Animal Arterivirus Infections

Guest Editors: Denis Archambault, Udeni B. R. Balasuriya, Raymond R. R. Rowland, Hanchun Yang, and Dongwan Yoo
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The arteriviruses (Family Arteriviridae, Genus Arterivirus) include an interesting group of enveloped positive stranded RNA viruses that infect domestic and wild animals and they share a strikingly similar genome organization and replication strategy to that of coronaviruses, but differ considerably in their genetic complexity and virion architecture. Until recently, arteriviruses included equine arteritis virus (EAV), porcine reproductive and respiratory syndrome virus (PRRSV), lactate dehydrogenase-elevating virus (LDV) of mice, and simian hemorrhagic fever virus (SHFV). Three of these viruses were first discovered and characterized a long time ago (EAV-1953, LDV-1960 and SHFV-1964), whereas PRRSV emerged first in Europe and North America in the late 1980s and early 1990s. In 2012, arteriviruses were expanded to include the newly identified wobbly possum disease virus (WPDV) that causes neurologic disease among free-ranging Australian brush-tailed possums (Trichosurus vulpecula) in New Zealand. Similarly, four new genetically divergent SHFV variants were recently identified in a single male colobus monkey (Procolobus rufomitratus tephrosceles) and in African red-tailed (guenon) monkeys (Cercopithecus ascanius) from Kibale National Park, Uganda.

The arteriviruses are highly species specific, but share many biological and molecular properties, including virion morphology, a unique set of structural proteins, genome organization and replication strategy, and the ability to establish prolonged or true persistent infection in their natural hosts. However, the epidemiology and pathogenesis of the infections caused by each virus is distinct, as are the diseases they cause. EAV and PRRSV cause respiratory and reproductive disease in equids and swine, respectively. The natural hosts of LDV are field and the laboratory mice, but unlike other arteriviruses, LDV is generally non-pathogenic. Indeed this virus is a significant adventitious agent in the laboratory mice, and causes an increase of lactate dehydrogenase enzyme, contamination of transplantable tumors, clinically silent infection with mild pathology, and immunomodulation. SHFV establishes persistent, perhaps life long infections without disease in Patas monkeys (Erythrocebus patas), baboons (Papio anubus), and African green monkeys (Ceropithecus aethiops). SHFVs of African origin are highly infectious and fatal in Asian rhesus monkeys (Macaca mulatta, M. arctoides and M. fascicularis). Although the human arterivirus is yet to be identified, there is
significant concern about cross species transmission of some of these arteriviruses following xenotransplantation. There is no indication that any humans are infected with SHFV or any other arterivirus during disease outbreaks. However, it is obvious that transplantation of humans with tissues from SHFV-infected baboons or PRRSV-infected pigs would result in transfer of a considerable amount of these viruses, which could result in selection of a variant(s) that can replicate in humans. In this respect, PRRSV and SHFV should be seriously considered as a threat to xenotransplantation of tissues and organs from pigs and baboons.

Of the five arteriviruses, EAV and PRRSV are the most economically important viruses and pose a significant threat to equine and swine industries, respectively. Both viruses are distributed throughout the world, but PRRSV infection has become the most serious infectious disease of the pigs costing over $560 million US dollars per year to the US swine producers. Genetic manipulation of full-length cDNA clones of these two viruses has become an especially important and widely used tool to study the biology, pathogenesis, and virulence determinants of both of these viruses. Due to the economic impact, there has been significant research interest in molecular characterization of PRRSV and its interaction with the host defense system. In conjunction with advances in PRRSV molecular virology, reverse genetics and immunology, more and more attention is being directed to development of safe and efficacious vaccines against PRRSV. However, development of effective vaccines against PRRSV infection has been a significant challenge due to the presence of two genotypes of the virus with rapid emergence of antigenic variants and delayed suboptimal neutralizing antibody responses to infection. Current modified live and killed vaccines have had limited success, prompting investigation of alternative approaches to design new and improved vaccines.

In this special issue of Animal Arterivirus Infections by BioMed Research International dedicated to recent advances in arterivirus research, we have assembled seven manuscripts that describe original research data on PRRSV and EAV. The articles cover characterization of innate immune response to PRRSV and EAV, genetic diversity of PRRSV strains circulating in China, interaction between the structural and nonstructural proteins of PRRSV with the host cellular factors, vaccination-induced protection against homologous and heterologous challenge, and development of a recombinant DNA vaccine against PRRSV. These articles provide new insight into better understanding immune responses to PRRSV and EAV in the context of disease as well as vaccine-induced protection, which will help to develop better vaccines to control the growing worldwide burden of disease from these agents in the future. Clearly a comprehensive research approach combining molecular virology, immunology, viral pathogenesis, and host genetics is required to unravel the complexities of virus-host interactions of these viruses. We believe that we have assembled basic knowledge that encompasses these complexities, describes technologies that have contributed to this knowledge, and identifies at least some of the major problems faced in attempting to further understand the virus-host interactions that result in disease. We would like to thank all the authors and co-authors who contributed to this special issue of Animal Arterivirus Infections, as well as the numerous reviewers who reviewed each and every paper in a timely manner and provided their input to improve the quality and clarity of the final product. We would also like to acknowledge that there are many other colleagues and scientists who are active in the field of arteriviruses whose expertise has not been represented here.

Denis Archambault
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Research Article

Equine Arteritis Virus Does Not Induce Interferon Production in Equine Endothelial Cells: Identification of Nonstructural Protein 1 as a Main Interferon Antagonist

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The objective of this study was to investigate the effect of equine arteritis virus (EAV) on type I interferon (IFN) production. Equine endothelial cells (EECs) were infected with the virulent Bucyrus strain (VBS) of EAV and expression of IFN-β was measured at mRNA and protein levels by quantitative real-time RT-PCR and IFN bioassay using vesicular stomatitis virus expressing the green fluorescence protein (VSV-GFP), respectively. Quantitative RT-PCR results showed that IFN-β mRNA levels in EECs infected with EAV VBS were not increased compared to those in mock-infected cells. Consistent with quantitative RT-PCR, Sendai virus- (SeV-) induced type I IFN production was inhibited by EAV infection. Using an IFN-β promoter-luciferase reporter assay, we subsequently demonstrated that EAV nsps 1, 2, and 11 had the capability to inhibit type I IFN activation. Of these three nsps, nsp1 exhibited the strongest inhibitory effect. Taken together, these data demonstrate that EAV has the ability to suppress the type I IFN production in EECs and nsp1 may play a critical role to subvert the equine innate immune response.

1. Introduction

Equine arteritis virus (EAV) is the causative agent of equine viral arteritis, a respiratory and reproductive disease of horses [1, 2]. EAV is a small enveloped virus with a positive-sense, single-stranded RNA genome of ~12.7 kb. It belongs to the family Arteriviridae (genus Arterivirus, order Nidovirales), which also includes porcine reproductive and respiratory syndrome virus (PRRSV), simian hemorrhagic fever virus (SHFV), and lactate dehydrogenase-elevating virus (LDV) of mice [3–5]. The EAV genome includes at least ten known functional open reading frames (ORFs 1a, 1b, 2a, 2b, 3, 4, 5a, 5b, 6, and 7) [5–7]. ORFs 1a and 1b are located at the 5′-proximal three-quarters of the genome and are translated to produce replicase polyproteins pp1a and pp1ab (1,727 and 3,175 amino acids, resp.). Translation of ORF1b depends on a −1 ribosomal frameshift located just before termination of ORF1a translation [8]. The two replicase precursor polyproteins are cleaved by three ORF1a-encoded proteases that reside in nsp1, nsp2, and nsp4, yielding at least 13 end-products, namely, nonstructural proteins (nsp) 1 to 12, including a recently described nsp7α and 7β [5, 9, 10]. The remaining eight ORFs (2a, 2b, and 3, 4, 5a, 5b, and 6–7) are located in the 3′ quarter of the genome and encode the structural proteins (E, GP2, GP3, GP4, ORF5a protein, GP5, M, and N, resp.) of the virus [5, 6, 11–13].
Type I interferon (IFN-α/β) is a key component of the host innate immune response to viral infection [14]. Recognition of pathogen-associated molecular patterns (PAMPs) in double-stranded RNA (dsRNA) by intracellular receptors, such as retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated antigen 5 (MDA-5) [15], activates protein signaling cascades that result in the activation of transcription factors, including interferon regulatory factor-3 (IRF-3) and nuclear factor-κB (NF-κB) [14]. The IFN-β promoter contains positive regulatory domains (PRDs), including the binding sites for different transcription factors, IRF-3 (PRDs I and III) and NF-κB (PRD II). Activation of these transcription factors triggers the formation of enhanceosomes in the cell nucleus and induces the expression of IFN-α/β [14, 16]. Both IRF-3 and NF-κB activation are mediated by mitochondrial antiviral signaling (MAVS) protein, which functions downstream of RIG-I and MDA-5 and upstream of the IκB kinase (IKK) complex and TANK-binding kinase-1 (TBK1) [17–20]. Among the various factors involved in type I IFN production, IRF-3 plays a critical role. IRF-3 is expressed in most cell types and resides in the cytoplasm in an inactive form. When stimulated, IRF-3 becomes phosphorylated and undergoes conformational changes, resulting in dimerization with the exposure of nuclear localization signal. In the nucleus, IRF-3 recruits coactivator CBP/p300 and forms a complex to bind IRF-3 responsive elements (PRDs I and III) of the IFN-β promoter [14]. In addition to IRF-3, NF-κB is also a critical regulator of host innate and adaptive immunity. It plays an important role in the regulation of cell proliferation as well as cell survival. Many viruses have evolved strategies to counteract key elements of the IFN response and prevent development of an antiviral response in the host [14]. Through evolution, viruses can either activate or inhibit the NF-κB pathway in order to replicate in host cells. Viruses such as African swine fever virus and influenza A virus block NF-κB activation to counteract the host innate immune response [21, 22]. In contrast, viruses such as hepatitis C virus, reovirus, and herpes simplex virus have developed mechanisms to directly activate NF-κB to support production of progeny viruses and intracellular spreading [23–25].

Until now, the innate immune response to EAV infection was poorly characterized and the information pertaining to type I IFN production was largely derived from studies of PRRSV and other nidoviruses [26–32]. Recently, van Kasteren et al. [33] reported that the EAV PLP2 has de-ubiquitylation function which suppresses RIG-I to control innate immune signaling in EAV-infected cells [34, 35]. In all these studies, the investigators have used recombinant proteins (e.g., nsp2 protein of EAV) and a specific immune suppression mechanism. However, the effect of the whole virus and involvement of other EAV proteins in the suppression of host cell immune responses are largely unknown. To elucidate the molecular mechanism of EAV involved in host immune suppression, the objective of this study was to investigate the effect of EAV on type I IFN production and to further identify the specific viral proteins responsible for the suppression of IFN-β activity.

2. Materials and Methods

2.1. Virus and Cells. Equine pulmonary artery endothelial cells (EECs [36], baby hamster kidney-21 (BHK-21 [ATCC CCL-10], Manassas, VA), and HEK293T (ATCC CRL-11228) cells were maintained in Dulbecco’s modified essential medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT), 100 U/μl per ml penicillin-streptomycin, and 200 mM L-glutamine (Gibco, Carlsbad, CA) in a humidified incubator with 5% CO₂ at 37°C. HeLa cells (NIH AIDS Research and Reference Reagent Program, Germantown, MD) were propagated in minimum essential medium (MEM) supplemented with 10% heat-inactivated FBS in a humidified incubator with 5% CO₂ at 37°C. MDBK (ATCC CCL-22) cells were grown in Eagle’s minimum essential medium with 10% ferritin-supplemented bovine calf serum (HyClone Laboratories, Inc., Logan, UT) and 100 U/μg per ml penicillin-streptomycin (Gibco, Carlsbad, CA). The virulent Bucyrus strain of EAV (EAV VBS, horse passage 15 pleural fluid; ATCC VR-796, Manassas, VA) was passaged once in EECs to obtain high-titered working stocks for the present study using the method described previously [37]. Sendai virus, the Cantell strain (SeV; ATCC VR-907, Manassas, VA), was propagated in embryonated chicken eggs. The virus titer was determined by hemagglutination inhibition (HI) assay using chicken red blood cells as described previously [38]. Vesicular stomatitis virus expressing green fluorescent protein (VSV-GFP [39]) was kindly provided by Dr. Adolfo Garcia-Sastre (Mt. Sinai School of Medicine, New York, NY).

2.2. Plasmids. Plasmids for expression of recombinant EAV nsp1 to nsp12 in mammalian cells were constructed as previously described [40]. Briefly, the coding regions of each of the twelve nsp were PCR-amplified from the EAV rVBS full-length infectious cDNA clone [41] and cloned into the eukaryotic expression vector pCAGGS [33]. The nsp5, nsp6, nsp10, and nsp12 were expressed as C-terminal FLAG-tagged fusion proteins. To express the recombinant EAV nsp5, BHK-21 cells were transfected with each individual plasmid containing an nsp coding region. Transfection was performed using FuGENE HD (Promega, Madison, WI) according to manufacturer’s instructions. The specificity of each recombinant protein was confirmed by immunofluorescence and Western blot analysis. Reporter plasmids expressing the firefly luciferase under the control of either the IFN-β promoter (p125-Luc) or an artificial promoter containing three IRF-3 binding sites (p55-CIB-Luc) were kindly provided by Yoneyama et al. [42]. The DNF-κB-Luc reporter plasmid (Strategene, La Jolla, CA) expresses the firefly luciferase under the control of a promoter with NF-κB-response element. The pRL-SV40 plasmid (Promega, Madison, WI) expresses a Renilla luciferase under the control of a simian virus (SV40) promoter. The pEFneo-RIG-I, pEFneo-MDA-5, and pEFneo-IKKε were kindly provided by Komatsu et al. [43]. The pcDNA3-TRIF and pCMV2-IKK2-WT were purchased from Addgene (Cambridge, MA). Construction of the pCAGGS-IRF-3 and pCAGGS-NSI plasmids was described previously [44].
2.3. Antibodies. To detect EAV antigens in infected cells, monoclonal antibodies (MAbs) against EAV nsp1 (Mab 12A4) and N protein (Mab 3E2) were used [45, 46]. Specific polyclonal rabbit antisera recognizing EAV nsp2 [47], nsp3 [48], nsp4 [47], nsp7-8 [47], and nsp10 [49] have been described previously. In addition, antisera against nsp9 and nsp11 were raised by immunizing rabbits with purified full-length recombinant proteins expressed in *E. coli* (J.C. Zevenhoven, D. D. Nedialkova, and E. J. Snijder, unpublished data). Anti-FLAG Mab (F3165) purchased from Sigma (St. Louis, MO) was used to detect FLAG-tagged EAV fusion proteins in immunofluorescence assay. Rabbit polyclonal antibodies for human IRF-3 (sc-9082) and NF-κB p65 (sc-7151) were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). Alexa Fluor 488-conjugated and Alexa Fluor 594-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA).

2.4. RNA Extraction and Quantitative Real-Time RT-PCR (qRT-PCR). Total RNA was extracted using MagMAX-96 Total RNA Isolation kit (Ambion, Austin, TX) in a MagMAX Express-96 magnetic particle processor (Applied Biosystems, Foster City, CA). RNA from each culture was treated with DNase to remove any contaminating genomic DNA (gDNA). The RNA concentration was assessed at OD\textsubscript{260} nm and purity was verified by the OD\textsubscript{260}/OD\textsubscript{280} ratio using NanoDrop (Thermo Scientific, Wilmington, DE). The reverse transcription reaction was performed with 1 μg of total RNA using RT random primers and a MultiScribe reverse transcriptase (High Capacity cDNA Reverse Transcription kit with RNase inhibitor (Applied Biosystems, Foster City, CA)) according to the manufacturer’s instructions. The following IFN-β primers and probe set were used for PCR amplification with an Applied Biosystems 7500 Fast Real-Time PCR System: EqIL-IFN-BF: 5’-AATGCCCTCTCCTGGTGTG-3’, EqIL-IFN-βR: 5’-CGGACAGTCTCGGTCACAGA-3’, and EqIL-IFN-β: probe 5’-FMCTTCACCCGGCTCT-NFQ-3’. For each sample, cDNA corresponding to the β-glucuronidase (β-GUS) gene was amplified and used as an endogenous control. All PCR efficiency values were determined using LinReg [50]. The relative concentration of target gene mRNA was equal to 2^{-ΔC_{T}} , where ΔC_{T} = [(Avg. gene of interest C_{T} − Avg. β-GUS C_{T}) sample – (Avg. gene of interest C_{T} − Avg. β-GUS C_{T}) calibrator]. The calibrator was calculated from the mean ΔC_{T} of mock-infected samples for each individual gene.

2.5. Interferon Bioassay. The interferon bioassay was performed using a recombinant vesicular stomatitis virus (VSV) that expresses green fluorescent protein (VSV-GFP) as previously described [31, 39, 51]. Briefly, EECs were either infected with EAV or Sendai virus (SeV) alone or dually infected with both EAV and SeV at an m.o.i. of 1 and incubated for 24 h at 37°C. Culture supernatants were collected and virus in supernatant was inactivated by ultraviolet (UV) irradiation for 30 min. Two-fold dilutions of supernatants were made in DMEM and used in IFN bioassays. MDBK cells were grown in 96-well plates to 70% confluency and incubated with two-fold dilutions of each of the supernatants. After 24 h incubation at 37°C, cells were infected with VSV-GFP at an m.o.i. of 0.1 and further incubated for 18 h. Cells were fixed with 4% paraformaldehyde and expression of green fluorescence protein was examined under an inverted fluorescence microscope.

2.6. Cytotoxicity Test of EAV nsp1 on HEK293T Cells. HEK-293T cells in 96-well plates were transfected with increased amount of plasmid expressing EAV nsp1 (0, 0.05, 0.1, 0.2, or 0.4 μg/well) using FuGENE HD (Promega, Madison, WI) transfection reagent (0.8 μL/well). At 24 h after transfection, cytotoxicity was determined by using a cell proliferation assay (CellTiter 96 Aqueous One Solution, Life Technologies, Grand Island, NY).

2.7. Luciferase Reporter Assay. HEK293T cells were seeded in 24-well plates and transfected with various combinations of plasmid DNAs: the pENeo-RIG-1, pENeo-MDA-5, pENeo-MKK6, pcDNA3-TRIF, or pCAGGS-IFR-3 was mixed with a plasmid expressing an EAV protein (or empty pCAGGS vector), a luciferase reporter plasmid, and the pRL-SV40 plasmid. Transfection was performed using FuGENE HD (Promega, Madison, WI) transfection reagent following the manufacturer’s instruction. For the SeV or IFN stimulation, HEK293T cells were transfected with a plasmid expressing an EAV protein (or empty pCAGGS vector), a reporter plasmid, and the pRL-SV40 plasmid. The plasmid pCAGGS-NSI expressing swine influenza virus NSI was used as positive control. At 20 h after transfection, cells were infected with SeV at 5000 HA unit/0.5 mL/well for 12–16 h or induced by treatment with 2000 IU/0.5 mL/well of IFN-α or IFN-β for 16 h. Cells were harvested at the indicated time points. Cell lysates were subjected to reporter gene assay using the dual luciferase reporter system (Promega, Madison, WI) according to manufacturer’s instruction. Firefly and Renilla luciferase activities were measured in a luminometer (Berthold Technologies, Oak Ridge, TN). Values for each sample were normalized using the Renilla luciferase values. Relative luciferase (RLU) activity is defined as the ratio of firefly luciferase reporter activity to *Renilla* luciferase activity.

2.8. Immunofluorescence Assay. Cells were seeded on coverslips and grown to 80% confluence. DNA transfection was performed in HeLa cells using Lipofectamine 2000 (Invitrogen; Carlsbad, CA) according to the manufacturer’s instructions. A transfection mix containing DNA and Lipofectamine 2000 in OPTI-MEM I (Invitrogen; Carlsbad, CA) was incubated at room temperature for 20 min and added to each well. After incubation, the transfection mix was replaced with fresh medium, and cells were incubated for 12 h to allow gene expression. For IRF-3 staining, cells were stimulated with 1 μg/mL of polyinosinic-polycytidylic ([poly(I:C)]; Sigma, St. Louis, MO) or untreated for 8 h. For NF-κB p65 staining, HeLa cells were treated with 20 ng/mL of TNF-α for 45 min or untreated. After incubation, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 10 min at room temperature (RT), and
then permeabilized using 0.1% Triton X-100 for 10 min at RT. After blocking with 1% bovine serum albumin (BSA) in PBS for 30 min, cells were incubated with primary antibody in PBS containing 1% BSA for 2 h followed by incubation with Alexa Fluor 488- and/or Alexa Fluor 594-conjugated secondary antibody for 1 h. Nuclear staining was performed with DAPI (4′,6-diamidino-2-phenylindole; Sigma, St. Louis, MO) for 3 min at RT. After washing with PBS, coverslips were mounted onto microscope slides using Fluoromount-G mounting medium (Southern Biotech, Birmingham, AL) and examined under the fluorescence microscope (Leitz Laborlux 12).

