determine the NK cell function. PBMC were used as effectors against K562 targets and the released LDH was measured using a LDH substrate. Each bar or the data point on the line graph represents the average % NK cells or percent NK specific lysis, respectively, from three pigs ± SEM. Asterisk denotes statistically significant difference between pigs inoculated with PRRS-MLV+/− *Mtb* WCL.

tion of IgG at PID 30 was detected from pigs that received PRRS-MLV+ *Mtb* WCL (Fig. 2Aii). The virus specific total IgA secretion was also less (but not statistically significant) at PID 30, and it was significantly reduced at PID 60 in pigs vaccinated with PRRS-MLV+ *Mtb* WCL compared to vaccine alone inoculated pigs (Fig. 2Bii and Biii). In contrast, PRRSV specific VN titers at PID 7, 21 and 35 were significantly higher in PRRS-MLV+ *Mtb* WCL vaccinated pigs compared to PRRS-MLV alone inoculated animals (Fig. 2C). Therefore, our results suggested that *Mtb* WCL has induced the increased PRRSV specific VN titers in PRRS-MLV inoculated pigs, and interestingly it was independent of total PRRSV specific antibodies.

3.3. Increased NK cell frequency and rescue in its cytotoxicity was mediated by Mtb WCL in PRRS-MLV inoculated pigs

Lung immune cells were immunostained to identify the frequency of NK cells. A significant increase in the frequency of NK cells at PID 15, and a moderate increase at PID 30 and 60 in the lungs of pigs vaccinated with PRRS-MLV+ *Mtb* WCL, compared to mock and vaccine alone received pigs was detected (Fig. 3 A-C). Further, a colorimetric NK cell cytotoxic assay was performed and detected a moderate rescue in the NK cell cytotoxicity only at E:T ratio1:100 in PRRS-MLV+ *Mtb* WCL received pigs at PID 60 (Fig. 3D). Increased frequency of NK cells at PID 15 and 30 did not result in the rescue of NK cell killing function (data not shown).

3.4. Mtb WCL mediated upregulation of Th1 cytokine and reduction of immunosuppressive cytokine response in pig lungs

Lung MNC of PRRS-MLV+ *Mtb* WCL vaccinated pigs secreted significantly higher levels of the Th1 cytokines (IFNγ and IL-12) and significantly reduced levels of the immunosuppressive cytokine IL-10 at PID 15 compared to pigs that received PRRS-MLV alone and mock-inoculated pigs (Fig. 4A–C). Also at PID 30 and 60, a significant increase in secretion of the cytokine IFN-γ and a moderately reduced secretion of the cytokine IL-10 by lung MNC were detected (Fig. 5A–D). However, a considerable difference in the secretion of the cytokine IL-12 was not observed between pig groups at PID 30 and 60 (data not shown).

3.5. Mtb WCL mediated increase in the lymphoid cell subsets in vaccinated pigs

The phenotype and frequency of various lymphoid immune cells in pigs were analyzed by flow cytometry. The frequency of mock-infected pig immune cells is shown separately (Table 1). The lungs from PRRS-MLV+ *Mtb* WCL vaccinated pigs had a higher frequency (but not significant) of total lymphocytes ($CD3^+$ T cells) and $CD4^+$ T cells at all PID (Table 2A and B). At PID 30, a significant increase in the frequency of $CD8^+$ T cells was detected in the lungs of PRRS-MLV+ *Mtb* WCL vaccinated pigs compared to lungs from pigs inoculated with PRRS-MLV alone (Table 2C). An increased (but not significant) frequency of $CD8^+$ T cells was also detected in the

