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Interleukin-1β expression by a recombinant porcine reproductive and respiratory syndrome virus

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ABSTRACT

The cytokine interleukin-1 beta (IL-1β) is a potent inflammatory mediator in response to infection, and can be used as an immunological adjuvant. In this study, we constructed a recombinant porcine reproductive and respiratory syndrome virus (vP129/swIL1β) expressing swine IL-1β from the separate subgenomic mRNA inserted between the ORF1b and ORF2 genome region. MARC-145 cells infected with vP129/swIL1β secreted 1947 pg of IL-1β per 2×10^5 cells at 36 h post-infection. *In vitro* growth kinetics analysis in MARC-145 cells showed that the vP129/swIL1β virus had a similar replication rate as that of parental virus. We further performed *in vivo* characterization of the vP129/swIL1β virus in a nursery pig disease model. The vP129/swIL1β infected pigs did not show visible clinical signs, while respiratory distress and lethargy were evident in pigs infected with the parental virus. The expression of various cytokines from peripheral blood mononuclear cells measured by fluorescent microsphere immunoassay showed that IL-1β, IL-4 and IFN-γ expression levels were up-regulated in pigs infected with vP129/swIL1β at 7 and 14 days post-infection. However, no detectable level of IL-1β was found in serum samples from pigs infected with either vP129/swIL1β or parental virus. In summary, this study demonstrated a recombinant PRRSV as a useful tool to study the role of different cytokines in disease progression and immune responses, which represents a new strategy for future therapeutic application and vaccine development.

1. Introduction

Cytokines are signaling molecules for intercellular communication such as the induction of immune responses by recruiting immune cells and regulating cell proliferation and differentiation. During viral infection, cytokines play a central role in the innate and adaptive immunity. For example, both Type I and II interferons (IFNs), and various interleukins (ILs) play crucial roles in the control of viral replication at the early stage of virus infection. The innate cytokines are also important in the induction of adaptive immunity specific to the infecting virus by inducing inflammation responses. The excessive inflammation due to viral infection-mediated cytokine production may also lead to immunopathic conditions and pathogenicity of the viral infections. Because of the important role that cytokines play in the immune response, cytokines have been studied for their application in therapeutic treatment and in vaccine formulations (Andersson and Tracey, 2011; Heegaard et al., 2011; Leong et al., 1994). However, since cytokines have a short half-life and require large dose administration systemically in order to be fully functional, new methods to deliver cytokines to the site of infection have been developed, including the creation of chimeric molecules, delivery vehicles, and the use of viral vectors to express cytokines (Ahlers et al., 2003; Yilma et al., 2010).

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small-enveloped virus containing a single positive-stranded RNA genome. PRRSV is classified in the Order *Nidovirales*, family *Arteriviridae* (Snijder and Meulenberg, 1998). Nucleotide sequence comparisons show that PRRSV can be divided into distinct European (Type I) and North American (Type II) genotypes, possessing only about 63% nucleotide identity at the genomic level (Allende et al., 1999; Nelsen et al., 1999). PRRSV causes mild to severe respiratory disease in infected newborn and growing swine, and reproductive failure in pregnant sows (Rossow et al., 1995; Rossow, 1998). PRRSV infection stimulates a minimal level of innate cytokine production. The inflammatory cytokines, including IL-1β,

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IL-6 and IL-8, have been detected in virus-infected pigs (Aasted et al., 2002; Suradhat et al., 2003; Suradhat and Thanawongnuwech, 2003; Thanawongnuwech and Thacker, 2003; Bassaganya-Riera et al., 2004; Thanawongnuwech et al., 2004; Lunney et al., 2010). However, in comparison to the other strong immunogenic swine pathogens, such as swine influenza virus and pseudorabies virus, the quantities of secreted cytokines appeared to be much lower. It is suggested that such poor innate anti-viral IFN and cytokine responses result in a weak adaptive immune response.

There are still no therapeutic drugs or completely effective vaccines to control PRRSV. Currently available modified live vaccines have shown the most promising efficacy but have only been partially successful in protection against a wide array of heterologous PRRSV isolates in the field (Bøtner et al., 1997; Mengeling, 2005). In the effort of new vaccine development, cytokines have been evaluated as adjuvants during the immunization of animals (Zuckermann et al., 1998, 1999; Somasundaram et al., 1999; Foss et al., 2002; Murtaugh et al., 2002; Boyaka et al., 2003; Petrovsky and Aguilar, 2004; Royaee et al., 2004). Interleukin-1 (IL-1) is a potent inflammatory mediator in response to infection. IL-1β has been tested as an immunological adjuvant because of its ability to influence a broad spectrum of biological activities within the immune system. It was reported that co-administration of recombinant bovine IL-1β and a modified live BHV-1 vaccine was able to enhance both the humoral and cellular immune response against BHV-1 (Reddy et al., 1990, 1993). A recombinant BHV-1 expressing bovine IL-1β showed that both cellular and secreted forms of recombinant IL-1β possessed biological activity, and the expression of IL-1β protein did not affect the *in vitro* growth efficiency of the virus (Raggo et al., 1996). In this study, we explored the strategy of manipulating the immunogenic properties of a virus by expression of a swine IL-1β (swIL1β) protein using a PRRSV reverse genetic system. A recombinant PRRSV, designated vP129/swIL1β, was generated. This recombinant virus was further tested in a nursery pig model to determine whether swine IL-1β could be successfully delivered *in vivo*. The effect of *in vivo* expression of swine IL-1β on the pathogenic property of the virus as well as the PRRSV specific immune response was further examined.