3. Results

3.1. Suppression of Type I Interferon Production by EAV. To investigate the effect of EAV on type I IFN production, IFN-β mRNA expression in infected cells was analyzed using qRT-PCR. Equine pulmonary endothelial cells (EECs) were infected with EAV VBS at an m.o.i. of 5 for 8 h, followed by infection with SeV for 3 or 6 hpi. SeV-alone-infected cells were used as positive control for type I IFN induction and mock-infected cells were used as negative control. As shown in Figure 1(a), IFN-β mRNA was barely detected in cells infected with EAV alone, whereas SeV infection induced a strong expression, approximately 190–200-fold increase of IFN-β mRNA expression level in comparison to that of mock-infected cells at 3 or 6 hpi. In contrast, SeV-induced IFN-β mRNA expression, at both 3 and 6 hpi, was significantly suppressed in cells previously infected with EAV. In parallel with the quantitative measurement of IFN-β mRNA, EAV-induced suppression of IFN production was confirmed by IFN bioassay using VSV-GFP, since VSV is IFN sensitive and presence of IFN-α/β blocks VSV replication. EECs were either infected with EAV or SeV alone or infected with both EAV and SeV. Mock-infected cells were used as negative control. Subsequently, MDBK cells were incubated with two-fold serial dilutions of virus-inactivated cell culture supernatant and then infected with VSV-GFP. The VSV infectivity was determined by monitoring the level of GFP expression. As shown in Figure 1(b), VSV-GFP replicated well in cell culture supernatant from mock-infected cells, whereas VSV replication was effectively inhibited in those MDBK cells that were preincubated with supernatant from SeV-infected EECs. No VSV-GFP replication was observed up to a dilution of 1:32 of the culture supernatant from SeV-infected cells. In contrast, VSV-GFP replication was not inhibited in MDBK cells that were preincubated with cell culture supernatant from EECs infected with EAV alone. Consistent with quantitative RT-PCR results, SeV-induced type I IFN production was significantly inhibited by EAV exposure since VSV-GFP replication was detected at a lower level compared to that from EECs only infected with SeV. Taken together, the data suggest that EAV has an ability to suppress the induction of IFN in infected cells.

3.2. EAV nspl, nsp2, and nsp11 Exhibited Strong Inhibition on IFN-β Promoter Activation. To investigate the role of EAV proteins as potential IFN antagonists, we focused on the nonstructural proteins of EAV. Each of the 12 nsp-encoding regions from the genome of EAV VBS was cloned individually into a mammalian expression vector, pCAGGS [40]. The expression of recombinant proteins in the plasmid DNA transfected cells was verified by immunofluorescence assay (Figure 2(a)). To determine whether these nsps have an effect on IFN-β activation, we used an IFN-β promoter-luciferase reporter assay. HEK293T cells were cotransfected with individual nsp-expressing plasmid and the luciferase reporter plasmid (p125-Luc) along with a Renilla luciferase expression plasmid (pRL-SV40) for normalizing purpose of sample expression levels. As a positive control, pCAGGS-NSp1 plasmid expressing swine influenza virus NS1 (sw-ns1) gene was used to cotransfect the cells with the reporter plasmid, since sw-ns1 is a known IFN antagonist [52]. At 24 h after transfection, cells were infected with SeV to induce luciferase production. The IFN-β promoter-luciferase reporter assay result is presented in Figure 2(b). As we expected, the expression of sw-ns1 significantly inhibited IFN-β promoter-driven luciferase expression. In contrast, a strong reporter signal was observed in cells transfected with empty pCAGGS plasmid after infection with SeV. IFN-β promoter activation by SeV infection was suppressed to various degrees by expression of several EAV nsps, among which nsp1, nsp2, and nsp11 showed strong inhibition of IFN-β promoter-driven luciferase expression. In particular, nsp1 exhibited the strongest inhibitory effect, followed by nsp11 and nsp2. The results suggest that several EAV nsps are capable of suppressing IFN-β promoter activation.

3.3. EAV nspl Interferes with IRF-3- and NF-κB-Mediated Signaling Pathways for IFN-β Production. Since the nsp1 of EAV showed the strongest inhibitory effect on the IFN-β promoter activation, we further determined the specific IFN production signaling pathway(s) associated with nsp1 expression. Toward this end, we tested nspl in IRF-3- and NF-κB-promoter-driven luciferase reporter systems. Cells were cotransfected with control plasmids or with plasmids expressing the EAV nspl protein, the plasmid pRL-SV40, and a luciferase reporter plasmid.

As shown in Figure 3(a), upon SeV stimulation, the level of IRF-3-dependent luciferase expression was significantly reduced in cells expressing EAV nspl and sw-ns1 compared to that in cells transfected with control plasmid (empty pCAGGS vector). Similarly, NF-κB promoter-dependent luciferase expression was suppressed in cells expressing EAV nspl (Figure 3(b)). These results suggest that EAV nspl suppresses IFN-β production by interfering with the IRF-3 and NF-κB signaling pathways. Furthermore, the viability of the HEK293T cells expressing EAV nspl 24 h after transfection was compared to that of untransfected control cells. As shown in Figure 3(c), the cell viability appeared to be not affected by the nspl protein expression. These data further suggest that the decreased type I IFN production is not due to cellular cytotoxicity, which further confirmed the important role of nspl in the interfering with IRF-3 and NF-κB signaling pathways.
Figure 1: Inhibition of type I IFN production after EAV infection. (a) Expression levels of IFN-β mRNA in EAV infected cells. EECs were mock-infected or infected with EAV VBS at an m.o.i. of 5 for 8 h. Subsequently, cells were infected with Sendai virus (SeV; 100 HAU/mL) for 3 h or 6 h. Total RNA was isolated and real-time RT-PCR was performed for the detection of equine IFN-β. Bar graph showing relative quantitation (RQ) values of IFN-β mRNA expression from three independent experiments are shown. (b) VSV bioassay for IFN production. EECs were mock-infected or infected with EAV VBS at an m.o.i. of 1 for 24 h. SeV was used as an IFN stimulator. Cell culture supernatants were collected and UV-irradiated for 30 min prior to use in the assay. MDBK cells were grown in 96-well plates and incubated with 2-fold dilution series of the supernatant up to 1/32. After 24 h incubation, cells were infected with VSV-GFP at an m.o.i. of 0.1, and 18 h after infection GFP expression was assessed by fluorescence microscopy. Each dilution was tested in duplicate.
3.4. Effect of EAV nsp1 on the IRF-3-Dependent Signaling Pathway. We further investigated specific steps in the IRF-3 signaling pathway that EAV nsp1 could possibly block. We tested each step in the signaling pathway of IRF-3 activation. First, we investigated whether the nsp1 was interfering with the mitochondrial antiviral signaling (MAVS) complex activity. Since MDA-5 or RIG-I is associated with the MAVS complex, cells were cotransfected with a plasmid expressing RIG-I, MDA-5, or MAVS protein, a plasmid expressing EAV nsp1, the plasmid pRL-SV40, and the p55-CIB-Luc reporter plasmid. As shown in Figures 4(a)–4(c), IRF-3 promoter-dependent luciferase expression was suppressed in the presence of EAV nsp1. These results suggested that EAV nsp1 might inhibit the MAVS-mediated IFN-β induction or downstream portion of the signaling pathway. Therefore, we further tested the effect of EAV nsp1 on TRIF- and IKKe-mediated IFN-β induction. The results showed that nsp1 had the ability to suppress TBK1- and IKKe-mediated reporter gene expression (Figures 4(d) and 4(e)). Similarly, overexpression of IRF-3 itself did not activate the transcription of the luciferase reporter gene either (Figure 4(f)). These results suggest that EAV nsp1 might block the signaling process downstream IRF-3 activation, possibly in the nucleus.

3.5. Effect of EAV nsp1 on the NF-κB -Dependent Signaling Pathway. Since EAV nsp1 also inhibited the activation of the NF-κB-dependent signaling pathway (Figure 3), the mechanism by which EAV nsp1 can inhibit the NF-κB signaling pathway was analyzed in detail. As shown in Figure 5(a), in the presence of EAV nsp1, NF-κB-dependent reporter gene expression was strongly inhibited with the stimulation of the TNF-α, a potent inducer for the activation of NF-κB signaling pathway. Subsequently, the effect of EAV nsp1 on the MAVS complex and Toll/interleukin-1 receptor domain-containing adaptor protein (TRIF-) and IkB kinase beta (IKKe-) mediated NF-κB activation was evaluated. Overexpression of any of these proteins induced activation of NF-κB-dependent reporter gene expression. However, when cells coexpressed the EAV nsp1 with one of these signaling
molecules, the expression level of luciferase reporter was significantly reduced (Figures 5(b)–5(d)). Similarly, overexpression of p65, a subunit of the NF-κB complex, had no effect on the activation of the transcription of NF-κB-driven reporter gene, but EAV nspl significantly reduced the expression level of luciferase reporter signal (Figure 5(e)).

3.6. Effect of EAV nspl Expression on Nuclear Translocation of IRF-3 and NF-κB. The mechanism of EAV nspl effect on IRF-3- and NF-κB-dependent gene expression was further examined by observing the nuclear localization of IRF-3 and NF-κB, respectively. EAV nspl transfected HeLa cells were stimulated with poly(I:C), and subsequently expression of IRF-3 was stained with anti-IRF-3 antibody. In the absence of stimulation, IRF-3 was homogeneously distributed throughout the cell, whereas upon poly(I:C) stimulation IRF-3 was mainly translocated into the nucleus. As shown in Figure 6(a), expression of EAV nspl did not block the nuclear translocation of IRF-3. The result suggested that nspl-mediated suppression of IFN production occurs downstream of the IRF-3 nuclear translocation. Similarly, the nuclear translocation of NF-κB p65 subunit was determined in cells expressing EAV nspl by immunofluorescence assay (Figure 6(b)). NF-κB remained largely not only in the cytoplasm but also in the nucleus to some extent in unstimulated cells, whereas TNF-α stimulation induced the nuclear translocation of p65. In cells expressing EAV nspl, TNF-α stimulation did not notably change the p65 nuclear...
translocation, and p65 normally remained in the nucleus, indicating that EAV nsp1 did not block p65 nuclear translocation. These results suggest that, in the NF-κB-dependent signaling pathway for IFN-β production, the activation of IFN-β transcription would be blocked by nsp1 somewhere downstream in the nucleus after NF-κB nuclear translocation occurred.

4. Discussion

Synthesis and secretion of type I IFNs, such as IFN-α and IFN-β, are critical aspects of the antiviral immune response [14, 53, 54]. Viruses use different mechanisms to inhibit interferon response in order to evade the host innate immune response. Many viruses encode more than one protein capable of inhibiting the interferon response, which act synergistically to ensure complete blocking of interferon activity. For example, Ebola, Nipah, and SARS-CoV encode multiple viral proteins capable of inhibiting interferon activity, suggesting important roles for these proteins in pathogenesis and disease outcome [55–58]. In this study, we investigated whether EAV has the ability to interfere with the host innate immune response, in particular, type I IFN production. Our results demonstrated that EAV infection in EECs significantly inhibited type I IFN production at both mRNA and protein levels, whereas infection with SeV stimulated a high level of type I IFN production. Furthermore, EAV infection significantly inhibited SeV-induced type I IFN production as well. Based
on IFN-\(\beta\) promoter-luciferase reporter assay results, three EAV nonstructural proteins, nsp1, nsp2, and nsp11, were identified as potential IFN antagonists. Previous studies reported that EAV nsp2-encoded papain-like proteinase (PLP2) is capable of inhibiting Ub- and ISG15-dependent innate immune responses [34, 35, 59]. The EAV nsp11 encodes NendoU endoribonuclease [60], which is highly cytotoxic upon its \textit{in vitro} expression. In PRRSV, the homologous nsp11 was proposed to be an IFN antagonist [26, 31, 61]. However, whether this effect is due to the cytosolic version of the enzyme targeting on the overall RNA population of the cell or the specific suppression of IFN production needs to be determined in the future. It could be possible that the three potential immune antagonists of EAV, nsp1, nsp2, and nsp11, target different parts of the host cellular immune system and their synergistic effect during the course of infection could be able to shut down the host cell innate immune response completely. This may explain why the induction of interferon and some immunomodulatory cytokines are inhibited during \textit{in vivo} EAV infection [62].

Among the three potential IFN antagonists of EAV, expression of nsp1 had the strongest inhibitory effect on IFN-\(\beta\) promoter activation. In comparison to our findings, previous studies on PRRSV also demonstrated that the EAV nsp1 homologous, PRRSV nsp1\(\alpha/\beta\) has the strongest ability to inhibit type I IFN synthesis [26, 31, 51, 63–67].
Further analysis revealed that EAV nspl inhibited two key signaling pathways for IFN-β activation, the IRF-3- and NF-κB-dependent signaling pathways. In our study, EAV nspl blocked each signaling step upstream of IRF-3 or NF-κB activation, suggesting that EAV nspl acts downstream of all those tested steps in both signaling pathways. Immunofluorescence microscopy analysis further showed that nspl did not have much effect on the nuclear accumulation of IRF-3 and NF-κB. Therefore, we postulated that EAV nspl might have an effect on the IFN-β promoter inside the nucleus. Previous studies have shown that PRRSV nsplα/β modulates type I IFN response by blocking dsRNA-induced IRF-3 activation and IFN promoter activities, but IRF-3 phosphorylation and its nuclear translocation occur normally in the presence of the nsplα/β [51]. Recent study reported that PRRSV nsplα blocks the IRF-3 activation by degrading the CREB (cyclic AMP response element binding)-binding protein (CBP) and subsequently inhibiting formation of enhanceosomes in the nucleus [51, 63]. EAV nspl was reported to be largely nuclear located, similar to PRRSV nsplβ [68], suggesting that it may have an effect on the formation of the transcription enhanceosome on the IFN-β promoter inside the nucleus. However, PRRSV nsplβ does not degrade CBP or interrupt the formation of enhanceosome [68]. Thus, EAV nspl may
have a unique function in modulating the IFN production in the nucleus. The EAV nsp1 does not contain the traditional nuclear localization signal [68], which suggests that nsp1 might be bound with cellular protein(s) and shuttled into the nucleus. Further studies are required to map the exact point(s) on the IFN induction pathway at which EAV nsp1 acts. It will be interesting to determine the requirement for nuclear localization of the EAV nsp1 that relates to its interferon antagonist function.

In summary, this study is the first report on molecular mechanisms of EAV involved in IFN antagonistic activity in its natural host cells. Our data indicate that several EAV replicase proteins, including nsp1, nsp2, and nsp11, possess IFN antagonistic activity and may have potential roles in the regulation of host innate immune responses. Among these proteins, nsp1 may play a key role as IFN antagonist. Further studies are needed to elucidate the detailed mechanisms that EAV proteins are involved in counteracting the host innate immune response.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**Authors’ Contribution**

Yun Young Go and Yanhua Li contributed equally to this work.

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Research Article

Differential Host Cell Gene Expression and Regulation of Cell Cycle Progression by Nonstructural Protein 11 of Porcine Reproductive and Respiratory Syndrome Virus

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Nonstructural protein 11 (nsp11) of porcine reproductive and respiratory syndrome virus (PRRSV) is a viral endoribonuclease with an unknown function. The regulation of cellular gene expression by nsp11 was examined by RNA microarrays using MARC-nsp11 cells constitutively expressing nsp11. In these cells, the interferon-β, interferon regulatory factor 3, and nuclear factor-κB activities were suppressed compared to those of parental cells, suggesting that nsp11 might serve as a viral interferon antagonist. Differential cellular transcriptome was examined using Affymetrix exon chips representing 28,536 transcripts, and after statistical analyses 66 cellular genes were shown to be upregulated and 104 genes were downregulated by nsp11. These genes were grouped into 5 major signaling pathways according to their functional relations: histone-related, cell cycle and DNA replication, mitogen activated protein kinase signaling, complement, and ubiquitin-proteasome pathways. Of these, the modulation of cell cycle by nsp11 was further investigated since many of the regulated genes fell in this particular pathway. Flow cytometry showed that nsp11 caused the delay of cell cycle progression at the S phase and the BrdU staining confirmed the cell cycle arrest in nsp11-expressing cells. The study provides insights into the understanding of specific cellular responses to nsp11 during PRRSV infection.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most significant infectious diseases for the pig industry worldwide and causes severe economic losses [1]. The etiological agent is PRRS virus (PRRSV), which belongs to the family Arteriviridae in the order Nidovirales [2] and possesses a single-stranded positive-sense RNA genome of 15.4 kb in size [3–6]. Two distinct genotypes have been reported for PRRSV: European (type I) and North American (type II) genotypes [7, 8]. The PRRSV genome contains 10 open reading frames (ORFs) including the newly identified ORF5a [9, 10]. ORF5a is translated to produce the PPIa polyproteins, but ORF5b is expressed as a fusion with ORF5a by ribosomal frameshifting and produces the PPIa/b fusion polyproteins. PPIa and PPIa/b are cotranslationally processed into 14 cleavage products. These products are nonstructural proteins (nsps) that are believed to participate in viral genome replication and subgenomic mRNA transcription [11–13]. Of these, nsp11 is a 223 amino acid protein and contains a nidovirus-specific domain, termed NendoU, in the C-terminal region. NendoU is known to contain an endoribonuclease activity and consists of two subdomains, A and B [4, 14–16]. Mutational studies using equine arteritis virus (EAV) nsp11, which is a homolog of PRRSV nsp11, show that three enzymatically catalytic sites reside in subdomain A, while two aspartic acids in subdomain B are responsible for the overall protein structure [16]. In EAV, nsp11 plays a key role in viral RNA synthesis and thus it may also be essential for PRRSV replication. Recently, PRRSV has been shown to modulate type I IFN response [17] and nsp11 has been suggested to participate in the modulation of IFN response [18].
Cellular transcriptional profiles during PRRSV infection have been studied to some extent [19, 20]. However, such studies do not identify specific viral proteins responsible for gene expressions changes, and thus the present study was conducted to understand the specific cellular response to nsp11 in cells stably expressing the protein using RNA microarrays. Based on the microarray data, five major cellular pathways were identified to be regulated by nsp11, and of the five pathways the cell cycle pathway was examined. We provide the evidence that PRRSV nsp11 protein participates in modulating the cell cycle progression at the S phase.

2. Materials and Methods

2.1. Cells. MARC-145 is a subcloned cell line of MA-104 which was derived from African green monkey kidney [21]. MARC-145 is the only established cell line permissive for PRRSV replication and thus widely used for the study of PRRSV in vitro. MARC-145 and MARC-nsp11 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Mediatech Inc., Manassas, VA, USA) containing 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA) in a humidified incubator with 5% CO₂ at 37°C.

2.2. Plasmids, Antibodies, and Chemicals. The nsp11 coding sequence was PCR-amplified from the FL12 strain of PRRSV and was inserted into the retroviral expressing vector pLNCX2 (Clontech) and mammalian expression vector pXJ41 with a FLAG tag at its N-terminus using the following primers: forward 5′-AAATCTGAGGCCCACCATTGGGG-TCGAGCTCCCCGCTCCC-3′ and reverse 5′-GCAGGC-GCTTACTTATCGTCGTCATCCTTGTAATCTTCAAGTGAAAATAGGC-3′. The translation initiation and termination codons were added to the nsp11 coding sequence. The anti-FLAG monoclonal antibody (MAB M2, Sigma) and the anti-BrdU antibody were purchased from Sigma (St. Louis, MO, USA). Bromodeoxyuridine (5-bromo-2′-deoxyuridine, BrdU) is a synthetic nucleoside that is an analog of thymidine and is commonly used in the detection of proliferating cells. Polyinosinic:polycytidylic (poly I:C) as a double-stranded RNA analog was purchased from Sigma. A donkey anti-rabbit antibody conjugated with Texas Red and a goat anti-mouse antibody conjugated with FITC were purchased from Invitrogen (Carlsbad, CA, USA). The nsp11-specific rabbit antibody was generated in our laboratory using recombinant proteins described as follows.

2.3. Recombinant Protein Preparation. Since wild-type nsp11 seemed to be toxic in E. coli [15], the NendoU nsp11 mutant (nsp11-K3779A), was subcloned into the E. coli expression vector pET-28a+ with the His-tag at both termini, and this plasmid was transformed into E. coli BL21. A 5 mL overnight culture was started using LB broth containing ampicillin (1 μg/mL) by inoculating with transformed bacteria at 37°C with vigorous agitation. In the following morning, 500 mL of 2xYT (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per L) containing ampicillin was inoculated with the 5 mL overnight culture. The culture was incubated at 37°C for approximately 4 h and when the optical density at 600 reached 0.6–0.8, protein expression was induced by adding IPTG up to 1 mM concentration. The culture was incubated for additional 2 h. Cells were pelleted by centrifugation at 7700 × g for 10 min at 4°C. The cells were resuspended in 12.5 mL of STE (5 mL of 1 M Tris-HCl, pH 8.0, 150 mM of 0.5 M NaCl and 1 mL of 0.5 M EDTA, pH 8.0 in 500 mL) containing aprotinin (1–10 μg/mL) and PMSF (1 mM) and pelleted again at 7700 × g for 10 min. The cells were resuspended again in 12.5 mL of 1X PBS, and DNase 1 (20 μg/mL) and lysosome (200 μg/mL) were added and treated for 1 h. Then, DTT was used to make a final concentration of 5 mM and incubate 5 min on ice. 20% of Sarkosyl solution was additionally added to a final concentration of 0.5%, followed by sonication to shear the genomic DNA at the setting scale of 4 for 10 s at least three times (Soniprep 150; Sanyo Gallenkamp PLC, Leicester, UK). After sonication, the samples were centrifuged at 12,000 rpm for 30 min (J2-21; Beckman Coulter, Brea, CA, USA), and supernatants and pellets were collected separately and subjected to SDS-PAGE individually to determine the presence of nsp11-K3779A protein for each fraction. Nsp11 was purified from the supernatants and concentrated to 1 mg/mL using the HisTrap column according to the manufacturer’s instruction (GE Healthcare Life Sciences, Piscataway, NJ, USA). A total of 2 mg of nsp11 was used to immunize a rabbit 5 times at 2-week intervals, intramuscularly using Freund’s incomplete and complete adjuvants, and an anti-PRRSV-nsp11 rabbit serum was generated (Immunological Research Center, University of Illinois, Urbana, IL, USA). The specificity of the antiserum was determined by immune-blots and immunofluorescence using PRRSV-infected MARC-145 cells.