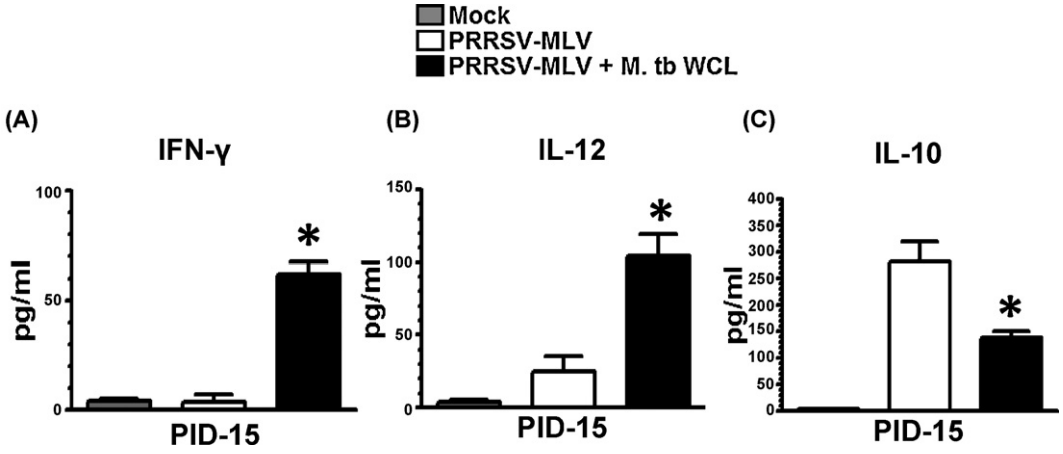

Fig. 4. Significantly increased Th1 and reduced immunosuppressive cytokines production was mediated by *Mtb* WCL to PRRS-MLV. Pigs were inoculated with PRRS-MLV+/− *Mtb* WCL, intranasally, and euthanized at PID 15. Supernatants harvested from lung MNC cultured in the presence of killed PRRSV antigens were analyzed for cytokines: (A) IFNγ; (B) IL-12; (C) IL-10 by ELISA. Each bar represents the average cytokines from three pigs ± SEM. Asterisk denotes statistically significant difference between pigs inoculated with PRRS-MLV+/− *Mtb* WCL.

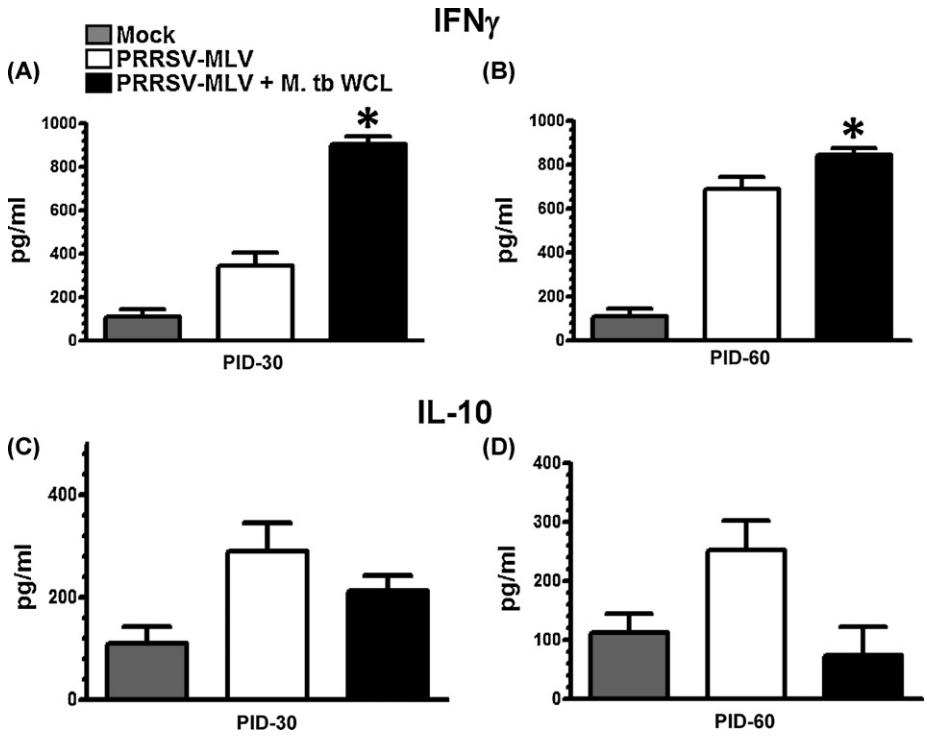

Fig. 5. Enhanced IFN-γ and reducedIL-10 production was mediated by *Mtb* WCL to PRRS-MLV. Pigs were inoculated with PRRS-MLV+/− *Mtb* WCL, intranasally, and euthanized on PID 30 and 60. Supernatants harvested from lung MNC cultured in the presence of killed PRRSV antigens were analyzed for cytokines: (A and C) IFNγ; (B and D) IL-10 by ELISA. Each bar represents the average cytokines from three pigs ± SEM. Asterisk denotes statistically significant difference between pigs inoculated with PRRS-MLV+/− *Mtb* WCL.

