Mycobacterium tuberculosis Whole Cell Lysate Enhances Proliferation of CD8 Positive Lymphocytes and Nitric Oxide Secretion in the Lungs of Live Porcine Respiratory and Reproductive Syndrome Virus Vaccinated Pigs

Cordelia Manickam,^{1,3} Varun Dwivedi,¹ Jayla Miller,¹ Tracey Papenfuss,² and Gourapura J. Renukaradhya^{1,3}

Abstract

Porcine respiratory and reproductive syndrome (PRRS) is an economically important disease of pigs worldwide. Currently used PRRSV vaccines provide incomplete protection. Recently, we identified *Mycobacterium tuberculosis* whole cell lysate (*Mtb* WCL) as a potent mucosal adjuvant to modified live PRRSV vaccine (PRRS-MLV). In this study, pigs were unvaccinated or vaccinated with PRRS-MLV plus *Mtb* WCL, intranasally, and challenged with either homologous (strain VR2332) or virulent heterologous (strain MN184) PRRSV; subsequently, euthanized at three time points post-challenge to evaluate lung immune responses. Microscopic examination of lung sections revealed reduced disruption of the lung architecture and less of interstitial pneumonia in vaccinated, compared to unvaccinated MN184 challenged pigs. The restimulated lung and peripheral blood mononuclear cells revealed increased proliferation of CD8⁺ lymphocytes, and in the lung homogenate increased secretion of nitric oxide was detected in vaccinated MN184 challenged pigs. In summary, the adjuvant effects of *Mtb* WCL to PRRS-MLV resulted in favorable anti-PRRSV immune microenvironment in the lungs to help better viral clearance.

Introduction

PORCINE REPRODUCTIVE AND respiratory syndrome (PRRS) is the most devastating problem plaguing the global swine industry within the last two decades. The annual economic losses due to PRRS is estimated to be \$664 million in the US (1). PRRS is characterized by both respiratory and reproductive abnormalities (2–4), with major losses from reproductive failure associated with stillbirths, mummifications, weak born piglets, and high preweaning mortality (5–7). The respiratory form leads to pneumonia, reduced feed intake with chronically recurring illness, and debilitation with potentially high mortality. The causative agent PRRS virus (PRRSV) is an *Arterivirus* having a positive sense RNA genome. The PRRSV is prone to constant genetic and antigenic variations. Based on its genetic diversity, the PRRSV can be divided into two genotypes, Type1 (European) and Type 2 (North American). The strains Lelystad and VR2332 are the reference prototype strains of Type 1 and Type 2 PRRSV, respectively. PRRSV replicates in alveolar macrophages and persists for up to 150 days in the lymphoid tissues (8). The porcine immune system is unable to mount an effective anti-

PRRSV immune response in the first few weeks post-infection (9,10); attributed to dampened production of anti-viral cytokines, virus neutralizing antibodies, and cell-mediated immune responses. In addition, immunosuppression caused by the virus makes infected pigs prone to secondary bacterial and viral infections (11).

To control PRRS outbreaks, commonly employed vaccines include modified live PRRSV (PRRS-MLV) and killed virus vaccines. Killed vaccine does not provide adequate protection. PRRS-MLV provides satisfactory protection against homologous infections, but protection against heterologous viruses and reinfections is incomplete. There are reports of results obtained using field isolates of PRRSV and PRRS-MLV administered by either parenteral or intranasal route showing suppression of innate NK cell cytotoxic function and IFN- α production (12–14), weak and delayed cell-mediated and virus neutralizing antibody responses (15,16), associated with prolonged immunosuppression (17). To improve the efficacy of PRRS-MLV in pigs, we explored the benefits of inducing anti-PRRSV mucosal immunity by intranasal co-administration of PRRS-MLV with a suitable mucosal adjuvant (17).

¹Food Animal Health Research Program, Ohio Agricultural Research and Development Center, and Departments of ²Veterinary Biosciences and ³Veterinary Preventive Medicine, The Ohio State University, Wooster, Ohio.