2.4. Establishment of nsp11-Expressing Cells (MARC-nsp11). MARC-145 cells were transduced with the nsp11 gene using the retroviral gene transfer system (Clontech). Briefly, 0.5 μg of the pLNCX2-FLAG-nsp11 plasmid was cotransfected with pSV5-G into the pantropic packaging cell line GP2-293 to produce infectious lentivirus containing the PRRSV nsp11 gene. After 48 h of incubation, culture supernatants were collected and used to infect MARC-145 cells. Nsp11 gene-integrated cells were selected using 1 mg/mL of G418 (Invitrogen) for approximately 2 weeks with fresh G418 every 4 days. When the majority of cells has died, G418-resistant cell colonies were picked using cloning cylinders and were amplified as putative nsp11-expressing cells. Seven clones were initially selected and individually amplified. One clone was chosen and designated as MARC-nsp11 for subsequent studies.

2.5. PCR, RT-PCR, and Quantitative PCR. For PCR, cellular DNA was extracted from MARC-nsp11 cells using QIAamp DNA kit (Qiagen) and PCR was performed to determine the nsp11 gene integration. For reverse transcription (RT), total cellular RNA was extracted using Trizol (Invitrogen) and was treated with RQ1 RNase-free DNase I (Promega) followed by RT using the nsp11-specific reverse primer and PCR using the primer set as described above. Quantitative (q) PCR
was performed using ABI Prism 7000 Sequence Detection System and Software (Applied Biosystems) in a final volume of 25 μL containing 2.5 μL of cDNA synthesized from the RT reaction, 2.5 pmol of each primer, 12.5 μL of SYBR Green Master Mix (Applied Biosystems), and 5 μL of water. The primer sequences were designed using Primer 5.0 Software (Invitrogen) or obtained from previous reports (Table 2). The amplification parameters were 40 cycles of two steps, each step comprised of heating at 95°C and extension at 60°C. The final mRNA levels of target genes were normalized using GAPDH as a housekeeping gene.

2.6. Immunoprecipitation. Typically, 100 μL of total cell lysates was incubated with 1 μL of the anti-nsp11 rabbit serum at 4°C overnight. Reactions were incubated with Protein A Sepharose beads (GE Healthcare) at 4°C for 4 h. Following centrifugation for 5 min, supernatants were aspirated and washed with the lysis buffer twice. The beads were mixed with the loading buffer, boiled, and subjected to 12% SDS-PAGE followed by transfer to Immobilon-P membrane (Millipore). After blocking membranes with 5% skim milk powder dissolved in TBS-T (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Tween 20) for 1 h at room temperature, membranes were incubated with the anti-FLAG antibody in TBS-T containing 5% skim milk powder at 4°C overnight. After 5 washes with TBS-T, membranes were incubated with a horseradish peroxidase-conjugated anti-mouse antibody for 1 h at room temperature. Membranes were washed 5 times again and proteins were visualized using the ECL detection system (Thermo, Minneapolis, MN, USA).

2.7. Dual Luciferase Reporter Assays. Double-strand RNA stimulation was conducted using poly (I:C). For nsp11-gene transfection, MARC-145 cells were seeded in 12-well plates and per well, 0.05 μg of pRL-TK, 0.5 μg of pIFN-β-luc, pIRF3-luc or pPRDII-luc, and 0.5 μg of pXJ41-FLAG-nsp11 were cotransfected using Lipofectamine 2000 according to the manufacturer’s instruction (Invitrogen). For MARC-nsp11 cells, pRL-TK and each of the three reporter plasmids were cotransfected with the same amount as that of the nsp11 gene transfection. Twenty-four hours after transfection, 0.5 μg of poly (I:C) was transfected into cells for 16 h. Cells were lysed using the passive lysis buffer (Promega), and supernatants were measured for luciferase activities using the Dual Luciferase Reporter Assay System (Promega) in the luminometer (Wallac 1420 Victor multilabel counter, Perkin Elmer, Waltham, MA, USA).

2.8. RNA Microarray Design and Data Analyses. MARC-145 and MARC-nsp11 cells were seeded one day prior to experiments and total cellular RNAs were extracted using Trizol (Invitrogen) and purified by RNasy mini kit (Qiagen). The quantity and quality of RNA were determined using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). The RNA samples were then subjected to microarray using Human Gene 1.0 ST arrays (Affymetrix UK Ltd., High Wycombe, UK) at Keck Biotechnology Center (University of Illinois, Urbana, IL). The microarray was repeated twice in triplicate each. For data analyses, quality control assessments, data processing, and statistical analyses were conducted using the package from the Bioconductor project [22] as indicated below. The Affymetrix’s Human Gene 1.0 ST array contained probes to interrogate 253,002 exons representing 28,536 annotated genes. Comparisons were made either on the exon- or gene-level to investigate alternative splicing or on the whole gene-level to summarize all transcripts of the gene. The individual probe values were background-corrected, normalized, and summarized into one value at both the exon- and gene-levels using the robust multiarray average (RMA) algorithm available from the oligo packages [23]. Testing for differential gene expressions between MARC-145 cells and MARC-nsp11 cells was conducted separately at the exon- and gene-levels by fitting a linear model including a term to account for the separate processing batches using the Linear Models for Microarray Data (Limma) package [24, 25]. The criteria for significance varied for the exon- and gene-levels. At the exon-level, the criteria were at least a 2-fold change and a raw P value < 0.02, resulting in 8,693 significant exons. At the gene-level, the Limma model was fit and raw P values were calculated using all genes on the array, but the correction for multiple hypothesis testing using the FDR (false discovery rate) method [26] was done for only the 9,241 genes that varied in expression across all the samples of at least a 1.5-fold change. The criteria used to select significant genes within the filtered dataset for upregulation and downregulation were FDR P value < 0.1 and fold change > 2 or < −2, respectively.

2.9. Flow Cytometry and Cell Cycle Analysis. Identical numbers of MARC-145 cells and MARC-nsp11 cells were seeded and grown for 24 h in DMEM containing 10% FBS. For flow cytometry, cells were collected by trypsinization, washed with PBS, and resuspended in cold PBS to 1 × 10⁶ cells per mL. The cell suspension was added dropwise to an equal volume of cold ethanol with continuous agitation. After overnight incubation at 4°C, its cellular DNA was stained with 10 μg/mL propidium iodide (PI) prepared in PBS containing 0.1% Triton X-100 and 10 μg/mL RNase A (Roche) for 30 min at room temperature in the dark. Samples were then analyzed by flow cytometry (BD AccuriC6, BD Accuri Cytometers, Ann Arbor, MI, USA), and the data were analyzed using FACS Express software supplied from Keck Biotechnology Center (University of Illinois, Urbana, IL, USA).

2.10. BrdU Incorporation and Immunofluorescence Assay. DNA synthesis in proliferating cells was determined using (BrdU) bromodeoxyuridine incorporation since its incorporation to DNA occurs during the S phase. Cells were seeded on cover slips at a density of 1 × 10⁵ cells/coverslip (10 mm × 10 mm) and allowed to rest for 24 h. The medium was removed and cells were incubated for 10 min, 20 min, or 24 h pulse in the BrdU labeling medium. For the 10 min and 20 min pulses, 10 μM of BrdU was applied, and for the 24 h pulse 100 nM of BrdU was applied. After BrdU incubation, cells were fixed in 2% paraformaldehyde in PBS for 15 min and washed with PBS three times. Cells were then permeabilized with 0.5% Triton X-100 for 7 min on ice.
followed by blocking with 1% normal goat serum (NGS) in PBS three times, 10 min each. To observe the nuclei of cells, an anti-lamin (1: 200) rabbit antibody was used as the primary antibody for 1 h in PBS containing 1% NGS, and cells were incubated with a donkey anti-rabbit antibody conjugated with Texas-red (1:1000) for 30 min. Followed by washing four times, 5 min each, 2% parafomaldehyde was used again to fix the bound antibodies. Cells were then incubated with 4N HCl for 25 min at room temperature to denature DNA and to identify the stained BrdU in the nuclei. After three 10 min washes with PBS and two 10 min washes with 1% NGS in PBS, an anti-BrdU monoclonal antibody (1: 500) was applied to cells for 90 min. Cells were then incubated with a goat anti-mouse antibody conjugated with FITC (1: 600) for 30 min. Washing with PBS was applied four times after every incubation with antibodies. The cover slips were mounted on microscope slides in the mounting buffer and visualized using a Zeiss Axio Imager z1 fluorescence microscope (Carl Zeiss Inc.) equipped with Chroma filters (Chroma Technology). Images were collected using AxioVision Software (Zeiss) and Hamamatsu ORCA cooled CCD camera. The BrdU-incorporated cells and a total number of lamin-positive cells were counted for both MARC-145 and MARC-nsp11 cells, and the % of BrdU incorporation was calculated using the formula as follows: % BrdU incorporation = (number of double-positive cells for BrdU and lamin)/(200 lamin-positive cells) × 100. The cell counting areas were selected randomly on slides and 200 lamin-positive cells were counted.

3. Results

3.1. Establishment of MARC-nsp11 Cells Stably Expressing nsp11 Protein. To study the regulatory role of PRRSV nsp11 in cellular processes, a cell line was established to constitutively express the nsp11 protein. MARC-145 was used as the parental cell line, since it is one of only few cell lines permissive for PRRSV infection. MARC-145 cells were infected with the lentivirus containing the nsp11 gene from PRRSV strain FL12 and G418 (neomycin)-resistant cells were selected. A total of seven drug-resistant clones were obtained and they were individually propagated for analyses. Cellular DNA was extracted from each clone, and PCR was conducted to determine gene integration. All clones were PCR-positive for nsp11 (Figure I(a)), and cell clone “a” was chosen for further characterization and designated MARC-nsp11. The chosen cell clone was examined for nsp11 mRNA by RT-PCR using primers indicated in Table 2, and a 660 bp fragment was specifically amplified (Figure I(b)). The nsp11 protein expression was also determined by immunoprecipitation using a rabbit anti-nsp11 antibody. A 26 kD protein was specifically identified in MARC-nsp11 cells at a low level (Figure I(c), lane 3), and the same size protein was identified in nsp11 gene-transfected cells (Figure I(c), lane 2), demonstrating the expression of nsp11 in these cells.

3.2. Suppression of Type I IFN Induction by nsp11. PRRSV nsp11 contains the NendoU domain which is a common motif associated with an endoribonuclease activity for viruses in the order Nidoviridae [14, 15, 27]. Furthermore, PRRSV nsp11 has recently been suggested as a potential type I interferon (IFN) regulator [17, 18]. Thus, the regulatory function of nsp11 for IFN induction was first examined in MARC-nsp11 cells and in MARC-145 cells transfected with the nsp11 gene. Cells were transfected with the pIFN-β-luc reporter plasmid and stimulated with poly (I:C) to examine the IFN induction (Figure 2(a)). While the cells transfected with the empty vector pXJ41 showed an efficient induction of luciferase activity of up to ~16-fold after stimulation, nsp11-expressing cells exhibited a strong suppression of the activity down to ~4-fold at the most (P < 0.01). The nsp11-mediated IFN suppression was dose-dependent (Figure 2(a)).

IFN expression is tightly regulated by IRFs (interferon regulatory factors), nuclear factor (NF)-κB, and activator protein (AP)-1 transcription factors. Among these, IRFs and NF-κB are major players regulating the formation of IFN enhanceosome and the IFN-β production, and thus we first examined the IFN regulatory activities of nsp11 in MARC-145 cells by gene transfection using pIFR3-luc and pPRDII-luc reporter plasmids. pIFR3-luc contains 4 copies of the IFR3-binding sequence, while pPRDII-luc contains 2 copies of the NF-κB binding sequence upstream of the luciferase gene. When cells were stimulated, the IFR3 reporter activity was increased by ~14-fold (Figure 2(b)). In the presence of nsp11, however, the IFR3 activity was decreased by 7-fold (P < 0.005) compared to the activity in the absence of nsp11 (Figure 2(b)). Similarly, the NF-κB reporter activity was increased by approximately 10-fold after stimulation, but in the presence of nsp11, this activity was decreased by 2-fold (P < 0.005) compared to the activity in the absence of nsp11 (Figure 2(c)). These results show the suppression of IFR3 and NF-κB induction by nsp11.

To examine whether MARC-nsp11 cells expressing nsp11 were biologically active, the IFN-β, IFR3, and NF-κB activities were determined after stimulation with poly (I:C) using the corresponding reporter constructs (Figure 2(d)). MARC-nsp11 cells (black bars) showed the decrease of luciferase activities compared to those of empty vector-transfected (gray bars) or mock-transfected (white bars) MARC-145 cells. The suppressive activities in MARC-nsp11 were less markedly than those in gene-transfected cells and this was probably due to the lower level expression of nsp11 in MARC-nsp11 cells. The reporter activities of IFN-β, IFR3, and NF-κB were reduced by ~2, 2, and 2.5-fold, respectively (P < 0.05). This indicates that nsp11 in MARC-nsp11 cells was biologically active and retained the regulatory activity for IFN induction.

3.3. Transcriptome Analysis in MARC-nsp11 Cells. To examine the transcription regulation of host cells by nsp11, an RNA microarray was conducted in MARC-nsp11 cells using human gene exon chips. These chips contained 253,002 exons from 28,536 annotated genes. After microarray analyses, genes were filtered by fold changes greater than 1.5, and 9,241 genes were initially identified to have been altered, among which 66 and 104 cellular genes were upregulated
and downregulated, respectively, under the criteria of a fold change of 2 or greater and a false discovery rate (FDR) of 10%. Based on the Database for Annotation, Visualization, and Integrated Discovery (DAVID), 79 of the significantly regulated genes were placed into 17 categories, some of which shared the common function. According to their functional correlations, the functional groups were summarized into five major cellular pathways that were regulated by nsp11: histone-related proteins, cell cycle and DNA replication pathways, MAPK signaling pathways, ubiquitin-proteasome pathways, and complementary pathways (Table 1).

For validation of the fold changes in the gene expression profiles, five genes (TNFSF10, DEPTOR, SH2, NOL6, and EGR1) were chosen according to their fold changes, and RT-qPCR was conducted. NOL6 and EGR1 were chosen to represent the group of upregulated genes, and TNFSF10 and DEPTOR were chosen to represent the group of downregulated genes, while SH2 was chosen as an unregulated gene. The results from RT-qPCR for these genes were in good agreement with their fold changes in the microarray and confirmed the fold change profiles for differential gene expression (Figure 3).

3.4. Regulation of Histone-Related Functions, Complement, MAPK Signaling, and Proteasome Pathways. Seventeen histone-related genes were found to be upregulated, whereas three genes (C1S, C1R, and C3) in the complement system were downregulated (Table 1). C1S and C1R were responsible for the activation of the classic pathway of the complement
system. C1R is autoactivated and then cleaves C1S for activation. Activated C1S cleaves C4 and C2, resulting in the activation of C3-convertase complex [28]. C3 is a central molecule in the complement system whose activation is essential for all the important functions performed by this system [29]. The downregulation of C1S, C1R, and C3 suggests the possible suppression of the complement system by nsp11.

Six genes (DUSP1, DUSP6, FOS, MYC, JUN, and SRF) related to the MAPK signaling pathways were found to be upregulated, and five genes in the proteasome pathways were found to be regulated, among which SUMO1 and SNCA were downregulated and PSMD3, PSMB10, and PSMA7 were upregulated (Table 1). DUSPs regulate the cellular localization and activity of MAPK which functions in the negative feedback loop of ERK regulation [30]. DUSP1 dephosphorylates ERK in the nucleus and allows its trafficking to the cytoplasm [31], while DUSP6 causes the cytoplasmic retention of ERK2 [31]. C-Jun/AP-1 and c-Fos genes were also upregulated in our study, which can be activated by JNK and p38 MAPK [32]. For the proteasome pathways, three (PSMD3, PSMB10, and PSMA7) out of five are proteasome subunits, and their
Table 1: Five major cellular pathways regulated by PRRSV nsp11.

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upregulation suggests an enhanced effect on the proteasomal pathways by nsp11. SUMO1 (small ubiquitin-like modifier 1) has multiple functions by attaching itself to substrates referred to as sumoylation. After sumoylation, protein may undergo degradation through the proteasome [33, 34].

3.5. Delay of Cell Cycle by nsp11. The microarray data suggested the regulation of cell cycle and DNA replication pathways by nsp11. A total of 10 genes related to these pathways were found to be regulated (Table 1). Among these, cell division cycle 45 (CDC45)-like and CDC25 homolog A are proteins controlling the cell cycle progression [35–37], whereas minichromosome maintenance complex 2 (MCM2), MCM4, and MCM5 are helicase components regulating DNA replication [38].

The MCM2-7 complex is assembled on the eukaryotic chromosomes during the G1 phase of a cell cycle, which is then activated during the S phase by MCM10, CDC45, and the GINS complex [39]. The regulation of MCMs and CDC genes suggests that nsp11 may perturb the normal host cell cycle. To examine this possibility, identical numbers of MARC-nsp11 and MARC-145 cells were seeded on plates, and 24 h later cells were collected for DNA staining and flow cytometry. In two independent experiments, the MARC-nsp11 cells at the S phase constituted 28.5% (Figure 4(b)) as compared to 12.6% for MARC-145 cells (Figure 4(a)), which was more than a 2-fold increase for nsp11-expressing cells indicating that MARC-nsp11 cells were accumulating at the S phase by 24 h.

To examine the nature of DNA accumulation at the S phase by nsp11, cells were pulsed-labeled for 10 min, 20 min, or 24 h with BrdU and stained for BrdU incorporation and lamins. BrdU is a nucleotide analog and thus can be incorporated into replicating DNA, whereas lamin proteins are major architectural proteins of the nuclear lining inside the nuclear membrane in cells. Thus, all cells are anticipated to be stained with an anti-lamin antibody, whereas only cells synthesizing new DNA in the S phase are presumed to be stained with an anti-BrdU antibody. A short pulse of 10 min or 20 min would detect BrdU incorporation in a single cell cycle, whereas a longer time incubation of 24 h would detect multiple cell cycles and thus the majority of normal cells would be positive for BrdU staining (Figure 5(a)). A total of 200 lamin-positive cells (in red) were randomly chosen for each slide, and BrdU positive cells (in green) out of the lamin-positive cells were counted to determine BrdU incorporation rates using the formula described in Materials and Methods. MARC-nsp11 cells exhibited less numbers of BrdU-positive cells after the 10 min and 20 min pulses compared to those of MARC-145 cells (Figure 5(a)), and their BrdU incorporation rates dropped from 47.07% (white bar) to 38.07% (black bar) (P < 0.005) and from 57.8% (white bar) to 44% (black bar) (P < 0.005), respectively (Figure 5(b)). After 24 h of labeling, a greater reduction of BrdU staining was observed for MARC-nsp11 cells, where the percentage of BrdU incorporation decreased from 92% (white bar) to 49.73% (black bar) (P < 0.001; Figure 5(b)). The intensity of BrdU staining in MARC-nsp11 cells was also significantly reduced after the 24 h pulse compared to that of MARC-145 cells (Figure 5(a)), demonstrating the substantial suppression of DNA synthesis by nsp11. Both flow cytometry and BrdU

### Table 1: Continued.

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### Table 2: Primer sequences for RT-qPCR for selected genes.

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Figure 3: Confirmation of differential gene expression by RT-qPCR in MARC-nsp11 cells. The same preparations that were used for RNA microarray were used for RT-qPCR. For quantitative PCR, two genes (NOL6 and EGR1) were chosen to represent upregulated genes by nsp11, and TNFSF10 and DEPTOR were chosen as the downregulated genes in the microarray assays (Table 2). SH2 was chosen as a nonregulated gene. Bars illustrate the differential gene expression determined by RT-qPCR. White bars represent MARC-145 cells and black bars indicate MARC-nsp11 cells. Numbers in the table show the fold changes.