2. Materials and methods

2.1. Cells and viruses

BHK-21 cells were used for initial transfection and recovery of recombinant virus. MARC-145 cells were used for recombinant virus rescue and subsequent experiments. These cells were maintained in Eagle's minimum essential medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics at 37 °C with 5% CO_2. Porcine alveolar macrophages (PAMs) were obtained by lung lavage of 6-week-old PRRSV-naıve piglets using a method described previously (Zeman et al., 1993). Peripheral blood mononuclear cells (PBMCs) were isolated from infected and control pigs as described previously (Meier et al., 2003) and plated in a 24-well plate (4×10^6 cells/ml). PBMCs were stimulated with 50 μl UV-inactivated PRRSV (2×10^5 $TCID_{50}$); parallel cultures were stimulated with the mitogen phytohemagglutinin (PHA), or medium, and served as positive and negative controls, respectively. Stimulated cultures were incubated at 37 °C for 24 h. Cell culture supernatants were collected from stimulated cells and stored at −70 °C for cytokine analysis.

The IL-1β expressing recombinant PRRSV (vP129/swIL1β) was generated from a North American Type II PRRSV full-length cDNA infectious clone, P129 (Calvert et al., 2002, 2003). The third passage of the MARC-145 cell-recovered parental (vP129) or recombinant virus was used for *in vitro* and *in vivo* experiments. Viral titers were determined by titration on MARC-145 cells and quantified as 50% tissue culture infective dose ($TCID_{50}$)/ml.

2.2. Construction of the plasmid (pCMV-S-P129-1bswIL1β) for generation of the IL1β expressing recombinant PRRSV (vP129/swIL1β)

The plasmid pCMV-S-P129-1bswIL1β was constructed using pCMV-S-P129-1bGFP2 (Kermit) (Calvert et al., 2009) in which GFP was inserted in the region between ORF1b and ORF2 with *AflII* and *MluI* enzyme sites (Fig. 1A). The expression of GFP in the construct was derived from the leader/junction (L/J) sequence for ORF2, and the ORF6 L/J was inserted downstream of the GFP gene to drive the expression of ORF2, so that GFP is expressed from the independent subgenomic RNA. The GFP gene was replaced with swine IL-1β gene that was amplified from PAM by RT-PCR using primer pairs of IL-1b-Afl-F (*5′-aattcttaagatggccatagtacctgaacc*) and IL-1b-Mlu-R (*5′-aattacgcgtttagggagagaggacttcc*). The amplicon was digested with *AflII* and *MluI* enzymes and cloned into the plasmid after it was digested with the same enzyme and removed the GFP gene.

The plasmid pCMV-S-P129-1bswIL-1β was transfected into BHK-21 cells as described previously (Calvert et al., 2002, 2003). To rescue the virus, cell culture supernatant obtained at 48 h post-transfection was passaged on MARC-145 cells. Rescue of infectious virus was determined by an indirect immunofluorescent assay (IFA) using PRRSV-specific monoclonal antibody (mAb) SDOW17 as we described previously (Fang et al., 2006, 2008). The expression of swIL1β recombinant protein from the vP129/swIL1β in MARC-145 cells and PAMs was confirmed by Western blot using the polyclonal antibody to the protein (R&D systems).

2.3. In vitro characterization of vP129/swIL1β recombinant virus

The growth kinetics of the recombinant and parental viruses were examined by infecting MARC-145 cells with viruses at a multiplicity of infection (MOI) of 0.5. Infected cells were collected at 0, 6, 12, 24, 36, 48, 60 and 72 h post infection (hpi), and viral titers were determined by immunofluorescent assay (IFA) on MARC-145 cells and quantified as fluorescent focus units (FFU)/ml. Plaque morphology of each virus was determined by plaque assay on MARC-145 cells as described previously (Fang et al., 2006). To determine the expression of swine IL-1β, the culture supernatant collected at different time points was analyzed using a fluorescent microsphere immunoassay (FMIA) as we described previously (Lawson et al., 2010).

2.4. Sequencing of deletion regions

To determine the stability of the IL-1β gene in the recombinant virus, cell lysate from virus-infected cells was harvested, and RNA was extracted using a QIAamp viral RNA kit (Qiagen) following the manufacturer's instruction. The corresponding insertion region was amplified by RT-PCR using primers p129-11931F (*5′-gggaggattacaatgatgcgtttcg*) and p129-12159R (*5′-gatggtgaagccaaacaaaatggcc*). PCR products were sequenced at the Iowa State University DNA sequencing facility (Ames, IA, USA).