Intranasal immunization of certain vaccines has shown great promise in eliciting protective immunity against various respiratory viral infections such as bovine herpes virus-1, influenza, and parainfluenza-3 (18,19). Mucosal tissues and associated lymphoid organs contain approximately 80% of total immune cells in the body, and stimulation of the mucosal immune system leads to generation of both systemic and mucosal immune responses, resulting in effective prevention of entry and establishment of pathogens (20,21). Recently, we have demonstrated that intranasal co-administration of PRRS-MLV and adjuvant *Mycobacterium tuberculosis* whole cell lysate (*Mtb* WCL) can overcome the vaccine induced immunosuppression with concomitant increase in host innate and adaptive immune responses, associated with better protection against a virulent heterologous PRRSV MN184 challenge (12,17,22,23). We have also shown that protective anti-PRRSV immune response elicited in pigs to PRRS-MLV was mediated by the adjuvant effects of *Mtb* WCL, as pigs vaccinated with only PRRS-MLV failed to elicit adequate immune responses (22). In continuation, this article deals with aspects of microscopic lung pathology, proliferation of PRRSV specific CD8⁺ lymphocytes, and production of an important innate molecule 'nitric oxide' in the lungs of pigs vaccinated (PRRS-MLV + *Mtb* WCL) and challenged with PRRSV.

Materials and Methods

Pigs and inoculations

Conventional Large White-Duroc crossbred specific-pathogen-free piglets weaned at 3 to 4 weeks of age were obtained from farms regularly screened and free from PRRSV, porcine respiratory coronavirus, transmissible gastroenteritis virus, and porcine circovirus type 2 were used in the study. Before beginning of our study, pigs were tested to confirm absence of PRRSV antibodies by ELISA. Throughout the duration of the study, pigs received food and water *ad libitum*. All inoculations including PRRSV challenge was performed by intranasal delivery. Animals were maintained, samples collected, and euthanized in our large animal BSL2 facility as per the protocol approved by the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC), The Ohio State University, Ohio. Forty pigs were allocated to one of five groups: group 1, mock pigs ($n=4$) inoculated with vehicle (normal saline) and DMEM; other four groups ($n=9$ per group), groups 2 and 3, unvaccinated; groups 4 and 5, vaccinated [PRRS-MLV (Boehringer Ingelheim Vetmedica, Inc.) + *Mtb* WCL]. Groups 2 and 4 were challenged with a homologous parenteral PRRSV strain VR2332 (2×10^6 TCID₅₀ per pig), and groups 3 and 5 were challenged with virulent heterologous PRRSV MN184 (24) (1×10^6 TCID₅₀ per pig) in 2 mL on day 21 post-immunization. Nine pigs each under groups 2 to 5 were euthanized at three time points at 15, 30, and 60 day post-challenge (DPC) ($n=3$ per group). Mock inoculated pigs ($n=4$) were euthanized together prior euthanizing any virus challenged animals.

Microscopic lung pathology

Lung tissue samples collected from pigs was fixed in 10% neutral buffered formalin and processed into paraffin blocks,

three μm sections were cut and stained with haematoxylin and eosin as described previously (25). The lung sections were examined in a blindfolded fashion by a board certified veterinary pathologist to determine the lung lesion scores by virus-induced pathology (26).