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<td>EGR1</td>
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Figure 4: Flow cytometric analysis for MARC-nsp11 cells. A total of $1 \times 10^6$ MARC-145 or MARC-nsp11 cells were seeded in 60 mm-diameter dishes and cultivated for 24 h. Cells were gently harvested by trypsinization and fixed with cold ethanol. Cells were stained with 10 $\mu$g/mL of propidium iodide (PI) and subjected to flow cytometry. Shown is the PI trace (red areas) indicating the proportion of cells with the 2N and 4N DNA contents. The red areas present relative percentages. G1, first gap period; G2, second gap period; S, DNA synthesis and chromosome replication.
Figure 5: BrdU incorporation and DNA synthesis in MARC-nsp11 cells. (a) Cells were labeled with BrdU and stained to determine the newly synthesized cellular DNA at the S phase. Cells were pulse-labeled with 10 μM of BrdU for 10 min and 20 min, or 100 nM for 24 h. Cells were fixed with 2% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 followed by staining with an anti-lamin antibody (shown in the red color). Cells were then incubated with 4N HCl to denature the DNA, and stained for DNA-incorporated BrdU using an anti-BrdU antibody (shown in the green color). The scale bar in white indicates 10 micron. (b) BrdU incorporation rates in MARC-nsp11 cells. A total of 200 lamin-positive cells were counted, and of the 200 cells BrdU-positive cells were counted. The BrdU incorporation rates were then calculated using the following formula: (number of double-positive cells for BrdU and lamin)/(200 lamin-positive cells) × 100. The experiments were repeated 4 times and the results were presented as the arithmetic means ± standard error (n = 4). One star (*) represents P < 0.005 and two stars (**) represent P < 0.001. MARC-145 cells are indicated in unfilled white bars and MARC-nsp11 cells are indicated in black bars.

staining data indicate that nsp11 slows down the cell cycle progression through the S phase.

4. Discussion

In the present study, MARC-nsp11 cells were established to constitutively express PRRSV nsp11, and an RNA microarray was conducted in these cells to study differential transcription responses to nsp11. The microarray studies identified 170 differentially regulated cellular genes with the threshold of 2. Of these, 104 genes were downregulated and 66 genes were upregulated, and many of these genes were able to be placed according to their functional relevance into 5 different pathways: histone-related proteins, cell cycle and DNA replication, MAPK signaling, ubiquitin-proteasome, and the complement system. Compared to previous studies [19, 20], the genes identified in our study were fewer in number and less in diverse. This is probably because the regulated genes identified in our study were exclusively nsp11-specific, whereas the genes in the previous studies were responders to the entire spectrum of viral proteins. Thus, nsp11-regulated genes were mostly included in the previously identified genes. Zhou et al. [20] also showed that the genes relevant to cell cycle and DNA replication were regulated by highly pathogenic (HP)-PRRSV in PAMs. Chromosome organizing proteins were also regulated by nsp11, and proteins regulating the complement system for tissueremolding and inflammation were also found in our study. c-Jun and c-Fos are two effectors of the MAPK signaling pathway, and they were specifically upregulated during PRRSV infection [19]. PRRSV-mediated activation of the MAPK signaling pathway and the increase of JNK and p38 phosphorylation have recently been demonstrated [40], which is also in support of our findings.

Of the possible pathways regulated by nsp11, the cell cycle pathway was chosen and explored further. It appears that the cell cycle progression was delayed at the S phase
in nsp11-expressing cells compared to MARC-145 cells. A similar observation was recently made for coronavirus in cells expressing nsp15, which is a coronavirus homolog of PRRSV nsp11 [41]. In that study, SARS-CoV nsp15 was shown to downregulate the retinoblastoma (Rb) activity which is responsible for cell proliferation. As a consequence, a higher percentage of cells was accumulating at the S phase when expressing nsp15, supporting our observation of slower cell cycle progression in nsp11-expressing cells. The S phase tardiness may be associated with an altered DNA replication, since several MCM proteins, which are components of DNA helicase, were upregulated (Table I). It is possible that the increase of helicase proteins might have caused a malfunction of the replication fork and thus the inhibition of DNA synthesis in MARC-nsp11 cells.

Virus-mediated cell cycle regulation is not uncommon and can be beneficial to viruses. In particular, it is true for DNA viruses replicating in the nucleus such as SV40, herpes simplex virus, and adeno-associated virus, in which by arresting the cell cycle of infected cells, the cellular DNA replicative machinery may be captured and utilized for viral DNA replication [42–44]. For RNA viruses, influenza virus replication has been shown to be regulated by helicase and the MCM complex consisting of MCM2-7 [45]. The interaction between the influenza virus PA polymerase and MCM complex increases the stability of RNA polymerase. In our study, MCM2, MCM4, and MCM5 were upregulated by nsp11. Even though the PRRSV replicates in the cytoplasm, the cell cycle regulation may be considered beneficial for the virus, since an available pool of cellular machineries can be maximally utilized towards the production of progeny at an early stage of infection. For PRRSV, nucleocapsid (N) protein has also been suggested to regulate the cell cycle progression by modulating the ribosomal RNA synthesis in the nucleolus [46]. Thus, it is possible that N and nsp11 may both regulate the cell cycle progression and facilitate virus production by targeting different cellular components modulating the host cell cycle. The N protein is an RNA-binding protein which contains the nuclear localization signal (NLS) and thus localizes in the nucleus and nucleolus. By yeast 2-hybrid screening, the inhibitor of MyoD family a (I-mfa) domain-containing protein was identified interacting with PRRSV N [47]. Since the I-mfa domain-containing protein interacts with cyclin T1 [48], which participates in the control of the cell cycle, this interaction suggests a regulatory role of N for the cell cycle [17]. Different from N, nsp11 resides in the cytoplasm and contains an endoribonuclease activity. Nsp11 may alter the function or expression of cytoplasmic cellular components such as mRNA modification, which may then result in the regulation of the cell cycle. Indeed, modification of cellular mRNA by nsp11 has been suggested previously [49]. In summary, our data show that the PRRSV nsp11 protein is responsible for the delay of the S phase and thus, together with N, may regulate the cell cycle progression. N functions in the nucleus and nsp11 functions in the cytoplasm.

In the current study, only a few immune-related cytokine genes were identified especially for IFN-related genes. This is probably due to the cell type and the treatments used in our study. MARC-145 cells are epithelial cells of the African green monkey kidney, and these cells are anticipated to produce only a minimal amount of cytokines, and their ability to produce IFN is limited unless they are stimulated. A study is in progress to compare the gene expression profiles in MARC-nsp11 cells before and after stimulation.

The microarray study allowed us to identify differential effects of the nsp11 protein on the cellular gene expression profiles. Studies are required to verify the significance of the differentially regulated gene expression. Analyses of relative protein modifications and activations especially for checkpoint proteins will help us understand the basis of the change of S phase caused by nsp11. Clearly, our data provide new insights into the understanding of cell–virus interactions and the pathogenic mechanisms of PRRSV and host responses to infection.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Comparison of Host Immune Responses to Homologous and Heterologous Type II Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Challenge in Vaccinated and Unvaccinated Pigs

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Porcine reproductive and respiratory syndrome (PRRS) is a high-consequence animal disease with current vaccines providing limited protection from infection due to the high degree of genetic variation of field PRRS virus. Therefore, understanding host immune responses elicited by different PRRSV strains will facilitate the development of more effective vaccines. Using IngelVac modified live PRRSV vaccine (MLV), its parental strain VR-2332, and the heterologous KS-06-72109 strain (a Kansas isolate of PRRSV), we compared immune responses induced by vaccination and/or PRRSV infection. Our results showed that MLV can provide complete protection from homologous virus (VR-2332) and partial protection from heterologous (KS-06) challenge. The protection was associated with the levels of PRRSV neutralizing antibodies at the time of challenge, with vaccinated pigs having higher titers to VR-2332 compared to KS-06 strain. Challenge strain did not alter the cytokine expression profiles in the serum of vaccinated pigs or subpopulations of T cells. However, higher frequencies of IFN-γ-secreting PBMCs were generated from pigs challenged with heterologous PRRSV in a recall response when PBMCs were re-stimulated with PRRSV. Thus, this study indicates that serum neutralizing antibody titers are associated with PRRSV vaccination-induced protection against homologous and heterologous challenge.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically important pandemic disease characterized by reproductive failure in sows and respiratory disease in young pigs. A recent study estimates that the total productivity losses in the U.S. swine industry due to PRRS are currently $664 million annually, an increase from the $560 million annual cost estimated in 2005 [1]. This indicates that not only does PRRS have a significant financial impact on the pork industry but also current strategies for reducing the burden of PRRS virus are not adequate.

PRRS is caused by porcine reproductive and respiratory syndrome virus (PRRSV), which is a member of the genus Arterivirus, family Arteriviridae, and order Nidovirales. PRRSV is known to mutate rapidly in both in vitro cell culture models and in vivo in natural field infections [2]. The ability of PRRSV to mutate rapidly creates genetically extensive and antigenic diverse strains in both North American and European field isolates [3]. The high genetic mutation rate
of PRRSV poses a challenge for PRRSV vaccine development [2]. Currently, both inactivated PRRSV vaccines and modified live virus (MLV) PRRSV vaccines are widely used to control the disease. However, inactivated vaccines as well as modified live vaccines have been shown to be ineffective in providing protective immunity to heterologous strains of PRRSV at the herd level [4–7]. Therefore, development of a broadly protective PRRSV vaccine will be one of the most efficient solutions to control the prevalence of PRRS worldwide.

It has been shown that pigs infected with PRRSV have inadequate immune responses, such as delayed onset of neutralizing antibody as well as weak interferon (IFN)-γ responses [2, 8]. Development of different types of vaccines aiming to increase host immune response and get broader protection from various field PRRSV infections has been proposed [9]. Currently, PRRSV-MLV is used to control the disease worldwide. However, the high incidence of genetic mutation during PRRSV transmission often results in vaccines based on strains of PPRSV isolated twenty years ago, such as MLV, having limited protection from new emerging viral strains. Disparity of immune responses elicited by different PRRSV strains was reported previously [10]. However, the role of humoral and cellular immune responses was not clearly elucidated in these reports with regard to the protection from virus challenge with different PRRSV strains. Therefore, dissecting the mechanisms of immune responses that are predictive of protection against heterologous PRRSV challenge will be valuable for the development of more efficacious vaccines. In this study, we investigated the differential profiles of host immune responses in naïve or vaccinated pigs challenged with homologous and heterologous PRRSV strains.

2. Materials and Methods

2.1. Cells and Virus. MARC-145 cells were maintained in Modified Eagle’s medium (MEM) supplemented with 7% fetal bovine serum (FBS) containing 100 U penicillin/mL and 100 μg streptomycin/mL at 37˚C with 5% CO2. Virus stocks were prepared and titered in MARC-145 cells and stored in aliquots at −80˚C until use. For virus infection and titration, MEM supplemented with 2% FBS was used. PRRS modified live virus vaccine (Ingelvac PRRS MLV) was purchased from Boehringer Ingelheim Vetmedica Inc. PRRS strains VR-2332, KS-06, or NVSL97-7895 have been described previously [11, 12].

2.2. Pigs, Vaccination, and Challenge. Twenty conventional large White-Duroc crossbred weaned specific pathogen-free piglets (3 weeks of age) were divided into four groups within the Large Animal Research Center (LARC) facility, Kansas State University. These piglets were confirmed seronegative for antibodies to PRRSV by ELISA and PRPSV–negative in the blood by RT-PCR. Pigs were allowed to acclimate for an additional week before initiation of the experiment. The first two groups were immunized intramuscularly on day postvaccination (DPV) 0 with vaccine (PRRS-MLV, 1 × 10⁶ TCID₅₀/pig). The other two groups were used as control groups before challenge and remained unvaccinated (Figure 1a)). After four weeks the pigs were challenged with 2 × 10⁶ TCID₅₀/pig of VR-2332 or KS-06 PRRSV. Necropsy was performed at 14 days postchallenge (DPC). Pigs were monitored for rectal temperature for the first 9 days after challenge and body weight once a week for the duration of this experiment.

2.3. Collection of Blood Samples for Analysis. Blood was collected on DPV 0, 7, 14, 21, and 28 and DPC 7 and 14. Serum was separated from clotted blood and preserved at −20˚C. Serum was used for evaluation of viral titers, serum neutralizing antibody titers, PRRSV-specific ELISA antibody titers (Herdcheck Porcine Reproductive and Respiratory Syndrome Antibody test Kit, IDEXX Laboratories), and cytokine expression as described previously [12]. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by Ficoll-Hypaque gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). PBMCs were used for ELISpot assay and flow cytometry analysis as described previously [12].

2.4. Gross Lung Lesion Analysis. Pigs were humanely euthanized on DPC 14 as approved by the Kansas state University Institutional Animal Use and Biosafety Committee. The lungs were macroscopically and microscopically evaluated as previously described [13]. Briefly, the dorsal and ventral surfaces of each lung lobe were given a score representing the approximate proportion that was consolidated. Individual lobe scores were used to determine an overall lung score representing the percentage of interstitial pneumonia. Sections of each of the 4 lobes of the right lung were fixed in 10% buffered neutral formalin, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H & E). Scoring of microscopic lung pathology was done in a blinded fashion by two veterinary pathologists in the Kansas State Veterinary Diagnostic Laboratory. Grading was on a 4 point scale as previously described [13].

2.5. Analysis of PRRSV Circulating in the Blood. Total RNA was extracted from pig serum and one-step SyBR Green real-time PCR (Bio-Rad) was performed to evaluate the PRRSV ORF7 expression level as previously described [14]. For quantification, total RNA of a known TCID₅₀ of virus was 10-fold serially diluted and was used to generate a standard curve. The virus quantities of unknown samples were determined by linear extrapolation of the Ct value plotted against the standard curve.

2.6. Virus Neutralizing Antibody Titer. Virus neutralizing antibody titers were assayed as previously described [12, 14]. Briefly, serum samples were heat inactivated (56˚C, 30 min) and serially diluted before the titration. The serial dilutions of serum samples were mixed with equal volume of PRRSV strains: VR-2332, KS-06, or NVSL97-7895 containing 200 TCID₅₀ of the virus. After incubation at 37˚C for 1h, the mixtures were transferred to MARC-145 monolayers in
96-well plates and incubated for an additional 72 h at 37°C in a humidified atmosphere containing 5% CO₂. Cells were then examined for cytopathic effects (CPE). CPE was used to determine the end-point titers that were calculated as the reciprocal of the highest serum dilution required to neutralize 200 TCID₅₀ of PRRSV in 90% of the wells.

2.7. ELISpot Assay. Half million PBMCs were plated in enriched RPMI in a 96-well multiscreen plate (Millipore, Billerica, MA) precoated overnight with capture IFN-γ mAB (BD Pharmingen, San Diego, CA). PBMCs were restimulated with three different strains of PRRSV (VR-2332, KS-06, or NVSL97-7895) at 0.1 MOI for 24 h at 37°C. IFN-γ-secreting cells were detected by biotinylated anti-pig IFN-γ detection antibody and visualized using the immunospot image analyzer (Cellular Technology, Cleveland, OH). We calculated the number of PRRSV-specific IFN-γ-secreting cells by subtracting the number of spots in unstimulated cultures (all samples were <10) from the count of PRRSV-stimulated cultures. Data were presented as the mean.

Figure 1: Vaccination with PRRSV-MLV induced complete protection from homologous PRRSV challenge and partial protection from heterologous challenge. (a) Experimental timeline. (b) Rectal temperature of pigs was monitored daily after PRRSV challenge. (c) Gross lung lesion scores present in all lung lobes on DPC 14 were scored using a 4-point scale. (d) PRRSV viral RNA in the serum throughout the study was determined by qPCR. Each bar represents the average of samples from five pigs ± SEM. * P < 0.05.
numbers of antigen-specific IFN-γ-secreting cells per 10^6 PBMCs from duplicate wells of each sample.

2.8. Flow Cytometry Analysis. Flow cytometry analysis was performed to determine different lymphocyte populations based on the cell surface marker phenotype: T-helper cells (CD3+CD4+CD8-), cytotoxic T lymphocytes (CD3+ CD4-CD8+), Th/memory cells (CD3+ CD4+CD8-), and γδ T cells (CD8+ TcR1N4+). Mouse anti-pig TcR1N4 antibody was purchased from VMRD (Pullman, WA), and the rest of the antibodies used in this study were purchased from BD Biosciences. Immunostained cells were acquired using a flow cytometer as described previously [12, 14]. Briefly, PBMC was treated with 2% pig serum to block Fc receptors. Cells were then stained with an appropriate Ab which was either directly conjugated to a specific fluorochrome or with a purified Ab to pig specific immune cell surface marker (TcR1N4). For cells stained with a purified Ab, labeled cells were treated with antispecies isotype specific secondary Ab conjugated with fluorochrome. Finally, cells were fixed with 1% paraformaldehyde before reading on a flow cytometer. Percentages of each lymphocyte population were analyzed by 100,000 unique events using FlowJo software (Tree Star, Inc., OR, USA).

2.9. Analysis of Cytokine Responses. Pig sera were collected at DPC7 to evaluate IL-4, IL-8, IL-10, IFN-γ, TNF-α (Life Technologies, Carlsbad, CA), and IFN-α (Abcam, Cambridge, MA) secretion profiles by ELISA. Procedures were performed as per the manufacturer’s instructions. For a given sample, the OD450 was then transformed to concentration by applying a linear regression formula calculated from the results of the standards provided in each kit.

2.10. Statistical Analysis. All data were expressed as the mean value of five pigs ± SEM. The differences in the level of body temperature, lung pathology score, humoral response, cytokine production, and viremia among each group were determined by the one-way analysis of variance (ANOVA) followed by post-hoc Tukey’s test using SigmaPlot 11 software (Systat Software Inc., San Jose, CA). The difference in the percentage of different T cell subpopulations was determined by the paired t-test using SigmaPlot 11 software.

3. Results

3.1. Vaccination with PRRSV-MLV Induced Complete Protection from Homologous PRRSV Challenge and Partial Protection from Heterologous Challenge. To compare host immune responses to challenge by different PRRSV isolates, pigs were either vaccinated with PRRSV-MLV or a mock vaccine (PBS) on day 0 and then challenged with homologous VR-2332 or heterologous KS-06 PRRSV on day 28 (Figure 1(a)). Clinically, the mean body temperature of unvaccinated pigs challenged with the KS-06 strain of PRRSV was higher compared to that in the other three groups at DPC 4 (Figure 1(b)). The body weight of all pigs was tracked throughout the study and weights of all groups were similar during the vaccination phase. Interestingly, pigs vaccinated with MLV and challenged with VR-2332 had a slightly higher weight gain than that of the other groups on DPC 14 (data not shown). Unvaccinated pigs that were challenged with either VR-2332 or the KS-06 strain had higher lung lesion scores on DPC 14 compared to those in vaccinated pigs (Figure 1(c)). Vaccinated pigs challenged with VR-2332 showed full protection against PRRSV with average lung scores being normal and no lung damage observed during pathological analysis. Additionally, vaccinated pigs challenged with the KS-06 strain had moderate protection as shown by decreased lung scores compared to that in unvaccinated-KS-06 challenged pigs (Figure 1(c)).

In addition, complete protection in vaccinated pigs against homologous challenge was confirmed with the absence of PRRS viral RNA in the serum on DPC 7. As shown in Figure 1(d), pigs vaccinated with MLV had efficiently cleared the VR-2332 challenge virus from the blood to undetectable levels. Vaccinated pigs challenged with the KS-06 strain had less circulating PRRSV in the blood than that in unvaccinated-KS-06 challenged pigs, but the difference was not statistically significant. By DPC 14, the levels of PRRSV virus circulating in the blood were reduced significantly in all vaccinated groups. Therefore, our results suggest that PRRSV-MLV can protect pigs from homologous challenge and provide moderate protection against heterologous PRRSV challenge.

3.2. Serum Neutralizing Antibody Titer Is Associated with PRRSV Vaccination-Induced Protection against Homologous and Heterologous Challenge. It has been shown that a vigorous anti-PRRSV antibody response in pigs is seen early after vaccination or PRRSV exposure [15]. To determine antibody responses, we analyzed PRRSV-specific ELISA antibodies in homologous- and heterologous-challenged pigs using commercial IDEXX ELISA kit. Serum samples were collected at various time points and used to determine the PRRSV-specific antibody levels. As shown in Figure 2(a), vaccinated pigs produced PRRSV-specific antibodies starting from DPV 14. Interestingly, the antibody titers in vaccinated pigs were not further enhanced by PRRSV challenge. Additionally, it was found that unvaccinated pigs challenged with the KS-06 isolate showed a faster onset and higher ELISA antibody titers than unvaccinated pigs challenged with VR-2332 (Figure 2(a)).

However, there is no evidence that early anti-PRRSV antibody response plays a role in the protection against PRRSV infection. In contrast, later appearing antibodies with PRRSV neutralizing activity have been shown to play a critical role in anti-PRRS immunity. A previous study showed that passive transfer of neutralizing antibodies with a titer of 8 to recipient piglets protected them from challenge-induced viremia, while transfer of serum titers of 32 produced sterilizing immunity [15], suggesting that neutralizing antibody titers over 8 can protect pigs from PRRSV. The ability of a vaccine (modified live or inactivated) to induce PRRSV neutralizing antibodies to specific PRRSV isolates influences the level of protection the vaccinated pig has to
Figure 2: Serum neutralizing antibody titer is associated with PRRSV vaccination-induced protection against homologous and heterologous challenge. (a) PRRSV-specific antibodies were detected in the serum using IDEXX ELISA kit. The threshold for positive sera was set at a sample to positive (s/p) ratio of 0.4 according to the manufacturer’s instructions. ((b)–(d)) Serum samples were titrated on MARC-145 cells and the levels of anti-PRRSV neutralizing Abs were determined as the reciprocal of the highest dilution that could inhibit CPE. Data were shown as mean ± SEM for 5 pigs per group. *P < 0.05.