2.5. Animals and infection groups

Eighteen 3-week-old, PRRSV-naıve pigs obtained from a certified PRRSV-negative herd were divided randomly into three groups and housed separately in animal isolation facilities at South Dakota State University (SDSU). After a four-day acclimation period, pigs from each group ($n = 6$) were inoculated with 2 ml (1×10^6 $TCID_{50}$,

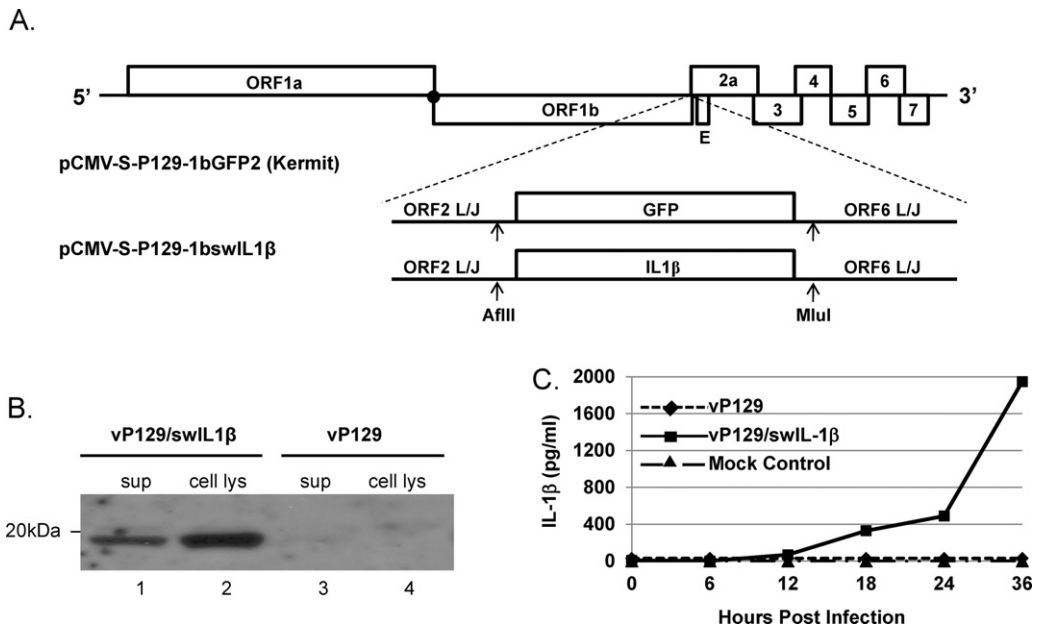

Fig. 1. Expression of swIL1β by vP129/swIL1β recombinant virus. (A) Construction of the plasmid, pCMV-S-P129-1bswIL1β. The GFP gene in pCMV-S-P129-1bGFP2 (Kermit) backbone was replaced by swine IL-1β gene. The IL-1β is expressed as an independent subgenomic RNA, which was derived from the leader/junction (L/J) sequence of ORF2. The ORF6 L/J was inserted downstream of the IL-1β gene to drive the expression of ORF2; (B) Western blot analysis of the detection of swIL1β in the supernatant fraction (sup) or cell lysates (cell lys) of MARC-145 cells infected with vP129/swIL1β or parental P129 virus. Cells were infected with the MOI of 1 and incubated for 24 h before sample preparation for Western blot analysis, and (C) kinetics of accumulation of swine IL-1β in culture supernatant of MARC-145 cells infected with vP129/swIL1β or parental virus. MARC-145 cells were infected in parallel at MOI of 0.5, and culture supernatant was collected at indicated time points. The swine IL-1β expression levels were determined by Luminex fluorescent microsphere immunoassay (FMIA).

given as 2 ml intranasally) of parental virus (group 1), recombinant virus (group 2) or mock-infected with cell culture medium (group 3).

2.6. Clinical signs and serum and tissue sampling

Clinical signs and rectal temperatures were monitored every day during the first five days post-inoculation. Clinical signs were recorded and quantified using a clinical scoring system adapted from Martínez-Lobo et al. (2011). The scoring system was designed to evaluate clinical respiratory signs including sneezing, nasal secretions, coughing and labored breathing. In addition, systemic clinical signs including hair roughness, lethargy and anorexia were also recorded and scored. Severity of clinical symptoms was evaluated on a scale of 0–5 whereby a score of 5 was considered to be the most severe. Blood samples were obtained from all pigs on days 0, 3, 7, 14, 21 and 28 dpi. Pigs were euthanized at 28 dpi to evaluate macroscopic and microscopic lung lesions. Lung lesions of the study animals were evaluated using a previously developed system (Halbur et al., 1995) based on the approximate volume that each lobe contributes to the entire lung: the left and right apical lobes, the left and right cardiac lobes, and the intermediate lobe each contribute 10% of the total lung volume, and the left and right caudal lobes each contribute 25%. These scores were then used to calculate the total lung lesion score based on the relative percentage contributions of each lobe. Blood and tissue [lungs, tonsils and lymph nodes (submandibular lymph node, lateral retropharyngeal lymph node, mesenteric lymph nodes)] samples were collected at the time of euthanasia. For tissue sampling, a representative tissue was collected from each lobe of the lung, while the entire tonsil and lymph node were collected from each pig.

All animal experiments were approved and guided by the Institutional Animal Care and Use Committee (IACUC) at South Dakota State University in accordance with Animal Welfare Assurance Guidelines.

2.7. Real-time RT-PCR quantification of viral load in infected animals

For the detection of viral RNA and determination of viral load, serum and tissue samples were examined using a real-time quantitative RT-PCR (Tetracore VetAlert PRRS), which is routinely performed at the South Dakota Animal Disease Research and Diagnostic Laboratory (SD-ADRDL) under strict quality assurance guidelines. The detailed method has been described in our previous publication (Wasilk et al., 2004).

2.8. Determination of humoral immunity

To compare the humoral antibody response of the treatment groups, all serum samples were evaluated using an IDEXX HerdChek PRRS 2XR ELISA following the manufacturer's instruction. The virus neutralization assay was performed as described previously (Yoon et al., 1994).