Carboxyfluorescein diacetate succinimidyl ester proliferation assay

To determine PRRSV specific proliferation of lymphocytes, lung mononuclear cells (LMNC) and peripheral blood mononuclear cells (PBMC) were isolated as described (27–29). Cells were stained with an irreversible cytosolic dye, (carboxyfluorescein diacetate succinimidyl ester, CFSE), as described previously (30). Briefly, five million cells were washed and stained with CFSE at $10 \mu\text{M}$ concentration in PBS and incubated for 15 min at room temperature. The labeling reaction was stopped using ice-cold RPMI containing 10% FBS, washed twice, and resuspended with the medium and incubated at 37°C for 30 min, and adjusted the cell numbers to 5×10^5 cells per well in a 96-well flat bottom plate. The cells were restimulated with semi-purified PRRSV antigens ($20 \mu\text{g}/\text{mL}$) of VR2332 and MN184 strains, obtained by ultracentrifugation of clarified PRRSV infected MARC 145 cell culture supernatant. Cells untreated or treated with Concavalin A were used as negative and positive control, respectively. Following 3 days of incubation, cells were washed and stained with R-phycoerythrin conjugated anti-pig CD8 antibody (clone 76-2-11, BD Biosciences). Finally, cells were fixed and 50,000 to 100,000 events acquired using the FACS AriaII (BD Biosciences) flow cytometer and analyzed for CFSE and PE stained lymphocytes. The cell proliferation data was analyzed by FlowJo software (Tree Star, Inc. OR) to obtain the proliferation index (PI) of each sample. The PI denotes the average number of cell divisions the responding lymphocytes underwent after subtracting from the background (initial population of cells at generation '0').

Griess assay

Nitric oxide production in the lungs was determined by measuring nitrite levels, which is formed as nitric oxide breakdown product using a commercial kit (Promega). Lung homogenates were prepared from the lung tissue samples as described previously (13). Samples were treated with a sulfanilamide solution for 5–10 min in the dark, and subsequently with *N*-1-naphthylethylenediamine dihydrochloride (NED) solution. The resulting reaction product was measured at an absorbance between 520 and 550 nm, and the absorbance was calculated by generating a nitrite standard reference curve.

Statistical analysis

All data were expressed as the mean $\pm$ SEM of three pigs. Statistical analyses were performed using two-sample *t*-test to establish differences between vaccinated and unvaccinated groups. Statistical significance was assessed as $p < 0.05$.

Results and Discussion

Porcine reproductive and respiratory syndrome virus has evolved into a major swine disease over the last 2 decades. We have recently demonstrated the benefits of intranasal

delivery of PRRS-MLV with a potent mucosal adjuvant, *Mtb* WCL, in stimulating favorable anti-PRRSV immune responses against virulent heterologous (MN184) PRRSV (12,22). As a potent mucosal adjuvant, we used mechanically lysed endotoxin-free WCL of *Mtb* which contains predominantly soluble intracellular bacterial proteins and is almost free from toxic cell wall lipid components (31) (Torrelles JB, personal communication). Clinically, pigs administered with *Mtb* WCL did not show any signs of side effects. In vaccinated (PRRS-MLV plus *Mtb* WCL) pigs, reduced gross lung pathology and significantly reduced viremia were attributed to adjuvant mediated innate and PRRSV specific adaptive immune responses (22). Here, in the same set of pigs, we are providing additional important information in the lung microenvironment with reference to status of PRRSV specific CD8⁺ lymphocytes, nitric oxide production, and microscopic lung pathology.

Histological examination has revealed reduced lung lesions (Fig. 1), indicated by reduced infiltration of mononuclear cells and interstitial pneumonic changes in vaccinated compared to unvaccinated MN184 challenged pigs (Fig. 1A). Further, in vaccinated, MN184 challenged pigs reduced (although not statistically significant) trend in lung lesion scores was detected at DPC 15 and 30 compared to unvaccinated pigs (Fig. 1Bi). Due to the low virulence nature of VR2332 strain, the inflammatory changes in the lungs were lesser compared to MN184 challenged pigs (Fig. 1Bi and Bii).