3.3. PRRSV-Dependent Cytokine Expression Patterns Are PRRSV Challenge Strain Specific. Compared to MLV vaccinated pigs challenged with the KS-06 strain, unvaccinated pigs displayed significantly higher IFN-α level in the serum (Figure 3(a)). In contrast, the difference in IFN-α production was not detected between vaccinated and unvaccinated pigs after they were challenged with VR-2332. Interestingly, vaccinated pigs produced significant higher levels of IL-8 compared to unvaccinated pigs after they were challenged with the specific challenge strain [15, 16]. Therefore, we analyzed the PRRS virus neutralizing antibody (VN) titers in the serum of different treatment groups. As shown in Figure 2(b), MLV vaccinated pigs began to develop VN titers to VR-2332 at DPV 28 and the titers were significantly higher at the end of the study as compared to those in unvaccinated pigs. It is worth noting that high titer of VN antibodies against the KS-06 strain was detected only in pigs vaccinated with MLV but not in unvaccinated pigs after both groups of pigs were challenged with the KS-06 strain (Figure 2(c)). To assay for broad neutralizing activity, another PRRSV strain, NVSL97-7895, was used to measure the VN titer of all serum samples. As shown in Figure 2(d), VN antibodies against NVSL97-7895 were developed only in vaccinated pigs, and the serum VN titers in vaccinated pigs challenged with the KS-06 strain were higher than those in vaccinated pigs challenged with the homologous VR-2332. This indicates that pigs receiving vaccination followed by challenge with a different strain of PRRSV may generate antibodies with a broader neutralizing spectrum.
with VR-2332 (Figure 3(a)). TNF-α expression levels were low in all pigs and there was no significant difference among treatment groups. Furthermore, serum IL-10 levels were significantly higher in unvaccinated pigs after KS-06 PRRSV challenge than those in vaccinated pigs (Figure 3(b)). In contrast, vaccinated pigs displayed a higher level of serum IL-4 after VR-2332 challenge compared to unvaccinated pigs (Figure 3(b)). There was no significant difference in serum levels of IFN-γ among all treatment groups.

Vaccination with PRRS-MLV has been shown to induce the production of IFN-γ-secreting cells as a mechanism of protecting pigs against PRRSV viremia [17]. Therefore, the frequency of IFN-γ-secreting cells in PBMCs was evaluated on DPC 14 in a recall response in which PBMCs were restimulated with VR-2332, KS-06, or NVSL97-7895 PRRSV. As shown in Figure 3(c), when restimulated with VR-2332, PBMCs from vaccinated pigs challenged with the KS-06 strain developed more IFN-γ-secreting cells than those from the other three groups. When restimulated with KS-06 or NVSL97-7895, PBMCs from KS-06 challenged pigs produced significantly higher amount of IFN-γ-secreting cells than that from pigs challenged with VR-2332. Finally, the ratios of IFN-γ-secreting cells in PBMCs restimulated with KS-06 PRRSV in all treatment groups were significantly lower than those in PBMCs restimulated with VR-2332 or NVSL97-7895.

3.4. T Lymphocyte Subpopulations Vary between Unvaccinated and Vaccinated Groups and Are Independent of PRRSV Challenge Strain. T lymphocyte subpopulations are reported to vary in pigs after challenge with different PRRSV strains [18]. In this study, we evaluated the changes in frequency of various lymphocyte populations before and after PRRSV challenge in all experimental groups. On DPV 28, the frequencies of T-helper cells (Figure 4(a)), cytotoxic T lymphocytes (CTLs;
Figure 4: T lymphocyte subpopulations vary between unvaccinated and vaccinated groups and are independent of PRRSV challenge strain. PBMCs were isolated from pigs at necropsy (DPC 14) and T cell subsets were determined by flow cytometry analysis according to their phenotypes. Shown are the percentages of (a) T-helper cells that were CD3+/CD4+/CD8−, (b) Cytotoxic T lymphocytes that were CD8+/CD4−CD3+, (c) Th/memory cells that were CD3+CD4+CD8+, and (d) γδ T cells that were CD8+TcR1N4+. Data were shown as mean ± SEM for 5 pigs per group. *P < 0.05.

4. Discussion

As one of the most prevalent diseases in swine, PRRS has caused vast economic losses to the pig industry worldwide. Adding to its devastation, the rapid evolution rate of PRRS virus generates countless genetically distinct field isolates, many of which have increased pathogenic ability [2, 10, 18]. Recent outbreaks of PRRSV in China were characterized by high morbidity/mortality and commercially available PRRSV vaccines offered no protection [19, 20]. This demonstrates that current commercial vaccines offer limited or no protection from newly emerging PRRSV field strains. Therefore,
studies on the difference of immune responses to homologous and heterologous challenge lay an important foundation for the development of effective vaccines and eradication strategies. The present study evaluated the differences of immune responses between vaccinated and unvaccinated pigs when challenged with homologous or heterologous PRRSV. Here we demonstrate that serum neutralizing antibody titers are associated with PRRSV vaccination-induced protection against homologous and heterologous challenge.

A recent review suggests that antibodies directed against both nonstructural and structural proteins including NSP2, GP2, GP4, and GP5 may possess PRRSV neutralizing activity [5], and the variability within GP5 may explain the deficiency in cross-protection of current vaccines against heterologous strains of PRRSV. VR-2332 (homologous) and KS-06 strain (heterologous), the PRRSV viruses used for challenge experiments in this study, share 99.7% or 90.2% similarity with the PRRSV-MLV vaccine strain based on GP5 amino acid sequence, respectively. From gross lung pathology and viremia results, homologous VR-2332 PRRSV infection was fully prevented after vaccination with PRRSV-MLV as evidenced by lack of virus in sera on DPC 7 and normal viremia results. From gross lung pathology scores (Figures 1(c) and 1(d)), the variability within GP5 may explain the deficiency in cross-protection of current vaccines against heterologous strains of PRRSV. VR-2332 (homologous) and KS-06 strain (heterologous), the PRRSV viruses used for challenge experiments in this study, share 99.7% or 90.2% similarity with the PRRSV-MLV vaccine strain based on GP5 amino acid sequence, respectively. From gross lung pathology and viremia results, homologous VR-2332 PRRSV infection was fully prevented after vaccination with PRRSV-MLV as evidenced by lack of virus in sera on DPC 7 and normal gross lung pathology scores (Figures 1(c) and 1(d)). Gross lung pathology scores in the vaccinated pigs challenged with the KS-06 strain were decreased compared to those in the unvaccinated pigs, which indicate MLV vaccination can lead to partial protection from heterologous PRRSV. These results allow us to compare the immune responses from pigs with complete, partial, and no (unvaccinated) protection against PRRSV challenge. By DPV 14, antibodies specific for N proteins of PRRSV, as measured by the IDEXX ELISA kit, were detected in vaccinated pigs and increased throughout the experimental period. PRRSV-specific antibodies were similar between vaccinated groups throughout the study, suggesting that anti-N protein antibodies are not predictive of PRRSV protection. Interestingly, we did observe that KS-06 PRRSV challenge induced a faster anti-PRRSV antibody response as compared to the vaccine strain, suggesting that more virulent strains could induce a stronger antibody response.

In contrast to anti-N protein antibodies, virus neutralizing antibodies (VN) have been shown to correlate with protection from PRRSV [15, 16, 21]. We found that VN to different PRRSV strains did not start to emerge until DPV 28 in the vaccinated pigs. At the time of PRRSV challenge (DPV 28), vaccinated pigs developed higher VN titers to VR-2332 (Figure 2(b)) than to KS-06 strain (Figure 2(c)), suggesting an association between PRRSV strain-specific VN titer and level of protection from PRRSV. Vaccinated pigs did not develop VN to KS-06 after vaccination but developed significantly higher VN titers to KS-06 as compared to the other three groups two weeks after challenge, which suggests that the KS-06 specific VN could be induced by KS-06 challenge (Figure 2(c)). Also, vaccinated and KS-06 challenged pigs developed a higher level of VN antibodies to the heterologous NVSL97-7895 PRRSV strain (Figure 2(d)). This result supports the notion that two vaccinations with different PRRSV strains can generate higher neutralizing Abs and broader cross-protection against various PRRSV field strains. Similar observation has been reported in influenza virus vaccination strategy studies [22].

It was reported that PRRSV can inhibit the expression of IFN-α [23]. However, we found that the level of IFN-α was increased in unvaccinated pigs challenged with KS-06 virus. Similar to previous reports, serum level of IFN-α is not associated with the PRRS virus clearance in pigs after viral challenges [18]. The serum level of inflammatory cytokine IL-8 in vaccinated pigs challenged with homologous VR-2332 virus was the highest among all treatment groups (Figure 3(a)). Our results are consistent with previous studies which have shown that low level of serum IL-8 is seen in persistent PRRSV infection, and elevated IL-8 levels in serum are correlated with the clearance of PRRS virus [24]. However, it remains to be determined how elevated IL-8 may contribute to the clearance of PRRS virus in vaccinated pigs and whether the level of serum IL-8 can be used to predict vaccination-induced protection in pigs.

The expression of IL-4 was significantly higher in vaccinated pigs as compared to that in unvaccinated pigs after KS-06 challenge. This and our previous study [12] and results from others [25] suggest that increased IL-4 expression may play a positive role in vaccination-mediated clearance of heterologous PRRS virus. However, IL-4 level in the serum may not have a direct role in protecting pigs from PRRSV infection since pigs challenged with homologous PRRSV (VR-2332) did not show increased IL-4 production. Thus, whether or not IL-4 plays an important role in the development of vaccination-induced protection against PRRSV has yet to be explored in future studies.

PRRSV infection has been shown to induce a strong immunosuppressive response characterized by promoting the secretion of IL-10 to antagonize the protective Th1 immune response [26]. In our study, we found that IL-10 production in the serum was increased in unvaccinated pigs, but not in vaccinated pigs, when they were challenged with the KS-06 strain (Figure 3(b)). In contrast, both unvaccinated and vaccinated pigs challenged with VR-2332 had similar levels of serum IL-10. The level of serum IL-10 in PRRS infection has been reported to be virus strain-dependent, which may be related to the virulence of each viral isolate [26]. Thus, the difference in IL-10 production between the two groups may be due to the fact that the KS-06 isolate is more virulent than the VR-2332 isolate.

IFN-γ is a key cytokine that is associated with host cell-mediated immunity (CMI) response, which is secreted by natural killer cells and several different T cell subpopulations. A report by Xiao et al. shows that the level of IFN-γ expression after PRRSV infection was variable and did not correlate with virus load [27]. Similar to their findings, we did not observe any changes to serum levels of IFN-γ among the four treatment groups (Figure 3(b)). In a recall response, IFN-γ-secreting cells from memory lymphocytes were calculated by stimulating PBMCs with different PRRSV isolates. MLV vaccination generated higher frequency of IFN-γ-secreting cells. However, PBMCs isolated from vaccinated and KS-06 challenged pigs generated more IFN-γ-secreting cells when restimulated with homologous or heterologous PRRSV as
compared to those from unvaccinated pigs (Figure 3(c)). We found that the lowest number of IFN-γ-secreting cells was from PBMCs restimulated with the KS-06 strain, as compared to another heterologous strain NVSL97-7895 or VR-2332 stimulation. This may be due to the fact that the KS-06 isolate is more virulent than the other two strains and can cause a stronger immunosuppression during infection [18]. Our results suggest that increased IFN-γ expression does not correlate with protection against PRRSV as evidenced by lower levels of IFN-γ in fully protected vaccinated pigs challenged with VR-2332 compared to partially protected vaccinated pigs challenged with KS-06 strain. Therefore, the role of IFN-γ in the protection from PRRSV infection needs to be further explored.

A high frequency of γδ T cells in pigs is considered to be related to the activation status of the innate immune system, and CD4+CD8+ double positive T cells possess memory, T-helper, and cytolytic functions [28, 29]. Although significant increases in the frequency of T-helper, Th/memory, and γδ T cells in PBMCs were observed in vaccinated pigs compared to that in unvaccinated pigs, and this may suggest a protective role of these cells against PRRSV infection, this parameter cannot predict the level of protection since changes in T cell subpopulations are similar between fully and partially protected groups of pigs.

5. Conclusion

Difference of immune responses between vaccinated and unvaccinated pigs challenged with either homologous or heterologous PRRSV has been presented in this study. A better understanding of immune response profiles leading to full and partial protection from PRRSV challenge will facilitate the development of more efficacious vaccines for a broader cross-protection as well as new strategies combating various circulating PRRS virus strains.

Conflict of Interests

The authors report no potential conflict of interests.

Acknowledgments

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References


Research Article

Genetic Diversity Analysis of Genotype 2 Porcine Reproductive and Respiratory Syndrome Viruses Emerging in Recent Years in China

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Porcine reproductive and respiratory syndrome virus (PRRSV) is characterized by its extensive genetic diversity. Here we analyzed 101 sequences of NSP2 hypervariable region, 123 ORF3 sequences, and 118 ORF5 sequences from 128 PRRSV-positive clinical samples collected in different areas of China during 2008–early 2012. The results indicated that the amino acid identities of the three genes among these sequences were 87.6%–100%, 92.5%–100%, and 77%–100%, respectively. Meanwhile, 4 novel patterns of deletion and insertion in NSP2 region or GP5 were first found. The phylogenetic analysis on these 3 genes revealed that the Chinese PRRSV strains could be divided into three subgroups; majority of genes analyzed here were clustered in subgroup 3 with multiple branches; the strains with 30-aa deletion in NSP2-coding region were still the dominant virus in the field. Further phylogenetic analysis on four obtained complete genomic sequences showed that they were clustered into different branches with the Chinese corresponding representative strains. Our analyses suggest that the genetic diversity of genotype 2 PRRSV in the field displays a tendency of increasing in recent years in China, and the 30-aa deletion in NSP2-coding region should be no longer defined as the molecular marker of the Chinese HP-PRRSV.

1. Introduction

Porcine reproductive and respiratory syndrome (PPRS) characterized as reproductive failure in sow and respiratory disorder in all-age pigs [1] is regarded as one of the major concerns for disease controlling in pig farms [2–5]. The first outbreak of PRRS in Western Europe and North America was almost concurrently documented during the late 1980s and early 1990s [6, 7]. Within the succeeding years, PRRS was an endemic disease in North America, Europe, and Asia [7–11]. Since then, PRRS has become the most economically devastating disease for global pig industry [4, 5].

The causal agent, porcine reproductive and respiratory syndrome virus (PRRSV), is classified into the order Nidovirales, family Arteriviridae, together with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV) [12, 13]. According to the genetic diversity, PRRSV can be divided into two genotypes: type 1 (European) PRRSV with prototype Lelystad and type 2 (North American) PRRSV with prototype VR-2332. Although the two types of PRRSV can cause similar syndrome to the infected pigs, they share only 55%–70% nucleotide and 50%–80% amino acid similarity in their various genes [14]. The single positive-strand RNA genome of PRRSV is approximately 15 kb in length, encoding at least 10 open reading frames (ORF) [15–18]. The ORF1a and ORF1b encode replication-related polymerase proteins, which can be autoproteolytically cleaved into at least 13 nonstructural proteins (NP) [19–22]. And the rest of ORFs 2 to 7 encode viral structural proteins [15, 17, 23, 24]. Among them, the largest nonstructural protein gene—NSP2, ORF3 encoding minor glycosylated structural protein—GP3, and ORF5 that encodes major envelope protein—GP5 are often selected for variation investigation and phylogenetic analyses for their
2. Materials and Methods

2.1. Sample Collection and Geographic Distribution. During the period from 2008 to early 2012, 128 clinical samples, including lung, brain, spleen, lymph node, and sera, which were positive for PRRSV by conventional laboratory detection, were collected from pig farms distributed in 18 regions of China. These samples were further used for PRRSV isolation or NSP2 HV region and ORF3 and ORF5 genes amplification and sequencing.

2.2. RNA Extraction and RT-PCR Amplification and Sequencing. Total RNA was extracted from 250 μL of tissue homogenates or serum by using TRIzol LS reagent (Invitrogen Corporation, Auckland, NY, USA). Then reverse transcription was performed by using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and specific antisense primers (Table 1). Resulting cDNA was amplified by using PrimeSTAR HS DNA polymerase (TaKaRa Biotechnology Co., Dalian, China) in the following process: 34 cycles of denaturation at 98°C for 12 s, annealing at 56°C for 10 s, and extension at 72°C for 1 min/kb. The PCR products were examined by gel electrophoresis and purified by using Agarose Gel DNA Extraction Kit (BioDev Co., Beijing, China) and then subjected to BGI (Beijing, China) for sequencing.

2.3. Cells and Virus. MARC-145 cells were grown at 37°C in Dulbecco’s minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The pulmonary alveolar macrophage (PAM) cells were prepared as described previously [33] and maintained in 10% FBS 1640 medium. Serum or supernatant of tissue homogenates from PRRSV-positive samples were used to inoculate the MARC-145 cells or PAM for PRRSV isolation.

2.4. Full-Length Genomic Sequencing of PRRSV Isolates. Fourteen pairs of primers for genotype 2 PRRSV (Table 1), covering the full-length genomes, were designed, based on JXwn06 (Accession number EF641008). Each fragment of the isolates was amplified and cloned into pEASY-Blunt vector (Transgen Tech Co, Beijing, China) as described previously [34]. The 5’ and 3’ ends region was amplified using 5’ and 3’ full RACE kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. The PCR products or plasmid with cloned PRRSV fragments was subjected to BGI (Beijing, China) for sequencing.

2.5. Sequence Alignment and Phylogenetic Analysis. The nucleotide and deduced amino acid sequences were aligned by ClustalW in software Lasergene (DNASTAR Inc., Madison, WI, USA) to determine sequence homology. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura, Peterson, Peterson, Stecher, Nei, and Kumar 2011), along with multiple sequences of representative PRRSV available in GenBank from various countries and areas (Supplementary Table S1, see Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/748068).

3. Results

3.1. Number of NSP2 HV Fragment and ORF3 and ORF5 Genes Amplified from PRRSV-Positive Clinical Samples. The fragments of NSP2 HV region and ORF3 and ORF5 genes amplified from PRRSV-positive samples were sequenced. The results showed that totally 101 NSP2, 123 ORF3, and 118 ORF5 sequences were successfully obtained from 128 PRRSV-positive samples collected during the period from 2008 to early 2012 (Table 2).

3.2. Sequence Alignment and Phylogenetic Analysis of NSP2 HV Region. The amplified NSP2 HV region exhibited various sizes in length (Table 2). Nucleotide and deduced amino acid sequences analysis revealed that 86 out of 101 NSP2 HV
Table 1: Primers used for amplification and sequencing of PRRSV genome and NSP2 HV region and ORF3 and ORF5 genes.

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Nsp2-R    CGATGAGTCAGTCGAGTGATAT  3178 1064/1154
ORF3-F    CAGGGTCAAATGTAACCATAGTG  12506
ORF3-R    GCCAAGAAGAAAAGCATGAGGAG  13457 952
ORF5-F    AGCCTGCTCTTTTTCGCTATCT   13654
ORF5-R    CTTTTTGAGGCGCTGCTATCTC  14335 682
ORF7F     TGATACACCGATGTTGCTG     14608
ORF7R     GCCATCCACCGACACATGTTCTC  15228 561

*The location is according to the genomic sequences of JXwn06 (GenBank Accession number: EF641008).

region sequences had the same length of 1014 nucleotide (nt), containing the same 30-aa deletion at aa 482 and aa 533–561 as JXwn06 and other HP-PRRSV strains, compared with the type 2 prototype VR-2332 and the Chinese early strains. The LN1101 and GZ1101 showed two novel deletion patterns in their NSP2 regions, whose nucleotide sequences length was 1050 nt and 1095 nt, respectively. The other 13 NSP2 sequences were 1104 nt in length, same as those of VR-2332.

Pairwise comparisons showed that those 86 sequences with 30-aa deletions in NSP2 shared 87.6%–100% amino acid similarity with each other. And their amino acid similarities with JXwn06 ranged from 91.7% to 99.4%, as well as 66.6% to 69.5% compared with VR-2332. Majority of the sequences without deletion shared high homology with HB-I(sh)/2002, showing the amino acid similarity of 98.1%–99.2%. Meanwhile, the JL1101 and GZ1101 displayed the highest homology with VR-2332, with amino acid similarities of 99.2% and 96.7%, respectively.

To further gain a better understanding of the genetic relationship, the phylogenetic analysis based on deduced
Table 2: Geographic origin and amplified sequence size from clinical samples in this study.

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*Each sample was named according to the region and collection year; *NA: not amplified.
The amino acid sequence of NSP2 HV region was conducted by using the 101 NSP2 sequences obtained in this study together with downloaded representative sequences (Supplementary Table S1). The phylogenetic tree revealed that all 101 NSP2 sequences belonged to genotype 2 of PRRSV and all Chinese PRRSV strains could be classified into three main subgroups (Figure 1). The JLI101 and GZI101 were located in subgroup 1 with the representative strains VR-2332, BJ-4, and RespPRRS MLV, the other 99 were clustered into the subgroup 3 with multiple branches, together with the representative strains HB-1(sh)/2002, JXwn06, JXA1, and JXA1 P80. No strains in this study were clustered into subgroup 2 with representative strain CH-1a, the earliest Chinese strain. This means that the genetic diversity of NSP2 still existed and the strains with 30-aa deletion in NSP2-coding region remain to be the dominant viruses in the field. Compared with the data from 2006 to 2007, the percentage of NSP2-deleted strains increased [25]. However, these subgroups did not appear to be associated with epidemiological features based on geography or date.