lungs at PID 15 and 60 (Table 2C). In addition, increased (but not significant) frequency of γδ T cells in the lungs of PRRS-MLV+ *Mtb* WCL vaccinated pigs was detected at all the PID (Table 2D). In contrast, the frequency of Tregs in the lungs of PRRS-MLV+ *Mtb* WCL vaccinated pigs was significantly less compared to the frequency of Tregs in the lungs of PRRS-MLV inoculated pigs at PID 15 (Table 2E).

In blood, a significant increase in the frequency of $CD4^+$ T cells was detected at PID 30 and 60 in PRRS-MLV+ *Mtb* WCL inoculated pigs compared to pigs inoculated with PRRS-MLV alone (Table 3A). Significant modulation of Treg populations was not observed in the blood as PRRS is predominantly a disease of the pig lung (Tables 2E and 3B).

3.6. Rescued arginase production in the pig lungs was mediated by Mtb WCL administered with PRRS-MLV

Information related to inhibition of arginase in the setting of respiratory viral infection and/or vaccination is limited. Here we report that pigs vaccinated intranasally with PRRS-MLV had significantly reduced intracellular arginase levels in their lungs at PID 60 compared to physiological levels from the mock pigs. In contrast, arginase levels in pigs administered PRRS-MLV+ *Mtb* WCL were completely rescued. Interestingly, the rescue was significantly higher than in pigs inoculated with PRRS-MLV at PID 30 and 60 (Fig. 6). Repeated analysis of arginase from pigs receiving PRRS-MLV+/− *Mtb* WCL at all the PID was also statistically significant with reference to increase or decrease in lung arginase levels, respectively (Fig. 6).

Table 1
Mock control pigs.

Immune cells phenotype	Lungs	Blood
$CD3^+$ – T cells	17.3 ± 0.9	52.9 ± 4.7
$CD3^+CD4^+$ – Th cells[a]	12.2 ± 2.5	12.8 ± 5.0
$CD3^+CD8^+$ – CTLs[a]	17.3 ± 0.9	32.6 ± 3.0
$TcR1N4^+CD8\alpha^+$ – γδ T cells	6.1 ± 2.1	4.0 ± 0.6
$CD4^+CD25^+Foxp3^+$ – Tregs[b]	1.9 ± 0.8	4.1 ± 0.1

Three mock-inoculated and nine pigs each inoculated with PRRS-MLV or PRRS-MLV+ *Mtb* WCL intranasally were euthanized on PID 15, 30 or 60 ($n = 3$ pigs at each PID), and lymphoid cell subsets were enumerated by flow cytometry.

[a] $CD3^+$ and $CD3^-$ cells were gated to enumerate CD4 and CD8α expression.

[b] $CD25^+$ cells were gated to enumerate CD4 and Foxp3 expression and the percent of total $CD4^+CD25^+Foxp3^+$ cells are shown. Each number is an average percent of immune cells from three pigs ± SEM.

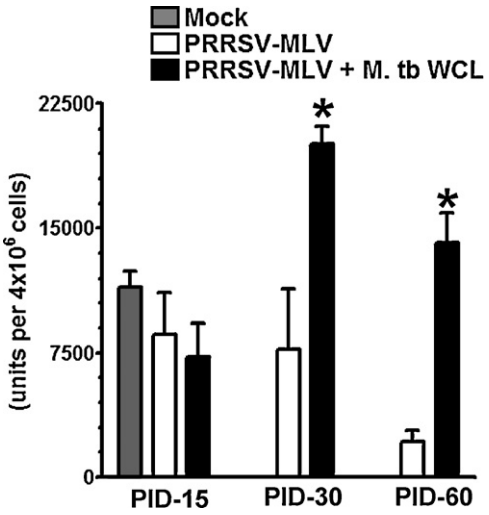

Fig. 6. *Mtb* WCL mediated rescue in arginase production in pig lungs to PRRS-MLV. Pigs were inoculated with PRRS-MLV+/− *Mtb* WCL or mock-inoculated, intranasally, and euthanized at indicated PID. BAL cells were lysed and the enzyme arginase was quantified by a colorimetric assay. Each bar represents the average units of arginase from three pigs ± SEM. Asterisk denotes statistically significant difference between pigs inoculated with PRRS-MLV+/− *Mtb* WCL.