The cell-mediated immunity against virus infections is contributed by different lymphocyte subsets, in particular contributed significantly by CD8⁺ cytotoxic T lymphocytes

(CTL) and activated Th/memory CD4⁺CD8⁺ T cells (19,32). PRRSV-specific lymphocytes present in the PBMC of infected pigs proliferate from 4-week post-infection (33,34). However, so far, knowledge related to the status of CD8⁺ lymphocytes in the lungs of PRRSV infected and/or vaccinated pigs is limited. Since the PRRSV replicates in the lungs of infected pigs, such information is important. In our study, increased proliferation of total PRRSV-specific lymphocytes in the lungs (LMNC) and blood (PBMC) of vaccinated compared to unvaccinated, and either PRRSV strain challenged pigs was detected (Fig. 2A and data not shown). Further, significantly increased proliferation of CD8⁺ lymphocytes in the PBMC of MN184 challenged and LMNC of VR2332 challenged pigs restimulated with MN184 antigens was detected (Fig 2B, Cii and Ciii). In pigs, apart from CD8⁺ T cells, CD4⁺CD8⁺ T lymphocytes are abundantly present, and they possess memory, activated T-helper and cytolytic properties (32,35–37). Since the antigen-specific proliferation occurs only by CD4⁺ and CD8⁺ T cells, our results indicated that PRRSV-specific proliferation occurred in both CD8⁺ and CD4⁺CD8⁺ T lymphocytes.

Nitric oxide (NO') is an important immune molecule involved in both innate and adaptive immunity. It helps in the clearance of intracellular pathogens by inhibiting viral replication and plays a major role in lung immunity and virus clearance. It has been shown to inhibit replication of influenza virus and herpes simplex virus type1 (38–40). A large amount of pulmonary NO' is produced by the epithelial lung inducible nitric oxide synthase (41), which acts on L-arginine to produce NO'. To determine the production of NO' in the

FIG. 1. Reduced infiltration of inflammatory cells and lung pathology in vaccinated PRRSV challenged pigs. Pigs were unvaccinated or vaccinated with PRRS-MLV plus *Mtb* WCL intranasally and challenged with PRRSV strain MN184 or VR2332, and euthanized at DPC 15, 30, and 60. Lung sections were stained with H&E: **(A)** (i) mock pigs; (ii) pigs unvaccinated and challenged with MN184; (iii) pigs vaccinated (PRRS-MLV plus *Mtb* WCL) and challenged with MN184. **(B)** H&E stained slides were examined microscopically for PRRSV induced lung pathology: **(i and ii)** scores of the lungs affected with inflammatory lesions.

FIG. 2. Vaccinated pigs had enhanced proliferation of CD8⁺ lymphocytes. **(A)** A representative overlaid histograms of proliferated PRRSV specific lymphocytes is shown. Pigs were unvaccinated or vaccinated (PRRS-MLV + *Mtb* WCL) and challenged with MN184 and euthanized at DPC 15. LMNC were stained with CFSE and restimulated with killed PRRSV antigens: **(i and ii)** MN184 and **(iii and iv)** VR2332, subsequently cells were analyzed by flow cytometry to determine total lymphocyte proliferation. **(B)** A representative overlaid histograms of proliferated CD8⁺ cells post-restimulation with either MN184 antigen or no antigen is shown in pigs unvaccinated or vaccinated (PRRS-MLV + *Mtb* WCL), challenged with MN184 and euthanized at DPC 30. PBMC were first gated for cells immunostained with R-phycoerythrin-conjugated pig specific CD8⁺ antibody and then analyzed for CFSE. Dilution of the dye CFSE indicates higher proliferation of cells. **(C)** PRRSV specific CD8⁺ lymphocyte proliferation data is shown. Pigs were unvaccinated or vaccinated and challenged as described above and euthanized at DPC 30. **(i and iii)** LMNC and **(ii and iv)** PBMC were stained with CFSE and subjected to either no stimulation (No Ag) or restimulated with indicated strain of killed PRRSV antigens. Each bar represents the average proliferation index of CD8⁺ lymphocytes from three pigs $\pm$ SEM. Asterisk denotes statistically significant difference ($P < 0.05$) between unvaccinated and vaccinated pigs.

pig lung, lung homogenates (which represent the lung parenchyma) were prepared and used in the analyses. A significant increase in the production of NO⁺ in the lungs of pigs received adjuvanted vaccine and challenged with virulent PRRSV MN184 at DPCs 30 and 60 was detected (Fig. 3A). Levels of NO⁺ production in PRRS-MLV alone vaccinated and MN184 challenged pigs was high and it was comparable to unvaccinated virus challenged animals at DPC 30 (data not shown).