Interestingly, a minor branch with JXA1 P80, the HP-PRRSV JXA1-derived vaccine strain, was observed in the NSP2 phylogenetic tree. Four strains HBII105, HBI1201, SCI101, and BJ1101, collected later than the year 2011 when the JXA1-derived vaccine was launched commercially, were also clustered in this branch, whereas the parental strain JXA1 was out of this branch, suggesting that there is the possibility that the four strains directly derived from the vaccine strain JAX1 P80. However few earlier strains were also clustered into this minor branch. Even though the analysis from this study does not fully reflect that a great number of emergence of PRRSV were due to the use of HP-PRRSV-derived MLV, the potential risk of the reversion of MLV to virulent strains, and the recombination between the vaccine virus and field viruses are worthy to pay more attention to in the future [35].

3.3. Sequence Alignment and Phylogenetic Analysis of ORF3 Gene. All the obtained ORF3 genes in this study had the same size of 725 nt. The sequences alignments indicated that they shared 92.5%-100% amino acid similarity with each other and 89.4%-95.3% amino acid similarity with JXwn06, as well as 80.7%-85.0% with VR-2332. The regions residues 33-46, 120-133, and 162-198 were conserved among these strains; otherwise, majority of amino acid substitutions were located in two hypervariable regions, the residues 58-71 and 216-226. Especially, 63 out of 123 contained the I66-T66 mutation, comparing with those in JXwn06 and VR-2332.

The phylogenetic analysis of deduced amino acid sequences of ORF3 indicated that all Chinese genotype 2 strains were distributed into three subgroups (Figure 2). Three genes JLI101, HBII103, and GZI101 were clustered into subgroup 1 with the representative strains VR-2332 and BJ-4, and no strains in this study were clustered into subgroup 2 with the representative strains CH-1a, HB-1(sh)/2002, and HB-2(sh)/2002. All the other strains were clustered into subgroup 3, which contained most Chinese strains collected later than 2004.

3.4. Sequence Alignment and Phylogenetic Analysis of ORF5 Gene. Except for the GZI101 which had one amino acid deletion at the position aa 34 in ORF5-coding region, the other 117 genes had the same size of 603 nt as that of VR-2332. Sequences alignments showed that the amino acid similarity among the 117 ORF5 genes ranged from 77.0% to 100%, and they shared 78%-99% amino acid similarity with VR-2332, as well as 86.5%-99% with JXwn06. Similar as previous report, the residue 3-39, the putative signal sequence was the most variable region, whereas, the regions 40-57, 67-90, 107-120, 138-160, and 165-184 were relatively conserved [25]. However a novel substitution E170-G170, which was conserved in the Chinese strains collected during the period from 2006 to 2007, was observed in recent strains.

The phylogenetic tree conducted by using the deduced amino acid sequences of ORF5 showed that the Chinese strains of genotype 2 PRRSV could be divided into 3 different subgroups (Figure 3). Three strains JLI101, HBII103, and GZI101 were in subgroup 1 with the representative strains VR-2332, BJ-4, and CH-1a, and the SD1003 was the only strain clustered in subgroup 2 with the representative strain MN184A; all other 115 strains were clustered into subgroup 3 with multiple branches, which were completely composed of Chinese strains with the representative JXwn06 and HB-1(sh)/2002. Similar to the NSP2 phylogenetic tree, a minor branch with the JXA1 P80 contained the strains collected both earlier and later than 2011.

3.5. Full-Length Genomic Analysis of 4 New PRRSV Isolates. Three strains, SD0901, LNI101, and GZI101, with characteristic deletion or insertion in NSP2 or ORF5 genes, and another strain BJ1102 were successfully isolated from the clinical samples using MARC-145 cells or PAMs. The four strains were subjected to full-length genomic sequencing after plaque purification of three rounds. The SD0901 (GenBank Accession number NJ256115) and BJ1102 (GenBank Accession number KF751237) shared same size of complete genome with 15,320 nt in length, excluding the ploy (A) tails. The genome sizes of LNI101 (GenBank Accession number KF751238) and GZI101 were 15,356 nt and 15,404 nt, respectively. The BJ1102 was isolated from clinical samples of Tibet mini-pig with acute PRRS symptom in a pig farm where HP-PRRSV-derived vaccine was used before importing Tibet mini-pig.

Sequence alignments indicated that the 5’UTR of the four strains shared nucleotide identities of 91.0%-100% with the representative genotype 2 PRRSV strains. A nucleotide ‘A’ insertion at the position nt 75 of GZI101 5’UTR was first observed in this study. It was shown that major variations were located in NSP2-coding region including 3-aa deletion at the position aa 593-595 in GZI101, 18-aa deletion at the position aa 482-499 in LNI101, 30-aa deletion at the positions aa 482 and aa 533-561 in BJ1102, and 31-aa deletion at the positions aa 468, aa 482, and aa 533–561 and an amino acid “P” insertion between aa 585 and aa 586 in SD0901 (Figure 4). In addition, a new deletion at the position aa 34 of GP5 was found in GZI101 (Figure 5). The individual homology analysis of the other genes was also summarized in Supplementary Tables S2–S5.

To further classify the evolutionary relationship of these 4 isolates, the phylogenetic tree was conducted based on their
full-length genomic sequence, together with both genotype 1 and genotype 2 representative strains. It was shown that the SD0901 and BJ1102 were clustered in the subgroup of Chinese HP-PRRSV and HP-PRRSV-derived vaccine virus, sharing high identity 98.7% and 98.4% with JXwn06, respectively; in addition, the LN1101 was the neighbor of HB-1(sh)/2002 in the same minor branch, which share 98.8% identity with each other. The GZ1101 was close to the minor branch with prototype VR-2332 and BJ-4 (Figure 6). The four strains exhibited 88.3%–97.8% nucleotide identity with each other.

The findings suggest that various PRRSV strains from different clusters simultaneously circulate and spread in pig farms in China.

4. Discussion

PRRSV is characterized of its extensive genetic/antigenic variation in the field [36]. Low replication fidelity of RNA polymerase, abundance of quasispecies, RNA recombination, and immune pressure selection are regarded as the
mechanisms of generating viral heterogeneity and diversity which promotes the evolution of PRRSV [37–39]. The emergence and reemergence of acute form PRRS is often influenced by the genetics of PRRSV [36]. Since the PRRS outbreak in China was first documented in 1995; this virus is always accompanied with the Chinese pig industry [27]. In 2006, an unparalleled, large-scale, atypical PRRS outbreak was reported in China [25, 30, 31]. In the following 1-2 years, the HP-PRRSV with 30-aa deletion in NSP2-coding region rapidly became the dominant in the field, meanwhile the classical and low-pathogenic strains could also be isolated from pig farms [25]. In 2011, the HP-PRRSV-derived MLV was licensed and widely used afterward in the field. This situation might greatly increase the immune selective pressure in pig herds to accelerate the variation and evolution of PRRSV [39]. Meanwhile, the European genotype 1 PRRSV strains also emerged in China [32], resulting in the complexity of PRRS. Therefore it is meaningful to continually survey the

Figure 2: Phylogenetic tree based on the deduced amino acid sequence of ORF3. The bootstrap consensus tree is shown. The sequence downloaded from GenBank had a suffix “ORF3.” The representative strains were labeled with “black triangle” and the vaccine strains were labeled with “black diamond.” The bootstrap values were shown close to the branches.
In this study we amplified and gained 101 NSP2 HV region sequences from 128 PRRSV-positive clinical samples. Out of them, 86 had the same 30-aa deletion in NSP2-coding region as that of JXwn06 and other early HP-PRRSV strains. The 86 new sequences shared 87.6%–100% amino acid similarity with each other, as well as 66.6%–69.5% with VR-2332, which were both lower than previous corresponding data, 93.4%–99.8% and 77.1%–77.8%, we obtained in 2006–2007 [25]. Meanwhile, 3 novel patterns of deletion or insertion in NSP2-coding region were first found in this study. These results suggest that the diversity of PRRSV NSP2 region has expanded from 2006–2007 to 2008–2012. The phylogenetic analysis on amino acid sequence of NSP2 indicated that all new strains in this study were clustered into 2 out of 3 subgroups: 2 strains in subgroup 1 with the representative strains VR-2332, BJ-4, and RespPRRS MLV and the other 99 in the subgroup 3 with the representative strains HB-1(sh)/2002, JXwn06, JXA1, and JXA1 P80, suggesting that the strains with 30-aa deletion in NSP2-coding region are still prevailing in the field. Among them, the BJ1102 with low pathogenicity (data not shown), which was closely related with vaccine virus, was clustered together with HP-PRRSV-derived vaccine virus in the same
VR-2332 NSP2

| Sequence homology ranged from 88.3% to 97.8%, and they were subjected to full-length genomic sequencing in order to include in Shi’s analysis [44,45].

Comparative analysis showed that their complete genome similarity of ORF5 among these strains was 77.0%, which was lower than the data (84.1%) in our previous research, supporting that the diversity of strains has increased since 2008. The phylogenetic tree based on deduced amino acid sequence of ORF5 showed that the Chinese PRRSV strains could be clustered into 3 different subgroups. Compared with Shi Mang’s phylogenetic result based on more than 8,000 sequences, subgroup I was composed of representative strains located in lineage 8 (VR-2332 and CH-1a), lineage 5.1 (VR-2332 and BJ-4), and lineage 7 (SP and prime Pac), subgroup 2 contained representative strains MN184a from lineage 1, and the other HP-PRRSV in subgroup 3 was late clustered into lineage 8 in Yanyan Ni’s modified phylogenetic tree, even if the information of Chinese HP-PRRSV had not been included in Shi’s analysis [44, 45].

Because of having novel genetic characterization or being isolated from special host Tibet mini-pigs, four strains, SD0901, LNI101, GZI101, and BJI102, in our study were subjected to full-length genomic sequencing in order to better understand their characteristics of whole genome. Comparative analysis showed that their complete genome sequence homology ranged from 88.3% to 97.8%, and they
Figure 6: Phylogenetic tree based on full-length genomic sequence of PRRSV. The strains isolated in this study were labeled with “black triangle.” The bootstrap values were shown close to the branches. The numbers below the scale bar indicate amino acid substitution (100x).

were clustered into different branches of genotype 2 PRRSV, further indicating that PRRSV strains with genetic diversity simultaneously exist in the field in China.

In this study, the molecular sequence data of PRRSV was utilized to characterize the epidemiology and evolutionary process in phylogenetic analysis, expecting that it could provide an important clue for modification of diagnosis methods and design of novel vaccine. Hopefully, these analyses will be useful for PRRS control strategy. Considering that the modern transportation in pork supply chains can easily spread the virus nationwide or even internationally, and meanwhile the wide use of attenuated PRRSV live vaccine will raise the risk of reversion to virulence and increase the possibility of recombination between vaccine strains and field strains, the PRRSV diversity will be continually expanded and the epidemic situation in the field will be more and more complicated. So if we try to gain a deeper view of the PRRSV epidemiology, the long-term investigation, linked observation between genetic diversity and phenotypic difference, and effort of explaining the mechanism of how HP-PRRSV strains gain the dominance in field should be first concerned in future.

5. Conclusion

Our analysis results indicated that the genetic diversity of PRRSV in the field further increased in recent years in China, due to the dramatic variations of NSP2 and ORF5 genes of PRRSV, and the 30-aa deletion in NSP2-coding region should be no longer defined as the only molecular marker of the Chinese HP-PRRSV as the PRRSV strain with same deletion and low pathogenicity emerged in the field and the attenuated live vaccines derived from HP-PRRSV were widely used in pig farms.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

A Novel Isolate with Deletion in GP3 Gene of Porcine Reproductive and Respiratory Syndrome Virus from Mid-Eastern China

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PRRSV strain SH1211 was isolated from the lung tissue of a piglet on a large-scale pig farm with approximately 30% morbidity and 50% mortality in mid-eastern China in 2012. The full-length genome of SH1211 was 15 313 nt in size, excluding the polyadenylated sequences, and shared 94.9% nucleotide sequence identity with the HP-PRRSV strain, JXA1. The GP2 and GP5 proteins of SH1211 shared only 91.5% and 85.1% amino acid sequence identities with those of the JXA1, respectively. A deletion at amino acid positions 68 and 69 was identified in the GP3 protein of SH1211, compared with the GP3 of Type-2 PRRSV isolates. A phylogenetic tree based on the nucleotide sequence of the complete genome showed that SH1211 is the most closely related to other HP-PRRSV strains isolated in China. However, phylogenetic analysis based on the GP2 and GP5 proteins showed that SH1211 is the most closely related to the QYYZ strain. A recombination analysis indicated that SH1211 might have been generated through recombination events between the JXA1 and QYYZ in which the GP2 and GP5 coding sequences were exchanged. Thus, SH1211 is a novel PRRSV strain with significant variation. Our analysis of SH1211 provides insight into the role of genetic variation in the antigenicity of PRRSVs in China.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), which causes great economic losses to the swine industry worldwide [1, 2], was first isolated in the Netherlands in 1990 [3] and was later identified in the USA [4]. The PRRSVs are divided into Type-1 (European, Lelystad prototype strain) and Type-2 (North American, VR-2332 prototype strain) genotypes, which vary in nucleotide sequence by approximately 60% [5, 6]. Along with equine arteritis virus, lactate dehydrogenase-elevating virus, and simian hemorrhagic fever virus, the PRRSVs are members of the order Nidovirales and family Arteriviridae [7, 8].

The PRRSV genome is 15 000 to 15 500 nt in length and consists of a 5'-untranslated region (UTR), at least 9 open reading frames (ORFs) that encode viral proteins, and a 3'-UTR [8, 9]. The ORFs 1a and 1b occupy the first two-thirds of the single-stranded, positive-sense RNA genome. These ORFs encode the ORF1ab replicase polyprotein, which is proteolytically cleaved into 13 small nonstructural proteins that are involved in virus replication and transcription [10, 11]. The PRRSV ORFs 2–7 encode a series of viral structural proteins that are associated with the virus envelope, including the GP2, E, GP3, GP4, GP5, M, and N proteins [8, 12–14]. A recent study identified ORF5a, which overlaps with the GP5-coding sequence and encodes a small hydrophobic protein [15, 16].

The PRRSV is characterized by extensive genetic variation, and a number of genetically/antigenically diverse strains have been identified [5, 17]. The coding region for the nonstructural protein 2 (nsp2) of PRRSV displays substantial genetic variation, including point mutations, insertions, and deletions [18–21]. The GP5 protein, a major component of the viral envelop, is thought to induce virus-neutralizing antibodies [22, 23] and displays the highest level of genetic variability among the PRRSV structural proteins [24, 25].
2. Materials and Methods

2.1. Clinical Samples. The lung tissue was obtained from a PRRSV-vaccinated piglet on a large-scale pig farm with approximately 30% morbidity and 50% mortality in Shanghai, China, in 2012. The piglet displayed clinical signs and symptoms that were typical of the porcine reproductive and respiratory syndrome (PRRS), including labored breathing, pyrexia, lethargy, and anorexia. The animal was diagnosed as PRRSV-positive using reverse transcription (RT) and polymerase chain reaction (PCR).

2.2. Virus Isolation. The lung tissue was homogenized and used to inoculate primary porcine alveolar macrophage (PAM) cells. The PAM cells were maintained in RPMI 1640 growth medium containing 10% heat-inactivated fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO₂ atmosphere until a cytopathic effect became visible. The infected cells were lysed using a freeze-thaw method and centrifuged at 2000 x g for 10 min. The supernatant was stored at −70°C.

2.3. RNA Extraction and RT. Total RNA was extracted from the tissue homogenate using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and suspended in nuclease-free water immediately before use. The RT procedure was performed using the SuperScript III First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Samples containing 8 μL of total RNA, 1 μL of 50 μM oligo (dT)₁₂ primer, and 1 μL of 10 mM dNTP mix were incubated at 65°C for 5 min for primer annealment and placed on ice for at least 1 min immediately afterward. For first-strand synthesis, 2 μL of 10× RT buffer, 4 μL of 25 mM MgCl₂, 2 μL of 0.1 M DTT, 1 μL of 40 U/μL RNaseOUT (Invitrogen), and 1 μL of 200 U/μL Superscript III reverse transcriptase were added to each sample, and the samples were incubated at 50°C for 50 min. The RT reaction was terminated by incubation at 85°C for 5 min, and the samples were placed on ice until complementary DNA (cDNA) synthesis was performed.

2.4. Primer Design and cDNA Synthesis. The ORF5 nucleotide sequence of SH1211 was compared with that of other PRRSV isolates available in GenBank database (NCBI) to identify similarities, upon which primers were designed. The SH1211 genome was divided into 7 overlapping fragments for amplification, and the 5’ and 3’ termini of the genomic sequence were synthesized using rapid amplification of the cDNA ends (RACE). The sequences of the primers used for the whole genome and the RACE procedure are provided in Table 1. The overlapping fragments were amplified by PCR using the Phanta Super Fidelity DNA Polymerase (Vazyme, China), and the thermal cycling was performed at 95°C for 3 min, followed by 35 cycles of 95°C for 10 s, 52°C to 56°C for 30 s, and 72°C for 2 min, with a final extension at 72°C for 10 min. The amplified PCR products were analyzed using agarose gel electrophoresis.

2.5. Genome Cloning and Sequencing. The PCR products were purified from the agarose gel using the AxyPrep DNA Gel Extraction Kit (Axygen, China) and cloned into the pEASY-Blunt Zero vector (Trans, China). Three clones were sequenced by a commercial service provider (Invitrogen, Shanghai, China).

2.6. Nucleotide and Amino Acid Sequence Analyses. The overlapping sequences of the PCR products were combined to obtain the full-length genomic sequence of the SH1211 strain. A nucleotide BLASTn analysis was used to compare the sequences of the SH1211 genes with those of the reference strains of PRRSV in the GenBank database (Table 2).

Table 1: Primers used for RT-PCR and RACE amplification for SH1211.

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The sequence alignments were generated using the Clustal W program. Phylogenetic trees of the full-length genomic, GP2, and GP5 nucleotide sequences were generated using the distance-based Neighbor-Joining method in the MEGA, version 5.05, software. The bootstrap values were calculated for 1000 replicates, and the evolutionary distances were computed using the Jukes-Cantor method. The amino acid sequences were obtained using the BioEdit program. Phylogenetic trees of the full-length genomic, ORF2, and ORF5 nucleotide sequences were generated using the Clustal X, version 1.83, program. The sequence alignments were generated using the Clustal X, version 1.83, program. The Recombination Detection Program (RDP4), version 4.1.3, was used to estimate the potential recombination events [29]. The GENECONV method was used to search for putative breakpoints [30].

### 2.7. Recombination Analysis

Multiple sequence alignments between SH1211 and the other PRRSV strains were performed using the Clustal X, version 1.83, program. The Recombination Detection Program (RDP4), version 4.1.3, was used to estimate the potential recombination events [29]. The GENECONV method was used to search for putative breakpoints [30].

### Table 2: Representative PRRSV Strains used in this study.

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### 3. Results

#### 3.1. Comparison of Full-Length Genomic Sequences of SH1211 and Other PRRSV Strains

The sequence analysis showed that the full-length genomic sequence of SH1211 was 15313 nucleotides in length, excluding the polyadenylated sequences, and included the following genes and UTRs: 5'UTR (188 nt), Rep (11792 nt), GP2 (771 nt), GP3 (759 nt), GP4 (537 nt), GP5 (603 nt), M (525 nt), N (372 nt), and 3'UTR (150 nt). The nucleotide sequence of the full-length SH1211 genome was compared with those of the other PRRSV isolates, including two North American strains (MN184C and VR-2332), one European strain (LV), and six Chinese strains (CH-1a, SY0608, QYYZ, JXA1, WUH4, and HB-1(sh)/2002) (Table 3).

The SH1211 strain shared a higher level of nucleotide sequence identity with the Type-2 PRRSV strains (81.8% to 95.1%), compared to that shared with the Type-1 PRRSV strain, LV (58.0%). The highest levels of shared nucleotide sequence identity (94.9% to 95.1%) were observed between the SH1211 strain and the WUH4, JXA1, and SY0608 strains, which had previously caused epidemics in China. Moreover, the 950-AA sequence of the protein produced from the Nsp2 gene (2850 nt) of the SH1211 strain contained 30 noncontiguous AA deletions, relative to that of the Nsp2 gene of the VR-2332 strain (Figure 1). These results indicated that the SH1211 strain is highly similar to a group of highly pathogenic (HP) strains of PRRSV previously isolated in China.

The nucleotide lengths of ORF1a and ORF1b of SH1211 were 7422 nt and 4383 nt, respectively. The ORF1a and ORF1b of SH1211 shared nucleotide sequence identities of 96.0% and 94.9% with those of the JXA1 strain, respectively, whereas it shared 53.2% and 63.0% with those of the LV strain, respectively. The predicted AA sequences of the proteins of SH1211 shared 97.2% to 99.4% identity with those of the JXA1 strain, except for the Nsp1β, Nsp2, and Nsp12 proteins, which were only 92.6%, 94.2%, and 93.4%, respectively. The Nsp1β protein of SH1211 shared 91.6% AA sequence identity with that of the SY0608 strain. These data indicated that a high level of variation occurred in the Nsp1β protein of the SH1211 strain.