2.9. FMIA for cytokine expression measurement

Serum samples and culture supernatant from PBMCs were evaluated for expression of selected swine cytokine proteins targeting immune markers for innate immunity: IL-1β, IL-8, IFN-α, TNF-α, IL-12; Th1 immunity: IFN-γ; Th2 immunity: IL-4; and regulatory T cell response: IL-10. Detailed FMIA procedure for cytokine protein detection was described in our previous publication (Lawson et al., 2010). Relative quantification of target cytokine expression was evaluated using mean fluorescent intensity (MFI) values, and each group's results were compared with the mean values for control pigs (or control cultures).

2.10. Statistical analysis

Statistical analysis was performed using GraphPad InStat version 3.06 (GraphPad Software). Comparison was performed by one-way analysis of variance with Tukey's multiple comparison tests to determine mean significance. Differences between groups of pigs at different times post-inoculation were considered statistically significant at $P < 0.05$ for all analyses.

3. Results

3.1. Expression of swIL1β by recombinant virus

The transfection of pCMV-S-P129-1bswIL1β plasmid DNA into BHK cells yielded the recombinant virus vP129/swIL1β. The vP129/swIL1β viruses were rescued by passage of the cell culture supernatant onto MARC-145 cells, which was evident by IFA staining with anti-nucleocapsid protein monoclonal antibody SDOW17. To detect the IL-1β protein expression in infected cells, Western blot was conducted. As shown in Fig. 1B, a protein band with apparent molecular weight between 17 and 20 kDa was recognized by anti-swIL1β antibody in the cellular and supernatant fractions of vP129/swIL1β infected cells, but not in the wild-type virus infected cells. To measure the amount of swIL1β protein secreted from infected cells, cell culture supernatant collected at 6, 12, 18, 24 and 36 hpi was analyzed (Fig. 1C). The concentration of swIL1β was 400 pg at 18 hpi and reached the maximum concentration of 1947 pg at 36 h, which corresponds to about 2 ng per 2×10^5 cells. We further assessed the stability of swine IL-1β gene in recombinant virus by serial passage of the vP129/swIL1β virus at least 10 times on MARC-145 cells. The IL-1β insertion region was analyzed. Total cellular RNA was isolated from cells infected with the passage 3 and 10 of the recombinant virus, and RNA was used as the template for RT-PCR amplification of the insertion region. The PCR product was sequenced, and results showed that the IL-1β gene remained intact as a full-length gene, and no mutation or deletion was detected.

3.2. In vitro growth characteristics of vP129/swIL1β recombinant virus

To determine whether the insertion/expression of swIL1β has an effect on viral replication, we compared the *in vitro* growth properties of the vP129/swIL1β with that of parental viruses. MARC-145 cells were infected with each of the viruses and harvested at 6, 12, 24, 36, 48, 60, and 72 hpi. Growth kinetics analysis showed that the vP129/swIL1β virus possessed a similar growth rate to that of the parental virus (Fig. 2A). Titers peaked at 48 hpi for both viruses. The peak titer of the vP129/swIL1β virus was 3.7×10^5 FFU/ml, compared to 3.0×10^5 FFU/ml for the parental virus. Plaque morphology of these viruses was also determined, and the plaque size produced by the vP129/swIL1β virus was similar to the parental virus (Fig. 2B). These results indicate that the insertion of swine IL-1β into the viral genome does not affect the viral replication *in vitro*.

3.3. In vivo growth and pathogenic property of vP129/swIL1β virus

A total of 18 three-week-old pigs, divided into three groups, were infected with vP129/swIL1β virus, parental virus or cell culture medium (mock infected). The clinical signs and body temperature were monitored during the first five days post-inoculation. As shown in Table 1, all pigs inoculated by parental virus developed clinical signs, showing respiratory distress (coughing, serious nasal discharge) and lethargy, and four pigs had rectal

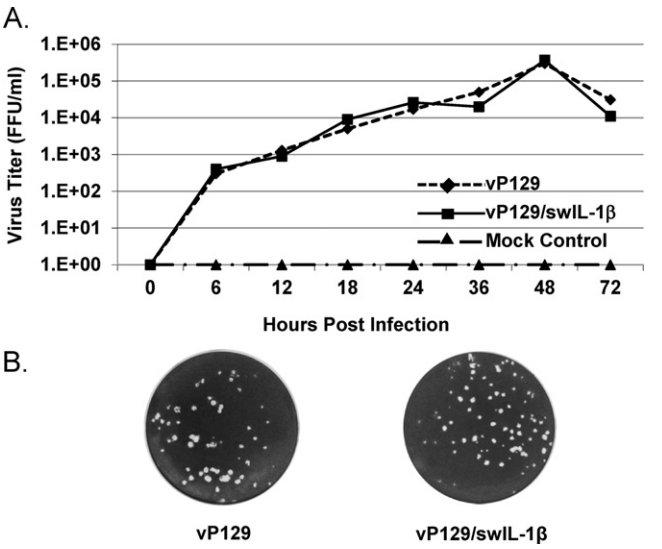

Fig. 2. *In vitro* growth characterization of vP129/swIL1β recombinant virus. (A) Growth kinetics comparison between vP129/swIL1β and parental virus in MARC-145 cells. MARC-145 cells were infected in parallel at MOI of 0.1 with the passage three of chimeric or parental virus. At 6, 12, 24, 36, 48, 60, and 72 h post-infection, cells were harvested, and the virus titers were determined by IFA on MARC-145 cells. The results were mean values from three replications of the experiment, and viral titers were expressed as fluorescence focus units per milliliter (FFU/ml), and (B) Plaque morphology of vP129/swIL1β and parental virus. Confluent cell culture monolayers were infected with viruses at a MOI of 0.1. After 2 h infection, cell culture supernatant was removed and an agar overlay was applied. Plaques were detected after five days incubation at 37 °C and stained by 0.1% crystal violet.