Table 2
Lungs.

		PID 15	PID 30	PID 60
(A)	$CD3^+$ – T cells			
	PRRS-MLV	32.4 ± 4.5	32.0 ± 3.0	32.0 ± 2.1
	PRRS-MLV + *Mtb* WCL	44.8 ± 7.8	38.5 ± 3.9	34.6 ± 1.3
(B)	$CD3^+CD4^+$ – Th cells[a]			
	PRRS-MLV	21.0 ± 2.5	15.1 ± 4.1	24.1 ± 2.1
	PRRS-MLV + *Mtb* WCL	26.7 ± 3.6	20.4 ± 2.9	31.4 ± 3.0
(C)	$CD3^+CD8^+$ – CTLs[a]			
	PRRS-MLV	42.4 ± 4.9	36.4 ± 2.1	30.4 ± 3.8
	PRRS-MLV + *Mtb* WCL	58.4 ± 11.4	50.6 ± 7.3*	41.3 ± 5.3
(D)	$TcR1N4^+CD8\alpha^+$ – γδ T cells			
	PRRS-MLV	5.0 ± 0.4	7.4 ± 3.3	9.8 ± 3.0
	PRRS-MLV + *Mtb* WCL	7.6 ± 2.6	10.7 ± 3.0	11.4 ± 2.1
(E)	$CD4^+CD25^+Foxp3^+$ – Tregs[b]			
	PRRS-MLV	58.4 ± 11.3	50.2 ± 7.3	41.3 ± 5.3
	PRRS-MLV + *Mtb* WCL	42.4 ± 4.9	36.4 ± 2.1*	30.4 ± 3.7

Three mock-inoculated and nine pigs each inoculated with PRRS-MLV or PRRS-MLV+ *Mtb* WCL intranasally were euthanized on PID 15, 30 or 60 ($n = 3$ pigs at each PID), and lymphoid cell subsets were enumerated by flow cytometry.

[a] $CD3^+$ and $CD3^-$ cells were gated to enumerate CD4 and CD8α expression.

[b] $CD25^+$ cells were gated to enumerate CD4 and Foxp3 expression and the percent of total $CD4^+CD25^+Foxp3^+$ cells are shown. Each number is an average percent of immune cells from three pigs ± SEM.

* Statistically significant difference ($P < 0.05$) between PRRS-MLV+/− *Mtb* WCL inoculated pig groups.

4. Discussion

A marked immunosuppressive response was observed in pigs inoculated intranasally with PRRS-MLV alone which was likely attributed to induction of Tregs, increased levels of IL-10, delayed and reduced secretion of IFN-γ, as well as low VN titers. Similarly, earlier studies also reported comparable immunosuppressive responses in pigs administered PRRS-MLV, also by parenteral route [44–46]. Apart from delaying the IFN-γ response, PRRSV preferentially promotes the synthesis of non-neutralizing antibodies [47].

Currently, development of mucosal vaccines against a variety of viral pathogens is gaining momentum to protect against predominant mucosal infections, such as influenza, parainfluenza, respiratory syncytial virus, rotavirus, and HIV/SIV [26,29,48,49]. Consistent with a high degree of compartmentalization, the mucosal immune system is populated by functionally distinct B cells, T cells, and accessory cell subpopulations comparable to populations present in systemic lymphoid tissues. The mucosal immune system is modulated by a variety of mechanisms involving innate immune cells such as DCs, NK cells, and mast cells which contribute significantly to host defense against pathogens [50,51]. Studies have also demonstrated the mechanisms of induction of mucosal immune responses to live and inactivated, viral and bacterial vaccines with the help of potent adjuvants [17,52,53].