In the lungs of pigs vaccinated and challenged with VR2332 strain, an opposite trend in the NO⁺ production was detected at DPC 15, and at DPC 30 and 60 the levels were comparable between the groups (Fig. 3B), which could be due to differential immune responses elicited by the avirulent VR2332 strain (42). Earlier reports have demonstrated the absence of NO⁺ in bronchoalveolar lavage fluid of a mild PRRSV strain SD23983 infected pigs (43), and production of inducible nitric oxide synthetase (iNOS) in alveolar macro-

phages (44). However, pulmonary epithelial cells in the respiratory viral infections contribute significantly in the production of NO⁺ (45). In our study, we used lung homogenates, and in pigs vaccinated with PRRS-MLV plus *Mtb* WCL and challenged with a virulent heterologous PRRSV strain MN184, significantly increased NO⁺ production was detected in the lung homogenate.

Earlier, we have shown an increased secretion of reactive oxygen species (ROS) by bronchoalveolar lavage fluid cells and PBMC of unvaccinated compared to vaccinated (PRRS-MLV plus *Mtb* WCL) MN184 challenged pigs at three DPCs (23). While ROS and reactive nitrogen species (RNS) are needed for antimicrobial activity, their overproduction leads to bystander cell death, inflammation, and tissue injury (46,47). NO⁺ is one of the RNS, and it reacts with oxygen radicals, leading to production of more ROS and RNS. A balance between NO⁺ and ROS will influence the outcome (48). Thus, it is possible that PRRS-MLV plus *Mtb* WCL

FIG. 3. Elevated nitric oxide production in vaccinated pigs. Pigs were unvaccinated or vaccinated (PRRS-MLV + *Mtb* WCL) intranasally and challenged with PRRSV strain: (A) MN184 and (B) VR2332. Lung homogenates were estimated for nitric oxide production using Griess reagent. Each bar represents the average amount of nitric oxide in mM concentration per gram of lung tissue from three pigs $\pm$ SEM. Asterisk denotes statistically significant difference ($p < 0.05$) between unvaccinated and vaccinated pigs.

vaccination optimally increased the NO' levels in the lungs to virulent MN184 challenge, which however did not lead to lung injury, rather helped in better anti-PRRSV immune response compared to control pigs (22).

In conclusion, intranasal co-administration of PRRS-MLV and *Mtb* WCL elicits favorable immune responses against a virulent heterologous PRRSV challenge; indicated by reduced gross lung pathology, early lung viral clearance, activation of virus specific immune cells, early virus neutralizing antibody generation, increased Th1, and reduced immunosuppressive cytokine production (22). In addition, in this study in the lungs of pigs vaccinated and MN184 challenged reduced microscopic lesions, increased proliferation of virus specific CD8⁺ T cells, and enhanced NO' production were detected. Thus, our results further supported the favorable anti-PRRSV immune responses in the pig lung microenvironment mediated by the adjuvant *Mtb* WCL to PRRS-MLV.

Acknowledgments

This research was supported by National Pork Board and USDA-NIFA PRRS CAP2 award 2008-55620-19132 to GJR. Salaries were provided by the state and federal funds appropriated to OARDC, The Ohio State University. We would like to thank Ms. Ruth Patterson for her help in the preparation of immune cells. Dr. Juliette Hanson, Todd Root, Mathew Weeman, Drs. Mahesh Khatri and Hadi Yassine for their help in animal studies. Drs. Eric Nelson and Michael Murtaugh provided PRRSV reagents.

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Dr. Gourapura J. Renukaradhya
Food Animal Health Research Program
Ohio Agricultural Research and Development Center
The Ohio State University
1680 Madison Avenue
Wooster, OH 44691
E-mail: gourapura.1@osu.edu