temperatures over 40 °C during the second and third day post-inoculation. In contrast, no apparent clinical signs were observed in those pigs inoculated by vP129/swIL1β virus, and rectal temperature was normal for this group of pigs through the time course of the study. All pigs given virus became infected, which was evident by *in vitro* virus isolation and positive real-time PCR results for the presence of viral RNA in serum. Viral load in serum peaked at 7 dpi (Fig. 3A) for both vP129/swIL1β and parental virus infected groups. Statistically, there was no significant difference in viral load between these two groups, although a slightly lower level of viral load in serum samples from those pigs infected with wild type virus was observed at 14, 21 and 28 dpi. Interestingly, in comparison to the group of pigs infected with wild type virus, a higher level of viral load was observed in the lymph nodes, tonsil and lung tissues from those pigs infected with vP129/swIL1β at 28 dpi (Fig. 3B). At necropsy (28 dpi), minimal lung lesions (Table 1) were observed in vP129 and vP129/swIL1β infected groups. Enlarged mandibular lymph nodes were observed in three of the pigs from the vP129/swIL1β infected group, and in two of the pigs from the vP129 infected group.

To determine the *in vivo* stability of the swIL1β gene in virus, serum samples were used for virus isolation in MARC-145 cells. Virus was recovered from serum samples collected on days 7, 14 and 21 dpi. For each dpi, we sequenced viruses isolated from six pigs, and sequencing was performed using both forward and reverse primers, resulting in a total of 12 sequences for each day analyzed. The results confirmed that the swIL1β gene remained intact as a full-length gene. However, sequencing results revealed that an A299G nucleotide mutation was consistently identified in two of the pigs, which resulted in the amino acid (aa) mutation of Glu to Gly at position 100 of IL-1β protein. Another T553C nucleotide (Cys to Arg) mutation was also found in the virus isolated from a pig at 7 dpi. These results suggest that selection occurred to generate mutations during the viral replication *in vivo*.

Table 1
Evaluation of clinical symptoms and lung lesions for each experimental group.

Clinical symptom[a] and lung lesion[b]	Group scores (mean ± SEM)		
	IL-1β	Parental	Mock
Rectal temp during 5 days post inoculation	39.6 (±0.1)	40.6 (±0.5)	39.7 (±0.3)
Clinical respiratory signs			
Sneezing	0	1.0 (±0.0)	0
Nasal secretions	0.3 (±0.1)	2.0 (±0.0)	0
Coughing	0	2.0 (±0.3)	0
Labored breathing	0	0	0
Systemic clinical signs			
Roughness of hair	0	0	0
Lethargy	0	3.0 (±0.0)	0
Anorexia	0	0	0
Gross lung lesion score	2.0 (±0.5)	2.2 (±0.5)	0

[a] Clinical signs were recorded and quantified using a clinical scoring system adapted from Martínez-Lobo et al. (2011). Severity of clinical symptoms was evaluated on a scale of 0–5 whereby a score of 5 was considered to be the most severe.
[b] Gross lung pathology was assessed by using a gross pig lung lesion scoring system (Halbur et al., 1995) where each lobe of the lung was evaluated for percent pneumonia and the percent pneumonia of each lobe was added for the entire lung.

3.4. *In vivo swIL1β protein expression and immune response in pigs*

We further investigated whether *in vivo* expression of swIL1β protein could modulate the viral specific host immune response. In an earlier study, swIL1β was detected in sera or peripheral blood mononuclear cells (PBMCs) (by the fluorescent microsphere immunoassay, FMIA) from PRRSV-infected pigs (Lawson et al., 2010). To compare with these earlier findings, samples were collected from control and virus-infected pigs at 3, 7 and 14 dpi. PBMCs were stimulated *in vitro* with parental virus, PHA, or medium for 24 h. The FMIA results showed that higher levels of swIL1β protein were expressed in PBMCs from pigs infected by PRRSV, either vP129/swIL1β or parental vP129 compared to control pigs at 3, 7 and 14 dpi (Fig. 4). The comparison between vP129/swIL1β and parental vP129 showed that a higher level of swIL1β protein was expressed in pigs infected by vP129/swIL1β at 14 dpi (Fig. 4). No significant difference in swIL1β protein expression was detected from both groups of pigs infected by vP129/swIL1β and parental virus at 3 and 7 dpi by the FMIA. Interestingly, no detectable level of swIL1β was observed in serum samples from all groups of pigs at 3, 7 and 14 dpi (data not shown). We further monitored the temporal expression of selected innate and cellular immune proteins in PBMCs from virus-infected pigs using FMIA. As shown in Fig. 4, besides IL-1β, IFN-γ expression levels were also significantly higher in pigs infected with vP129/swIL1β compared to pigs with vP129 at 14 dpi. A significantly higher level of IL-4 and TNF-α was also observed in vP129/swIL1β infected pigs compared to pigs with vP129 at 14 dpi. IL-8 and IL-12 expression were higher at an early time point (3 dpi) but lower at 7 dpi in vP129/swIL1β infected pigs compared to pigs infected with vP129. The expression of IL-10 was lower at an early time point (3 dpi) but was higher at 7 dpi in vP129/swIL1β infected pigs compared to pigs infected with vP129. The comparison between virus infected groups (either vP129/swIL1β or parental vP129) and the control group showed that the expression of IL-12 or IL-10 in PBMCs was either up- or down-regulated by virus infection at 3 and 14 dpi.