The innate NK cell is one of the major players in antiviral defense. We detected an increased frequency of NK cells in PRRS-MLV+ *Mtb* WCL inoculated pigs; however, the NK cell frequency was comparable to mock pigs in PRRS-MLV alone received pigs. These findings suggest that PRRSV modulates NK cell function rather than its frequency in pig lungs. NK cells play two major roles in antiviral defense such as secretion of cytokines (predominantly IFN-γ), and lysis of infected and transformed cells. PRRSV infection suppresses the NK cell cytotoxic function in pigs [5]. In a recent study, 25 pigs experimentally infected with a PRRSV showed approximately a 50% reduction in their NK cell killing function just two days post-infection (Dwivedi and Renukaradhya 2011, unpublished data). Now we report that PRRS-MLV delivered intranasally also suppresses the NK cell cytotoxic function; however, when PRRS-MLV was administered with *Mtb* WCL, an increased frequency with moderate rescue in their function was observed. Enhanced production of theTh1cytokine IL-12 in the lungs of PRRS-MLV+ *Mtb* WCL inoculated pigs was detected early (PID 7 and 15). IL-12 is critical for augmenting the innate NK cell function and is responsible for the induction of protective mucosal immunity against intracellular pathogens [9,54]. Direct NK-DCs crosstalk in mucosally vaccinated animals with adjuvant resulted in enhanced innate and adaptive immune responses [55].

Neutralizing antibody response is a major component of the protective immunity to PRRSV infection [56,57]. It has been demonstrated that PRRSV infection induces a polyclonal activation of B cells in pigs [4,58], leading to generation of non-neutralizing as well as auto-antibodies [59]. Also in PRRSV-MLV administered pigs, enhanced production of non-neutralizing antibodies directed mainly to PRRSV-nucleocapsid protein (internal viral protein), and also antibodies directed to anti-decoy neutralizing epitopes of the virus was detected [7,57].

Table 3
Blood.

		PID 15	PID 30	PID 60
(A)	$CD3^+CD4^+$ – Th cells[a]			
	PRRS-MLV	9.3 ± 2.6	12.9 ± 2.6	16.4 ± 0.5
	PRRS-MLV+ *Mtb* WCL	9.4 ± 1.1	38.5 ± 3.9*	28.9 ± 2.4*
(B)	$CD4^+CD25^+Foxp3^+$ – Tregs[b]			
	PRRS-MLV	8.6 ± 0.4	11.6 ± 1.3	24.5 ± 0.6
	PRRS-MLV+ *Mtb* WCL	10.3 ± 1.6	14.4 ± 2.1	30.5 ± 2.2

Three mock-inoculated and nine pigs each inoculated with PRRS-MLV or PRRS-MLV+ *Mtb* WCL intranasally were euthanized on PID 15, 30 or 60 ($n = 3$ pigs at each PID), and lymphoid cell subsets were enumerated by flow cytometry.

[a] $CD3^+$ and $CD3^-$ cells were gated to enumerate CD4 and CD8α expression.

[b] $CD25^+$ cells were gated to enumerate CD4 and Foxp3 expression and the percent of total $CD4^+CD25^+Foxp3^+$ cells are shown. Each number is an average percent of immune cells from three pigs ± SEM.

* Statistically significant difference ($P < 0.05$) between PRRS-MLV+/− *Mtb* WCL inoculated pig groups.

In contrast, our study shows a reduction in the total PRRSV specific antibodies with increased VN titers mediated by *Mtb* WCL to PRRS-MLV. Generation of antibodies against mucosal pathogens and soluble protein antigens is dependent on $CD4^+$Th cells [60,61]. Consistent with that information, a polarized Th1-based response mediated via $CD4^+$and $CD8^+$T cells, and enhanced secretion of Th1 and pro-inflammatory cytokines were detected in PRRS-MLV+ *Mtb* WCL inoculated pigs. Generally, the Th1 and Th2 immune responses mutually suppress each other when either of one is significantly upregulated. Therefore, our results clearly suggest that the mucosal adjuvant *Mtb* WCL suppressed the secretion of PRRSV specific non-neutralizing antibodies and at the same time promoted the secretion of neutralizing antibodies critical for PRRSV clearance.