The comparisons of the sequences of ORF2 through ORF6 of SH1211 showed that the SH1211 strain shared 84.4% to 97.5% nucleotide sequence identity with the Type-2 PRRSV strains. The ORF2 and ORF5 of SH1211 shared nucleotide sequence identities of 96.0% and 94.9% with those of the JXA1 strain, respectively, whereas it shared 53.2% and 63.0% with those of the LV strain, respectively. The predicted AA sequences of the proteins of SH1211 shared 97.2% to 99.4% identity with those of the JXA1 strain, except for the Nsp1β, Nsp2, and Nsp12 proteins, which were only 92.6%, 94.2%, and 93.4%, respectively. The Nsp1β protein of SH1211 shared 91.6% AA sequence identity with that of the SY0608 strain. These data indicated that a high level of variation occurred in the Nsp1β protein of the SH1211 strain.

The nucleotide lengths of ORF3a and ORF3b of SH1211 were 7422 nt and 4383 nt, respectively. The ORF3a and ORF3b of SH1211 shared nucleotide sequence identities of 96.0% and 94.9% with those of the JXA1 strain, respectively, whereas it shared 53.2% and 63.0% with those of the LV strain, respectively. The predicted AA sequences of the proteins of SH1211 shared 97.2% to 99.4% identity with those of the JXA1 strain, except for the Nsp1β, Nsp2, and Nsp12 proteins, which were only 92.6%, 94.2%, and 93.4%, respectively. The Nsp1β protein of SH1211 shared 91.6% AA sequence identity with that of the SY0608 strain. These data indicated that a high level of variation occurred in the Nsp1β protein of the SH1211 strain.

The comparisons of the sequences of ORF2 through ORF6 of SH1211 showed that the SH1211 strain shared 84.4% to 97.5% nucleotide sequence identity with the Type-2 PRRSV strains. The ORF2 and ORF5 of SH1211 shared nucleotide sequence identities of 96.0% and 94.9% with those of the JXA1 strain, respectively, whereas it shared 53.2% and 63.0% with those of the LV strain, respectively. The predicted AA sequences of the proteins of SH1211 shared 97.2% to 99.4% identity with those of the JXA1 strain, except for the Nsp1β, Nsp2, and Nsp12 proteins, which were only 92.6%, 94.2%, and 93.4%, respectively. The Nsp1β protein of SH1211 shared 91.6% AA sequence identity with that of the SY0608 strain. These data indicated that a high level of variation occurred in the Nsp1β protein of the SH1211 strain.

#### 3.2. Phylogenetic Analyses

Phylogenetic trees were produced based on the nucleotide sequences of the full-length genome, ORF2, and ORF5 of SH1211 and the various PRRSVs
Table 3: Nucleotide and deduced amino acid identities of SH1211 compared with those of WUH4, QYYZ, HB-1(sh)/2002, JXA1, SY0608, MN184C, VR-2332, CH-1a, and LV (%).

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Figure 1: Alignment of partial nsp2 amino acid sequence of SH1211 with several representative PRRSV strains. The amino acid deletions are shown with grey bar.
Figure 2: Phylogenetic analysis based on nucleotide sequences of the full-length genome (a), ORF2 (b), and ORF5 (c) of 30 fully sequenced PRRSV isolates. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. The evolutionary distances were computed using the Jukes-Cantor method and were in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5.
3. These data indicated that significant variation occurred in ORFs 2 and 5 of SH1211 and QYYZ, compared with the other PRRSVs.

3.3. Recombination Analysis. As shown in Figures 2(b) and 2(c), based on the ORF2 and ORF5 nucleotide sequences, the QYYZ strain displayed the closest phylogenetic relationship with the SH1211 strain. The SH1211 genome was subjected to a recombination analysis, and the potential breakpoints with optimal \(P\) values based on the \(\chi^2\) analysis were identified using the GENECONV method. Four potential recombination breakpoints were identified at nucleotide positions 11 697, 12 768, 13 819, and 14 472 (Figure 3), and the approximate \(P\) values for the fragments corresponding to nucleotide positions 11 697–12 768 and 13 819–14 472 were \(8.681 \times 10^{-24}\) and \(3.178 \times 10^{-35}\), respectively. The GP2 and GP5 genes of the SH1211 strain were located within these two recombinant regions. These data suggest that the JXA1 and QYYZ strains recombined to form the SH1211 strain.

3.4. Sequence Analyses of the UTRs. The 188-nt \(5'\) UTR of SH1211 shared a higher level of sequence identity (96.8\%) with JXA1 than that shared with LV (53.3\%). The nucleotide alignments revealed a deletion in the \(5'\) UTR of SH1211 at nucleotide position 119, relative to the JXA1 and WUH4 strains, and positions 119 and 120, relative to the CH-1a strain. Point mutations were identified in the \(5'\) UTR of SH1211 at positions 29 (C \(\rightarrow\) G), 57 (C \(\rightarrow\) U), 62 (G \(\rightarrow\) C), 83 (A \(\rightarrow\) G), and 156 (U \(\rightarrow\) C), relative to the \(5'\) UTR of the JXA1 strain. Furthermore, the transcription regulatory sequence, UUAACC, was identified at the \(3'\) boundary of the \(5'\) UTR in the SH1211 genome. The \(5'\) UTR of the SH1211 genome shared 84.9\% to 98.0\% sequence identities with the Type-2 isolates. The \(3'\) UTR of the SH1211 genome had a deletion at nucleotide position 19, relative to that of the CH-1a strain (Figure 4(b)), and two point mutations at positions 32 (A \(\rightarrow\) U) and 40 (G \(\rightarrow\) A), relative to the \(3'\)-UTRs of the JXA1 and WUH4 strains.

3.5. Amino Acid Analysis of GP3. As shown in Figure 5, the GP3 gene of SH1211 had a 6-nt deletion at positions 203–208 (corresponding to AA positions 67 and 68), relative to the GP3 gene of the Type-2 PRRSV strains. A two-AA deletion (E\(^{67}\) and P\(^{69}\)) was identified in the antigenic region of the predicted GP3 polypeptide, relative to that of the other PRRSVs examined. The AA substitutions, I\(^{64}\) \(\rightarrow\) A\(^{66}\), Y\(^{67}\) \(\rightarrow\) L\(^{67}\), R\(^{71}\) \(\rightarrow\) K\(^{69}\), S\(^{72}\) \(\rightarrow\) P\(^{70}\), L\(^{73}\) \(\rightarrow\) F\(^{71}\), Y\(^{79}\) \(\rightarrow\) H\(^{77}\), E\(^{83}\) \(\rightarrow\) G\(^{81}\), D\(^{85}\) \(\rightarrow\) N\(^{83}\), and P\(^{86}\) \(\rightarrow\) V\(^{82}\), were identified in the main antigenic region of the predicted GP3 polypeptide of SH1211, relative to the GP3 AA sequence of the RespPRRS MLV strain. In addition, the predicted GP3 polypeptide of SH1211 shared seven potential N-glycosylation sites, N\(^{29}\), N\(^{42}\), N\(^{50}\), N\(^{129}\), N\(^{150}\), N\(^{158}\), and N\(^{333}\), with the Type-2 PRRSV strains [31].

3.6. Amino Acid Analysis of GP5. Most of the AA substitutions identified in the predicted GP5 polypeptide of SH1211 were located within residues 1 to 31 in the N-terminal signal sequence, residues 32 to 60 in the hypervariable region of the ectodomain, and residues 189 to 200 in the C-terminal endodomain (Figure 6). Two AA substitutions, H\(^{39}\) \(\rightarrow\) Y\(^{38}\) and F/L\(^{39}\) \(\rightarrow\) S\(^{30}\), were identified in the primary neutralizing epitope (PNE), S\(^{37}\) H(F/L)QLIYNL [32], of the SH1211 strain, compared with that in the VR-2332 strain and its attenuated vaccine derivative, RespPRRS MLV. No AA mutations were found in the decoy epitope of the predicted GP5 protein of SH1211. Residues 13 and 151 in the GP5 proteins of PRRSVs have been shown to be associated with the virulence [6]. However, the AA substitutions, H\(^{13}\) \(\rightarrow\) R\(^{12}\) and K\(^{151}\) \(\rightarrow\) E\(^{150}\), were identified in the predicted GP5 protein of the SH1211 strain. To gain further insight into the genetic evolution of SH1211, we also analyzed variation in potential N-glycosylation sites. The predicted GP5 protein of the SH1211 strain shared three N-glycosylation sites with the CH-1a strain at AA positions 34, 44, and 51, and the deletion of two N-glycosylation sites at positions 31 and 35 was identified, relative to those in the GP5 protein of the JXA1 strain.

4. Discussion

The PRRS continues to be a serious threat, causing a significant economic impact on the swine industry worldwide. Although commercial vaccines against PRRSV are available, traditional control strategies and conventional vaccines have failed to provide sustainable disease control. Surveillance of the recently emerged strains has become necessary because of the considerable genetic and antigenic diversity identified in these HP-PRRSV isolates. In our current study, a novel variant PRRSV strain was isolated from a piglet in a PRRSV-vaccinated pig herd with high morbidity and mortality in mid-eastern China.

The GP3 protein of PRRSVs is a minor structural protein. The 254-AA GP3 protein is encoded by ORF3 and has a molecular mass of approximately 42 kDa [33, 34]. The GP3 protein is one of the most variable structural proteins among...
the PRRSVs, with only 54% to 60% AA sequence identity shared between the Type-1 and Type-2 strains [24, 35]. In the SH1211 strain, the length of ORF3 was 759 nt, and it had a six-nucleotide deletion at positions 203–208, which correspond to AA positions 67 and 68, relative to the Type-2 PRRSV strains. In the GP3 protein, four consecutive overlapping epitopes, corresponding to AA positions 61–105, were shown to be strongly immunoreactive to 85% to 100% of the anti-PRRSV sera tested [36]. These epitopes are located in the most hydrophilic region within the GP3 protein, and are considered to comprise an important immunoreactive region of the North American strains of PRRSV [36].

The AA sequence, Q^{61}AAQRQLEPGRN^{73}, of the antigenic region has been shown to be the target of virus-neutralizing antibodies [37]. It is notable that a two-AA deletion (E^{68} and P^{69}) occurs in the antigenic region of SH1211. Multiple AA substitutions were also identified in the primary antigen region of the GP3 protein of the SH1211 strain, relative to that of the RespPRRS MLV attenuated vaccine strain. A recent study demonstrated that an N-glycan moiety in GP3 is responsible for glycan shield interference, which can influence the ability of the host to produce neutralizing antibodies [38]. However, the two-AA deletion in the GP3 of the SH1211 strain did not affect any of the seven potential N-glycosylation sites identified. Future studies of the sequence diversity in the SH1211 strain are warranted to determine its relationship with immunogenicity.

The envelope protein, GP5, is the most variable protein of the PRRSVs, with only 51% to 55% sequence identity shared between the Type-1 and Type-2 strains [28, 35]. Hypervariability in GP5 is likely responsible for the low level of immunological cross-reactivity observed between the PRRSVs [17]. Thus, GP5 has become the focus of analyses of the genetic diversity of PRRSVs [27, 39, 40]. Our analysis of the GP5 of the SH1211 strain revealed significant variation, with only 84.4% nucleotide identity shared between the ORF5 of SH1211 and that of the representative Chinese HP-PRRSV strain, JXA1. The PNE is also an important domain of GP5 with regard to virus neutralization, and the H^{38}(L/F)^{39} residues in this domain are considered to be critical to the immunogenicity of this epitope [32, 41]. The PNE of the SH1211 strain had the H^{38}→Y^{38} and F/L^{39}→S^{39} AA substitutions in the PNE, which probably contributed to the ability of the isolate to escape neutralizing antibodies induced by PRRSV vaccines used in China, including the attenuated RespPRRS/Repro vaccine strain, the CH-1R vaccine strain (attenuated vaccine strain derived from CH-1a), and the attenuated live vaccine strain derived from JXA1.

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**Figure 4:** Alignment of nucleotide sequences of 5′ UTR (a) and 3′ UTR (b) of SH1211 with several representative PRRSV strains. The nucleotide deletions and the key nucleotide mutations within the different strains are shown with gray bar and blue bar, respectively. The transcription regulatory sequence (TRS) UUAACC is indicated by a black box.
The decoy epitope of GP5 is comprised of (A/V)\textsuperscript{27}LVN near the PNE, and may delay the production of virus-neutralizing antibodies [32]. However, no AA mutations were found in the decoy epitope of the GP5 of the SH1211 strain. A recent study identified two identical signal peptide cleavage sites in the GP5 of PRRSVs, which results in a mixture of GP5 proteins in virus particles, one with and one without the decoy epitope [42]. By comparing the cleavage of wild-type GP5 to that of mutant viruses in which cleavage site 1 or 2 was blocked, the majority of GP5 was found to be cleaved at site 2, which deleted the decoy epitope. Thus, other factors may play vital roles in delaying the production of virus-neutralizing antibodies [42]. Residues 13 and 151 of GP5 are associated with the virulence of PRRSVs. The R\textsuperscript{13}→Q and R\textsuperscript{151}→G AA substitutions in VR-2332 resulted in the attenuation of the RespPRRS MLV vaccine strain [6]. The GP5 of the SH1211 strain contained H\textsuperscript{13} and K\textsuperscript{151} at these key residues. Future studies of the virulence of the SH1211 strain are warranted to determine the influence of these mutations in GP5 on pathogenicity.

The number of the N-linked glycans on the envelope surface, which provide a barrier to antibodies. Variation in the glycan shield, therefore, presents a possible mechanism for reduced antigenicity [44]. In our current study, the GP5 of the SH1211 strain had N-glycosylation sites at positions 34, 44, and 51 only, which are identical to those in the classical PRRSV strain, CH-1a. However, the high morbidity and mortality in the SH1211-infected herd indicate that other important factors contribute to the virulence of the virus. A recent study showed that an ORF5a wobble position in PRRSV's is used as a highly selective codon usage mechanism that conserves the RQ-motif in the ORF5a protein, despite significant selective pressure on the GP5 N-linked glycosylation motifs [45]. Future studies of the relationship between the variations in ORF5a protein and the N-glycosylation sites in GP5 of the SH1211 strain are warranted.

The ORF2 and ORF5 of the SH1211 strain shared high levels of sequence identity with the QYYZ strain. However, the remaining ORFs of SH1211 shared higher levels of sequence identity with the JXA1 strain. In the phylogenetic trees based on the ORF2 and ORF5 nucleotide sequences, the SH1211 strain clustered with the QYYZ strain, whereas the phylogenetic tree based on the complete genome sequence...
showed substantially different relationships between the SH1211 strain and other PRRSVs. Therefore, we analyzed possible recombination events in the SH1211 strain, and the results indicated that the nucleotide sequences from positions 11 697–12 768 and 13 819–14 472 of SH1211 were derived from the QYYZ strain, while the remaining segments were from the HP-PRRSV strain, JXA1. The two recombinant QYYZ-like regions contained the ORFs encoding the GP2 and GP5 proteins. Thus, these recombination events might have contributed to the virulence of the SH1211 strain. Because the sequence identities shared between the SH1211 and QYYZ strains for the two recombinant regions were 92.9% and 92.8%, the statistical significance of the recombination breakpoints indicates that the SH1211 strain is a recombinant virus derived from the HP-PRRSV strain, JXA1, and the wild-type PRRSV strain, QYYZ.

The ORF1a and ORF1b of PRRSVs encode the long non-structural polyproteins, PP1a and PP1ab, with the expression of the latter depending on a ribosomal frameshift signal in the ORF1a/ORF1b overlap region [46]. The PP1a and PP1ab polyproteins are cleaved by viral proteases to release 14 nonstructural proteins, which include four proteases (Nsp1\(\alpha\), Nsp1\(\beta\), Nsp2, and Nsp4), an RNA-dependent RNA polymerase (Nsp9), a helicase (Nsp10), and an endonuclease (Nsp11) [46–48]. The Nsp1\(\beta\) protein functions in the inhibition of interferon (IFN)-\(\beta\) transcription [49], suppresses both IRF3- and NF-\(\kappa\)B-mediated IFN gene expression [49–51], and interferes with IFN-induced JAK-STAT signaling [50, 52]. A recent study indicated that the Nsp1\(\beta\) blocks the nuclear translocation of interferon-stimulated gene factor 3 (ISG3) by inducing the degradation of karyopherin-\(\alpha1\) and that V\(^{19}\) in the Nsp1\(\beta\) protein correlates with the inhibition of ISG3 translocation [53].

Among the nonstructural proteins of the SH1211 strain of PRRSV, the Nsp1\(\beta\) shared the lowest level of sequence identity (92.6%) with JXA1. Whether the high frequency of mutations in the Nsp1\(\beta\) protein contributes to the suppression of the host innate immune response to the SH1211 strain remains unclear. In addition, the 5' and 3' UTRs of PRRSVs have been shown to be important regulatory elements [54, 55]. Future studies are warranted to determine whether the nucleotides deletions in the 5' and 3' UTRs affect the replication, transcription, and virulence of the SH1211 strain.

5. Conclusions

The SH1211 strain represents a recently emerging virus with a genome structure that is typical of PRRSVs with unique genetic variation, including a deletion in the GP3 gene and apparent recombinations involving the coding sequences for the GP2 and GP5 proteins. Our sequence analysis of the SH1211 strain provides insight into the role of genetic variation in the antigenicity of PRRSVs in China. Future studies of the immunogenicity and pathogenicity of the SH1211 strain are warranted to identify the mechanisms underlying the contribution of genetic variation in PRRSVs to host-pathogen interactions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References


Research Article

Potential Role of Porcine Reproductive and Respiratory Syndrome Virus Structural Protein GP2 in Apoptosis Inhibition

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Porcine reproductive and respiratory syndrome virus (PRRSV) is a serious threat to the pork industry, and its pathogenesis needs further investigations. To study the role of two structural proteins of PRRSV in virus-host cells interactions, two stable cell lines (MARC-2a and MARC-N) expressing GP2 and N proteins, respectively, were established. We induced apoptosis in these cells by treating them with staurosporine and found a significant reduction in the number of apoptotic cells in MARC-2a as compared to MARC-N and MARC-145 cells. In addition, we found significantly higher activities of transcriptional factors (NF-κB and AP-1) in both cell lines as compared to MARC-145 (parent cells). Overall, our data suggest that, although both stable cell lines activate NF-κB and AP-1, GP2 triggers the antiapoptotic process through an intermediate step that needs to be further investigated.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) along with Lactate dehydrogenase-elevating virus, Equine arteritis virus, and Simian hemorrhagic fever virus belongs to the family *Arteriviridae* of order *Nidovirales*. It causes one of the most economically significant diseases in the swine industry. In PRRSV, the full-length single stranded genomic RNA is almost 15.4 kb in length with a 5’ cap and 3’ polyadenylation. It encodes ten ORFs (1a, 1b, 2a, 2b, 3, 4, 5a, 5b, 6, and 7), flanked by 5’ and 3’ untranslated regions [1, 2]. ORF 1a and 1b constitute the majority of the genome and encode viral nonstructural proteins while the rest codes for structural proteins: GP2, E, GP3, GP4, GP5, 5a, M, and N. A lipid bilayer envelope surrounds the viral RNA embedded within nucleocapsid (N) [3]. Of the structural proteins, GP2, GP3, GP4, and GP5 are glycosylated and present on the viral envelope, along with the nonglycosylated M and E proteins. GP5 is known as the major envelope glycoprotein, based on its abundance in the virion, whereas the GP2, GP3, and GP4 are the minor envelope glycoproteins [1]. The 3’-proximal genome part has a compact organization, and most of the genes have overlapping sequences. For instance, ORF 2b, encoding the E protein, is partially overlapping ORF 2a that encodes the GP2 protein.

Apoptosis is an important mechanism by which virus-infected cells are eliminated from the host; therefore, many viruses have evolved strategies to prevent or delay apoptosis in order to provide a window of opportunity in which virus replication, assembly, and egress can take place. Interfering with apoptosis may also be required for establishment and/or maintenance of persistent infections.

PRRSV induces apoptosis both *in vitro* and *in vivo* [4–6]. However, it is debatable whether PRRSV induces apoptosis directly (in infected cells) or indirectly (in bystander cells). Lee and Kleiboeker [7] demonstrated that PRRSV induced apoptosis in infected MARC-145 cells through a mitochondria-mediated pathway. In addition, Costers and colleagues [8] showed that both anti- and proapoptotic activities take place in PRRSV-infected MARC-145 cells and macrophages. It appears that, early in infection, PRRSV stimulates antiapoptotic pathways, whereas infected cells die by apoptosis later.

Previous studies demonstrated that PRRSV infection causes an activation of NF-κB and AP-1 transcription factors [9–11]. Moreover, the ERK signaling pathway is also activated...
in PRRSV-infected cells [12, 13], and activation of JNK is required for the virus-induced apoptosis [14]. However, the molecular mechanisms behind these events are poorly understood. Previous studies showed that PRRSV N [11, 15] and nonstructural protein 2 [16] contribute to NF-κB activation. In the present study, we investigated the role of the PRRSV GP2 and N proteins in apoptosis inhibition and NF-κB and AP-1 signaling pathways activation using stable cell lines MARC-2a and MARC-N, expressing the GP2 and N proteins, respectively.