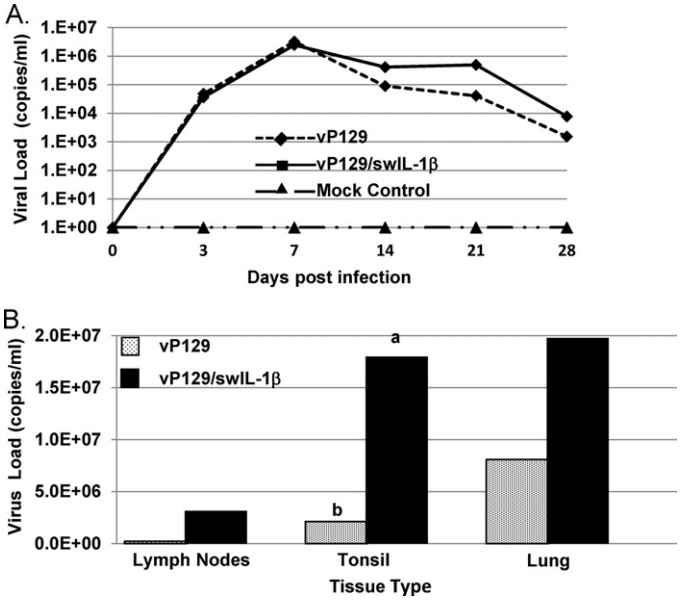

Fig. 3. Viral load in pigs infected with vP129/swIL1β or parental virus. Viral load was quantified by real-time RT-PCR, and the result was interpreted as RNA copy numbers per ml. (A) Viral load in serum samples, and (B) Viral load in tissue samples. Different letters ("a" and "b") represent mean viral load of different groups differs significantly ($P < 0.05$).

Further analysis of host humoral immune response showed that both vP129/swIL1β and parental virus infected groups had seroconverted by 14 dpi (Fig. 5A). Interestingly, a higher level of neutralizing antibody response was observed in the group of pigs infected with vP129/swIL1β at 28 dpi (Fig. 5B). Three of the six pigs (50%) in the vP129/swIL1β virus infected group had detectable neutralizing antibody titers by 28 dpi, while two of the six pigs (33%) in the parental virus infected group developed neutralizing antibodies by 28 dpi. Taken together, these results suggested that *in vivo* expression of swIL1β significantly modulated the PRRSV specific immune response.

4. Discussion

PRRSV infection appears to elicit poor innate IFN and cytokine responses unlike the other strongly immunogenic viral pathogens. Its effects result in a weak adaptive immune response as demonstrated by prolonged viremia, short duration of cell-mediated immune responses and slow development of virus-specific IFN-γ secreting cells. Anti-PRRSV neutralizing antibodies are generated slowly and their titers remain low. There is an extensive body of literature describing the innate interferon and cytokine effect on PRRSV replication and cytokine-mediated events that occur during PRRSV infection. In an earlier study, inoculation of pigs with porcine respiratory coronavirus, a potent inducer of innate cytokines, provided protection from a subsequent PRRSV infection (Buddaert et al., 1998). Overend et al. (2007) showed that recombinant swine IFN-β protects swine alveolar macrophages from infection with PRRSV. Royaee et al. (2004) showed that using an expression