Apart from the effective humoral response, robust CMI response is critical for protective PRRSV immunity [8,56,57,62,63]. Pathogen specific mucosal $CD8^+$ T cells are required for the clearance of intracellular pathogens at both enteric and respiratory mucosal sites [64–66]. Consistent with those details, in PRRS-MLV+ *Mtb* WCL received pigs there was an increased frequency of $CD8^+$ T cells and secretion of Th1 cytokines in pig lungs.

Studies have reported enhanced immunosuppressive responses in PRRSV infected pigs mediated through increased IL-10 and reduced IFN-γ production [67,68]. In pigs, IL-10 reportedly inhibits IFN-γ production by T cells [69]. Infiltration of Tregs in the infected pig lung microenvironment contributes to secretion of high levels of IL-10 and TGFβ [70]. In PRRSV infected pigs, an increased frequency of Tregs was detected indicating their involvement in disease progression [71–73]. The immunosuppressive nature of PRRSV-MLV was also reported to be mediated by increased Tregs and IL-10 which results in delayed and reduced IFN-γ secretion [44–46].

To elicit a protective CMI response to PRRS-MLV, it is important to counteract the virus-induced immunosuppressive response. In our study, *Mtb* WCL mediated an increased frequency of $CD4^+$ and $CD8^+$ T cells with enhanced secretion of Th1 cytokines (IFNγ and IL-12), counteracted the virus induced immunosuppressive response by reciprocally downregulating the frequency of Tregs and secretion of immunosuppressive cytokines. Therefore, it is possible to protect pigs from both PRRSV outbreaks and poly-microbial infections by adapting mucosal vaccination strategies with the use of a potent adjuvant.

The pro-inflammatory cytokine, IL-6, plays an important role in initiating the adaptive immune response by influencing the proliferation of professional antigen presenting cells such as macrophages [74,75]. In our study, at an early time point (PID 7), significantly increased secretion of IL-6 mediated through *Mtb* WCL was detected in pig lungs. Associated with that finding, there was a significant increase in the myeloid cell population ($CD172^+$) in TBLN of pigs vaccinated with PRRS-MLV+ *Mtb* WCL. As a lymphoid organ, TBLN is a site where sensitization and activation of immune cells take place; therefore, increased frequency of myeloid cells in TBLN might indicate the *Mtb* WCL-mediated initiation of the CMI response.

Arginase helps in the metabolism of L-arginine into L-ornithine and subsequently in the generation of L-proline, L-glutamate, and L-glutamine [13]. A physiological level of arginase is essential in the body as arginase knockout mice die by 2 weeks after birth [76]. Glutamine is an important fuel for lymphocytes and macrophages, and plasma glutamine is decreased in HIV infected individuals, where a decrease in the levels of glutamine impairs lymphocyte function [77]. The amino acid proline promotes the growth and differentiation of B-cells and also NK cell activity [78,79]. Therefore, one of the immune dysregulation mechanisms of PRRSV in pig lungs appears to be mediated through downregulation of arginase, because reduced intracellular arginase was detected in pigs inoculated intranasally with PRRS-MLV. Therefore, mucosal immunization with *Mtb* WCL has the potential to rescue an important molecular mechanism mediated by the enzyme arginase in pigs vaccinated with PRRS-MLV. Considering the modulation of function of arginase by PRRS-MLV, the direct role of arginase on anti-PRRSV specific mucosal immunity needs to be investigated in detail.

In conclusion, *Mtb* WCL is considered as a potent mucosal adjuvant for PRRS-MLV in the pig respiratory tract. This adjuvant has the ability to potentiate innate, humoral, and CMI responses by modulating both cellular and molecular mechanisms required for generation of anti-PRRSV specific protective immunity. Further, in continuation of this study, adjuvanticity of *Mtb* WCL was also confirmed in post-challenge studies which detected a cross-protective immunity to a genetically variant PRRSV MN184 [80]. Considering the fact that PRRS-MLV by itself has the ability to reduce PRRSV viremia and is capable of providing partial protection, it may be a fruitful approach to use this vaccine along with *Mtb* WCL intranasally. The ability of *Mtb* WCL to overcome immunosuppression and to augment the virus specific CMI response opens up new avenues to explore its mucosal adjuvanticity against other viral infections.

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