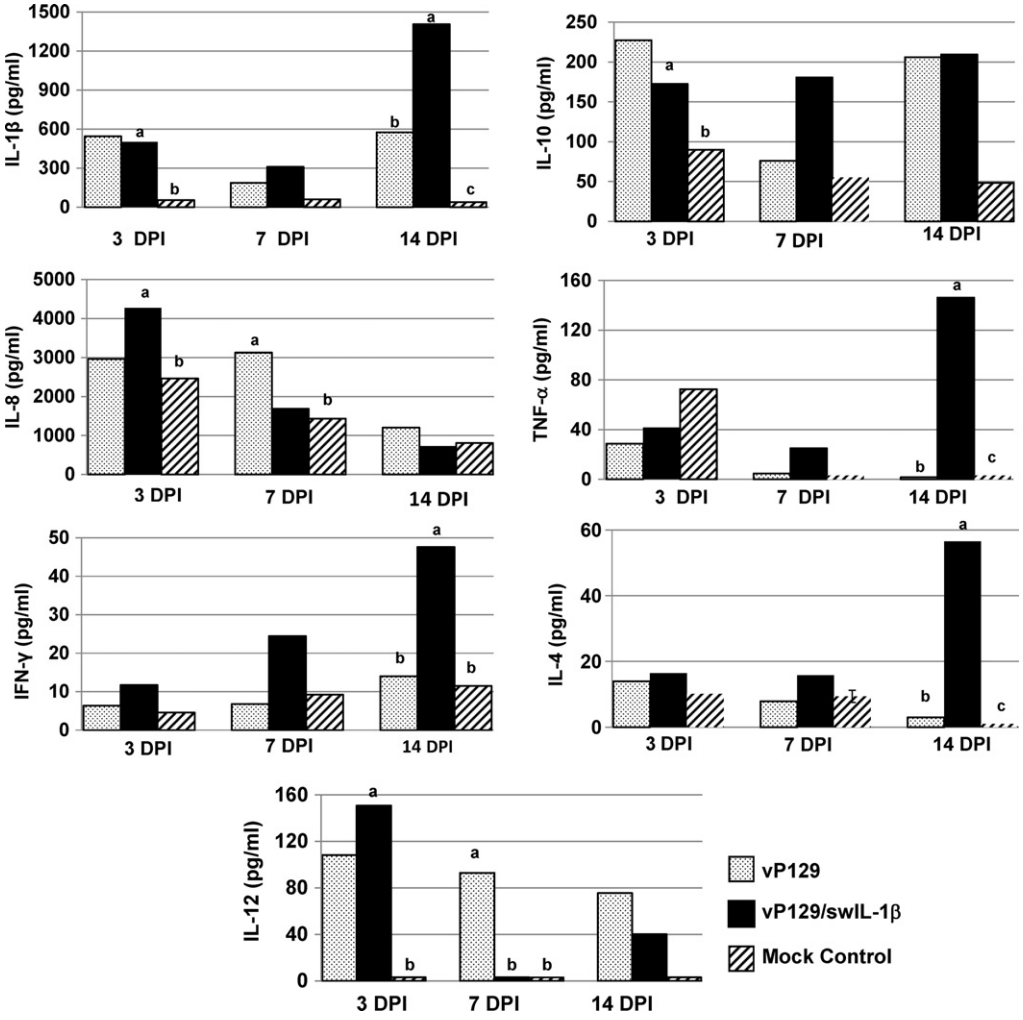

Fig. 4. Comparison of cytokine expression levels in activated PBMCs from pigs infected by vP129/swIL1β with that of parental virus. PBMCs were harvested at 3, 7 and 14 dpi and stimulated in culture with PHA or medium. Cell culture supernatants and pellets were harvested at 24 h post-stimulation. Cytokine protein expression was determined by Luminex fluorescent microsphere immunoassay. Each data point was interpolated using a five-parameter logistic regression curve and displayed as a mean fluorescent unit from three animals with the standard deviation. Different letters ("a", "b" and "c") represent mean cytokine level of different groups differs significantly ($P < 0.05$).

plasmid encoding porcine IFN-α as an adjuvant resulted in a temporary increase in the frequency of PRRSV specific IFN-γ secreting cells in the vaccinated animals. These results all support an important role of IFNs and cytokines in the initial innate immune response and the subsequent activation of adaptive immunity. However, with PRRSV infection, innate immune genes and proteins expression is suppressed and delayed (Van Reeth et al., 1999, 2002; Murtaugh et al., 2002). This initial suppression of the innate

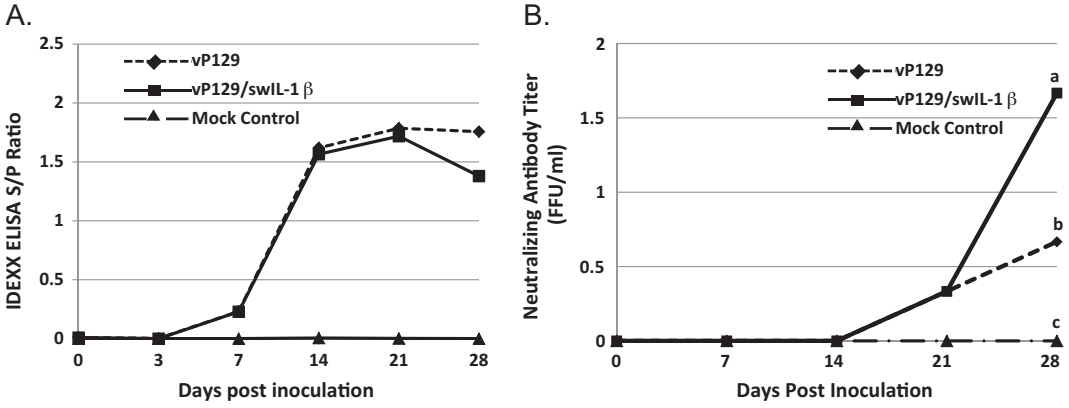

Fig. 5. Antibody response in pigs infected with vP129/swIL1β or parental virus. (A) PRRSV-specific serum antibodies measured by IDEXX HerdChek® PRRSV ELISA 2XR kit. S/P ratios of greater than 0.4 are considered positive. (B) Serum neutralizing antibody response determined by fluorescent focus neutralization assay. Results were interpreted as a 90% reduction of the viral infection, and the neutralizing antibody titers were presented as mean value ($n = 6$) and expressed on a $\log_2$ scale. The parental virus was used for the viral neutralizing assay. Different letters ("a", "b" and "c") represent mean neutralizing antibody titer of different groups differs significantly ($P < 0.05$).

immune response, leading to the delayed induction of protective cellular and humoral immunity, provides a window of time that allows PRRSV to replicate, shed and transmit to other contact naïve animals. Therefore, current strategies for vaccine development are directed at constructing a PRRS vaccine capable of inducing a high level of innate and cellular immune responses and robust neutralizing antibody activity within a short period of time after vaccination.

The use of cytokines as adjuvants for enhancing the viral specific immune response has been investigated in both subunit and modified live vaccines (Heath and Playfair, 1992; Gao et al., 1995; Godson et al., 1995; Ahlers et al., 2003; Yilma et al., 2010; Heegaard et al., 2011). In particular, IL-1β has been tested as a potent immune modulator in vaccine development. The recombinant bovine IL-1β has been tested as an adjuvant to co-administrate with a modified live BHV-1 vaccine, and an *in vivo* study showed enhanced humoral and cellular immune response. Although the study demonstrated the effect of IL-1β on the enhancement of host immunity, the systemic administration of IL-1β to develop an adjuvant effect requires a large dose of recombinant IL-1β and multiple injections at a local site. This could result in severe side effects and is not cost-effective in veterinary medicine. The use of recombinant virus as a vector to express the cytokines, such as IL-1β, has the potential to overcome these drawbacks. The viral vector system allows delivery of the cytokine to the specific site of infection. It reduces the dose requirement and thereby decreases the cost and side effects. A number of cytokines have been tested by inserting into recombinant viruses (Bembridge et al., 1998; Karaca et al., 1998; Giavedoni et al., 1997; Leong et al., 1994; Flexner et al., 1990). For example, expression of IFN-γ by recombinant respiratory syncytial virus attenuated the virus *in vivo* and protected animals from subsequent challenge with wild type virus (Bukreyev et al., 1999). In vaccinia virus, expression of IL-2 attenuated the virus while maintained the immunogenicity *in vivo* (Flexner et al., 1990). In our study, a recombinant PRRSV (vP129/swIL1β) was constructed, which expresses the IL-1β gene as a separate mRNA from an additional transcriptional product placed between ORF1b and ORF2 genome region. The recombinant virus produced high levels of IL-1β protein in cell culture. *In vitro* growth kinetics analysis indicated that the production of swIL1β was not toxic to the cells and did not affect viral replication. In comparison to the parental virus, the vP129/swIL1β virus grew to a viral titer similar to that of the parental virus in MARC-145 cells. This property is important for recombinant vaccine development. Genetic manipulation/attenuation of the live virus are typically accompanied by reduced viral titer. However, to achieve a satisfactory level of immunogenicity, the virus titer must be adequately maintained. The ability to produce high titers of vP129/swIL1β virus *in vitro* makes it easy to produce sufficient quantities for large-scale vaccine production. In addition, the large amount of recombinant IL-1β protein produced by the recombinant virus could be an alternative method for the *in vitro* production of recombinant cytokines.

We further demonstrated that the recombinant PRRSV could be used as an *in vivo* cytokine delivery system to study the mechanism of different cytokine effects on disease progression. First, the expression of IL-1β by vP129/swIL1β did not show a difference in viral load in the sera compared to those by the parental virus in animals. Also, our data showed that the IL-1β gene is maintained in the virus isolated from serum samples of the vP129/swIL1β infected pigs. Most importantly, the *in vivo* co-expression of swIL1β attenuated the pathogenic property of the virus. The parental virus P129 is known to be a highly pathogenic virus (Calvert et al., 2002, 2003). Results from our study demonstrated that parental P129 virus infected pigs showed significant symptoms of lethargy and respiratory distress. In contrast, the swIL1β expressing vP129/swIL1β virus infected pigs did not show apparent clinical symptoms. It is not clear why the expression of IL-1β by vP129/swIL1β resulted in attenuated symptoms in the animals. Initially, we hypothesized that vP129/swIL1β might be more virulent than the parental virus because of the expression of the proinflammatory cytokine IL-1β. It is possible that the attenuation may be related to the site (alveolar region) of virus replication and the expression of the cytokine where the anti-inflammatory mechanism is strong (Spight et al., 2005). Another interesting phenomenon related to this is that although the viral load in blood was similar, a higher level of viral load in tissues (tonsil, lymph nodes, and lung) was observed in pigs infected with vP129/swIL1β at 28 dpi. In this case, the viral load at the local tissues did not correlate with the disease progression, since the vP129/swIL1β infected pigs did not develop apparent clinical symptoms and no significant lung lesion was observed. It is possible that the continued expression of IL-1β in virus infected cells may promote virus replication by recruiting monocytes to differentiate into macrophages in the local tissues. Currently, we are investigating the potential mechanism of the attenuation by IL-1β in virus-infected animals.

Although no detectable level of swIL1β was observed in sera from animals infected with vP129/swIL1β or parental vP129, there were significantly higher levels of IL-1β detected in vP129/swIL1β infected pigs in PBMCs (Fig. 4). Further analysis of a time course pattern of cytokine proteins expression using a FMIA suggested that the co-expression of IL-1β could lead to a strong T cell response. In comparison to the parental virus, infection with vP129/swIL1β resulted in a significant increase of both Th1 marker (IFN-γ) and Th2 marker (IL-4) in PBMCs at 14 dpi. The result of the humoral immune response showed that both vP129/swIL1β and parental virus infected groups had seroconverted by 14 dpi at the same levels (Fig. 5A). However, a higher level of neutralizing antibody response was observed in the group of pigs infected with vP129/swIL1β compared to that with parental virus at 28 dpi (Fig. 5B), which may be due to the strong T cell response by vP129/swIL1β.

In summary, our study demonstrated that the recombinant PRRSV with the reverse genetics system can be used as a potential *in vivo* cytokine delivery system to study the role of different cytokines in disease progression and immune responses. This study represents a new approach for future *in vivo* application of therapeutic cytokines and development of genetic engineered vaccines for controlling PRRS.

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