2. Materials and Methods

2.1. Cells. MARC-145 (MA-104 clone, African green monkey kidney cell line) cells were maintained in minimal essential medium (Hyclone) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 10 mM nonessential amino acids, and 100 U of gentamycin.

2.2. Antibodies. NF-κB rabbit monoclonal antibody (MAb) and c-Jun mouse MAb were purchased from Cell Signaling Technology. Anti-N mouse MAb (SDOW-17) was purchased from Rural Technologies. Fibrillarin mouse MAb was purchased from Santa Cruz. Alpha-tubulin antibody was purchased from Sigma-Aldrich. Cy2-conjugated AffiniPure goat anti-mouse and goat anti-rabbit immunoglobulin G (IgG) were purchased from Jackson ImmunoResearch. Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Bio-Rad.

2.3. Generation of Stable Cell Lines. Synthesis of the codon-optimized N gene (PRRSV strain VR2332) was ordered from EZBiolab. A 380 bp fragment, amplified by primers (N-FOR-B-CGgaatccATGCATATATGGGGGAATTCAATATGGTAA and N-REV-B-CGgaatccCTAAGCGGATGGAGAGC) was digested with BamHI and inserted into BamHI site of the plasmid pIREShyIA-2a [17] creating pIREShyIA-N.

The pUC57-GP2 plasmid containing a codon-optimized sequence for GP2 gene (PRRSV strain VR2332) was ordered from GenScript and the pIREShyIA-GP2 plasmid for the eukaryotic expression of GP2 protein was constructed as follows: first, a 780bp fragment was amplified using primers containing BamHI sites (sORF2pUC57-ATgatcATGAAATATGCTGGAAATTCAATATGGTAA and asORF2pUC57-ATgatcCTAAGCGGATGGAGAGCAG) and pUC57-GP2 plasmid DNA as a template. Second, this fragment was digested with BamHI and inserted into BamHI site of pIREShyIA vector, creating pIREShyIA-2a. Note, natural GP2 gene (ORF 2a) contains an additional ORF (ORF 2b) encoding E protein. To avoid the expression of E protein, the initiation codon of ORF 2b in the synthetic GP2 gene was mutated from ATG to GTG.

To produce the stable cell lines (MARC-2a and MARC-N), subconfluent monolayers of MARC-145 cells were transfected with DNAs of pIREShyIA-2a and pIREShyIA-N, respectively, using Lipofectamine-2000 reagent (Invitrogen) and cell clones were selected by hygromycin B (300 μg/mL).

2.4. NF-κB and AP-1 SEAP Reporter Assay. To monitor the activation of NF-κB and AP-1 signal transduction pathways, the plasmids (pNFκB-SEAP or pAP1-SEAP) were purchased from Clontech and were transiently transfected into MARC-145, MARC-2a, or MARC-N cells by the use of Lipofectamine LTX (Invitrogen). Briefly, 4 μg of each plasmid DNA, 12 μL lipofectamine LTX, and 4 μL Plus reagent were used to transfect cells, seeded in a well of 6-well plate. Since these transfected plasmids contain the secreted alkaline phosphatase (SEAP) gene as a reporter, culture supernatants were collected 72 h after transfection, and SEAP activity was detected in by the Great Escape SEAP Chemiluminescence Assay kit (Clontech) using a GloMax 20/20 Luminometer (Promega). The chemiluminescence emitted by a SEAP-activated substrate (CSPD) was measured in relative luminescence units.

2.5. Nuclear and Cytoplasmic Fractionation. MARC-145, MARC-2a, and MARC-N cells were seeded (1 × 10^6 per well) on 6-well plates. Next day, cells on the plates were washed twice with ice-cold phosphate-buffered saline (PBS) before being scrapped and collected at 1000 rpm for 10 minutes. Cells were resuspended in 450 μL buffer A (100 mM HEPES, pH 7.9, 100 mM KCl, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], and 1 mM phenylmethylsulfonyl fluoride [PMSF]), allowed to swell on ice for 30 minutes, and vortexed for three times/30 s each. Extracts were collected by centrifugation at 14,000 rpm for 10 min and the supernatant was the cytoplasmic fraction. To remove any residual cytoplasmic extracts contamination, cells were washed using buffer A, followed by PMSF added PBS. The pellet was then resuspended and incubated for 20 minutes in 100 μL buffer B (200 mM HEPES, pH 7.9, 500 mM KCl, 5.0 mM EDTA, 1.0 mM EGTA, 1 mM DTT, and 1 mM PMSF) and centrifuged at 14,000 rpm for 5 min at 4°C. Now, the supernatant was the nuclear fraction. Protein concentrations in both fractions were estimated by Bradford assay, and the purity of the fractions was tested by Western blotting for tubulin and fibrillarin to define the cytoplasmic and nuclear fractions, respectively. Equal amounts of nuclear fractions were analyzed to allow the comparison of protein expression levels in different cells.

2.6. Western Blot Analysis. Denatured protein samples prepared from nuclear and cytoplasmic fractions were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-10% PAGE) and transferred to nitrocellulose membrane. To block nonspecific binding sites on the membrane, it was placed over Tris-buffered saline (0.1M Tris [pH 7.6], 0.9% NaCl) containing 0.1% Tween 20 and 5% skim milk for 1 h at room temperature. The membranes were incubated at 4°C overnight with appropriate primary antibodies diluted (1:1000) in 5% bovine serum albumin (BSA) or 1% skim milk prepared in 0.1% TBST as per manufacturer's instructions. HRP-labeled anti-rabbit IgG or anti-mouse IgG detection antibodies diluted (1:2000) in 1% skim milk prepared in 0.1% TBST were then added at room temperature for 1 h and signals were detected with the enhanced chemiluminescence method (Bio-Rad).
The band intensities were measured densitometrically. The band intensities data were normalized with fibrillarin and MARC-145 value was adjusted to 1 for comparison and data were represented as fold changes. For this, the unsaturated bands on the X-ray films were scanned and saved as 8-bit grayscale JPEG files and were analyzed by using the public domain software ImageJ from the National Institutes of Health.

2.7. Immunofluorescence Staining. MARC-N and MARC-145 cells at 85–95% confluency in 2-well Lab-Tek chamber slides were fixed with absolute methanol for 10 min at −20°C. After rehydration with PBS, cells were incubated with anti-N monoclonal antibody SDOW-17 (dilution 1:500) for 1 h at room temperature. Cells were rinsed three times with PBS and incubated with Cy2-conjugated AffiniPure Goat Anti-Mouse IgG (dilution 1:200). The cells were examined using a Zeiss Axiovert 200 M inverted fluorescent microscope.

2.8. Assessment of Apoptosis Using Annexin V/PI and Hoechst Staining. A commercially available annexin V apoptosis detection kit (Invitrogen) and flow cytometry were used to determine the annexin V-binding cells. Appropriate cells were grown on 35 mm disc. Set of wells were treated with 1 µM staurosporine (an apoptotic inducer), for 24 h, keeping another set as an untreated control. After collecting and washing twice with PBS, treated or untreated cells were resuspended in the binding buffer (500 µL), followed by the addition of FITC-annexin-V (5 µL) PI (5 µL) sequentially. The samples were then incubated for 15 min in the dark at room temperature and subjected to flow cytometric evaluation. The experiment was performed in triplicate and repeated three times.

Nuclear morphology of control and staurosporine treated cells was observed by staining cell nuclei with Hoechst 33342 (Invitrogen). Briefly, cells were incubated with Hoechst 33342 (10 µg/mL) for 15 min at RT and examined and counted manually (at least 200 per slide) under a fluorescence microscope by using the DAPI filter. Apoptotic cells were characterized by the condensation of chromatin and/or nuclear fragmentation.

2.9. MTT Assay. MARC-145, MARC-2a, or MARC-N cells were split up into 96-well plates at a density of 10⁵ cells per well. Next day, staurosporine (3 µM) was added. After 24 h, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay for mitochondrial activity, an indicator of cell viability, was carried out by adding
Apoptotic cells (%)  

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Living cells (%)  

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**Figure 2:** Inhibition of apoptosis in MARC-2a cells. After incubation for 24 h with 1 μM staurosporine, MARC-145, MARC-2a, and MARC-N cells were stained with FITC-annexin V and PI (a) or Hoechst 33342 (b). The percentage of viable cells was determined by MTT assay (c). The data represent the results of three independent experiments in triplicates, and error bars indicate standard deviations of the means. *P < 0.05* (**); *P < 0.01** (**).

25 μL of MTT (5 mg/mL) to each well and incubating the cells for 2 h in a CO₂ incubator at 37°C. Finally, 100 μL of lysis buffer (20% SDS in 50% dimethylformamide, pH 4.7) was added, and the cells were further incubated overnight before measuring the optical density at A595 with an ELISA reader. The percentage of living cells was calculated using the following formula: (A<sub>595</sub> treated cells)/(A<sub>595</sub> nontreated cells) × 100. The assay was performed in triplicate, and the experiment was repeated three times.

2.10. Statistical Analysis. All data were analyzed using the GraphPad Prism (Version 5.03) software. Differences among all groups were examined using the one-way ANOVA followed by Tukeys test. Differences were considered significant if *P < 0.05*.

3. Results

3.1. Construction of MARC-2a and MARC-N Cell Lines. To analyze molecular and pathological roles of GP2 and N proteins, we constructed two stable cell lines MARC-2a and MARC-N, expressing PRRSV GP2 and N proteins, respectively. The parent cell line used for this purpose was MARC-145 cell line because it supports infection and propagation of PRRSV.

The plasmids pIREShyIA-2a and pIREShyIA-N contained the gene sequences for GP2 and N, respectively, under the control of the human cytomegalovirus promoter, and they also contained the hygromycin B phosphotransferase gene as a selectable marker fused to the internal ribosome entry site (IRES) sequence at the 5' end. The IRES permits the translation of two open reading frames from one mRNA. MARC-145 cells were transfected with these engineered plasmids and grown under the selection pressure of hygromycin B. Hygromycin-resistant cell clones were further expanded and maintained in the presence of hygromycin B for several days. Cells from each of the maintained clones (MARC-2a and MARC-N) were used for the extraction of total nucleic acids. PCR analysis with the primers specific to the 2a and N genes revealed the amplified products of 380 bp and 780 bp,
and condensed chromatin material (brighter granulated blue fluorescence) from apoptotic cells with fragmented nucleus allowed discriminating normal live cells (blue homogenate treated cells were stained with Hoechst. This nucleus stain to MARC-145 (9.5%) and MARC-N (8.3%) cells (were positive for annexin V-FITC (apoptotic) in comparison a significantly low percentage of MARC-2a cells (1.5%) that a nalyze d at 24 h after treatment (Figure 2(a)). We found an significantly low percentage of MARC-2a cells (1.5%) that were positive for annexin V-FITC (apoptotic) in comparison to MARC-145 (9.5%) and MARC-N (8.3%) cells ($P < 0.05$).

For further verification of these findings, staurosporine treated cells were stained with Hoechst. This nuclear stain allowed discriminating normal live cells (blue homogenate fluorescence) from apoptotic cells with fragmented nucleus and condensed chromatin material (brighter granulated blue color fluorescence). In agreement with the annexin V staining, MARC-2a cells had significantly lower percentage of apoptotic cells (Figure 2(b)).

Finally, after treatment with staurosporine, the percentage of viable MARC-2a cells was higher than the percentage of living cells in MARC-145 and MARC-N cell lines (Figure 2(c)).

Taken together, these data indicate that MARC-2a cells are more resistant to programmed cell death than to parent MARC-145 cells or MARC-N cells.

3.3. NF-κB and AP-1 Activation. NF-κB and AP-1 activation in MARC-2a and MARC-N cells was confirmed using the SEAP reporter gene system. The principal behind this assay is the following. If the NF-κB or AP-1 signal transduction pathway is induced, endogenous NF-κB/AP-1 binds to the k/κ enhancer element, located in the promoter region of the pNFκB-SEAP or pAPI-SEAP vector, thus activating the transcription of the SEAP reporter gene.

As shown in Figure 3, MARC-2a and MARC-N cells had significantly increased levels of SEAP than MARC-145 cells, which indicate the NF-κB and AP-1 activation in these cells. Untransfected cells did not show any SEAP activity.

Additionally, these data were confirmed by Western blot analysis (Figure 4). For biological functions, transcription factors have to go to the nucleus. Thus, we have analyzed the nuclear fractions of MARC-145, MARC-2a and MARC-N cells for the presence of NF-κB and c-Jun (part of AP-1) proteins. Fibrillarin, the nucleolar protein, was used as the loading control. From ImageJ analysis, there was found a 6-fold and 8.5-fold increase in the expression of NF-κB in MARC-2a, and MARC-N cells, respectively, as compared to MARC-145 cells (Figure 4(a)). In case of AP-1, the fold increase was 4.5 and 4, respectively (Figure 4(b)).

4. Discussion

Many viruses, including PRRSV, utilize strategies of delaying the process of apoptosis for their benefit. Apoptosis can be triggered by a variety of stimuli including death receptors on the cell surface (extrinsic pathway) and intracellular signals (intrinsic pathway). Staurosporine, a strong inhibitor of protein kinases [18], triggers intrinsic pathway which results in the release of cytochrome c from mitochondria, formation of the apoptosome (cytochrome c, APAF-1, and caspase-9), and activation of caspase-9. Both the extrinsic and the intrinsic processes congregate at the activation of the downstream effector caspsases, which are responsible for inducing the morphological changes observed in an apoptotic cell. The apoptotic events are regulated by the interplay of pro- and antiapoptotic proteins which are members of the Bcl-2 family [19].

Many viruses encode homologue of Bcl-2 proteins that can prevent apoptosis, thus helping the viruses to complete their life cycle in the host cells [20]. For instance, human adenovirus E1B 19 K protein inhibits apoptosis by forming heterodimers with a variety of proapoptotic proteins of the
Bcl-2 family [21]. However, our attempts to detect interactions between GP2 and proapoptotic members of the Bcl-2 family were not successful (data not shown).

On the other hand, the cell survival in many viral infections is regulated by the NF-κB activity. For instance, expression of the human hepatitis C core protein activates the NF-κB pathway which results in an antiapoptotic activity [22]. Also, interferon (IFN) signaling activates the NF-κB that integrates into the IFN receptor pathways and promotes cell survival [23]. It is also proved that IL-15 has an antiapoptotic effect [24, 25]. The antiapoptotic property of IL-15 and its regulation by NF-κB signaling in PRRSV infection hinted a possible association with the cell survival strategy of PRRSV. On the other hand, AP-1 has an evolutionary origin from the avian sarcoma virus with cell proliferation and differentiation property that support its role in cell survival signaling [26].

5. Conclusions

The main goal of our research is to understand the role of the PRRSV structural proteins in the viral pathogenesis. In the current study, we have explored the role of PRRSV GP2 and N proteins in activation of the NF-κB and AP-1 signaling pathways. The role of the N protein in activation of NF-κB has been established earlier, whereas activation of cell genes transcription by GP2 has never been previously reported. To our best knowledge, this study is the first report, describing genes transcription activation function of GP2 protein. We have also demonstrated that N activates the AP-1 pathway in addition to NF-κB, which is a novel finding too.

The GP2 protein likely plays a role in apoptotic inhibition by PRRSV. Possibly, the transcription factors activated by GP2 protein are involved in this activity by activation of antiapoptotic and suppression of proapoptotic genes expression. However, the mechanism of this GP2 function needs to be further investigated.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References


**Research Article**

**Construction and Immunogenicity of DNA Vaccines Encoding Fusion Protein of Porcine IFN-λ1 and GP5 Gene of Porcine Reproductive and Respiratory Syndrome Virus**

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Porcine reproductive and respiratory syndrome virus (PRRSV) has been mainly responsible for the catastrophic economic losses in pig industry worldwide. The commercial vaccines only provide a limited protection against PRRSV infection. Thus, the focus and direction is to develop safer and more effective vaccines in the research field of PRRS. The immune modulators are being considered to enhance the effectiveness of PRRSV vaccines. IFN-λ1 belongs to type III interferon, a new interferon family. IFN-λ1 is an important cytokine with multiple functions in innate and acquired immunity. In this study, porcine IFN-λ1 (PoIFN-λ1) was evaluated for its adjuvant effects on the immunity of a DNA vaccine carrying the GP5 gene of PRRSV. Groups of mice were immunized twice at 2-week interval with 100 μg of the plasmid DNA vaccine pcDNA3.1-SynORF5, pcDNA3.1-PoIFN-λ1-SynORF5, and the blank vector pcDNA3.1, respectively. The results showed that pcDNA3.1-PoIFN-λ1-SynORF5 can significantly enhance GP5-specific ELISA antibody, PRRSV-specific neutralizing antibody, IFN-γ level, and lymphocyte proliferation rather than the responses induced by pcDNA3.1-SynORF5. Therefore, type III interferon PoIFN-λ1 could enhance the immune responses of DNA vaccine of PRRSV, highlighting the potential value of PoIFN-λ1 as a molecular adjuvant in the prevention of PRRSV infection.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS), characterized by severe reproductive failure in sows and respiratory distress in piglets and growing pigs, is one of the most economically significant viral diseases of swine [1–5]. Since firstly reported in the United States in 1987 and in Europe in 1990 [6, 7], PRRS has been gaining gradually increased attention because of its large-scale outbreak and tremendous losses in the global swine industry.

PRRSV, the causative agent of PRRS, is a small, enveloped, single-stranded, positive-sense RNA virus belonging to the family Arteriviridae. The PRRSV genome with a size of approximately 15 kb contains 9 open reading frames (ORFs). ORFs 1a and 1b encoded for nonstructural proteins and ORF 2–7 encoded for structural proteins [8–10]. Among them, the ORF5, that encoded major envelope glycoprotein (GP5), is one of the key immunogenic proteins of PRRSV and is the leading target for the development of the genetic engineering vaccines against PRRS [11–20]. The modified GP5 which
used three methods to modify, immunogenicity, particularly in the ability to induce neutralizing antibody responses and cellular immune responses, compared to the native GP5 [21]. Consequently, this modified GP5 may be useful to facilitate the development of the new generation of vaccines, such as DNA vaccines, live attenuated chimeric virus vaccines, and live virus-vector vaccines, against the highly pathogenic PRRSV in the future.

Type III interferon, a new interferon family, was firstly reported in 2003 and different from the types I and II interferon, including IFN-α1, IFN-α2, and IFN-α3. IFN-α and IFN-β, belonging to type I interferon, were confirmed to be adjuvants to improve the vaccines’ immune responses [22–24]. In addition, previous studies have shown that type III interferon has almost the same biological activity of other interferons, such as anti-viral, antitumor, and immune regulation but when compared with type I interferon, its side effects are obviously little. Thus, the research on type III interferon will play a role in promoting the control of animal diseases and medical treatment of human disease.

In view of the above information, in this study, we constructed the DNA construct units encoding pcDNA3.1-PoIFN-λ1-SynORF5 and find that pcDNA3.1-PoIFN-λ1-SynORF5 could induce stronger cellular and humoral immune responses than the responses induced by pcDNA3.1-SynORF5. Therefore, PoIFN-λ1 might be a promising candidate molecular adjuvant to develop more effective vaccines.

2. Material and Methods

2.1. Plasmids and Cells. pcDNA3.1-SynORF5, which was based on the native ORF5 gene of highly pathogenic PRRSV strain (constructed and kept in our lab), pcDNA3.1, Helacells, and Marc-145 cells were kept in our lab.

2.2. Experimental Animals. 6-week-old BALB/c mice were purchased from Yang Zhou University. The mice were randomly divided into 3 groups and acclimated under controlled specific pathogen-free (SPF) conditions for 1 week prior to the start of the experiment.

2.3. Cloning and Sequencing of PoIFN-λ1 Gene. The primers were designed for amplifying PoIFN-λ1 based on gene sequence of porcine IFN-λ1 gene (GenBank accession number FJ853390). PoIFN-λ1F: 5′-TTGTGATCCGCCACC-ATGCTACAGCTTGATCAGTG-3′, PoIFN-λ1R: GAGGGTACCCTTACCACACGAGTGTCGAA-GCTCCACTGTTA-3′. PCR reaction was performed in the thermocycler with the following program: denaturation at 95°C for 5 min, 30 cycles were comprised of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min and was ended with the final extension of 10 min at 72°C. PCR products obtained with primers PoIFN-λ1F and PoIFN-λ1R were inserted into vector pMD18-T, generating plasmids pMD18-T-PoIFN-λ1. cDNAs encoding PoIFN-λ1 were obtained subsequently by RT-PCR, using mRNAs from porcine peripheral blood mononuclear cells (PBMC). The sequence of the insert was confirmed by sequencing.

2.4. Construction of pcDNA3.1-PoIFN-λ1-SynORF5 Plasmids. The cloning product was inserted into pMD18-T vector and then sequenced. Based on the sequencing result, the PCR production and pcDNA3.1-SynORF5 were digested with a similar pair of restriction enzymes Nhe I/Kpn I; then the corresponding restriction fragments were linked using T4 DNA ligase. The standard molecular biological techniques to construct the pcDNA3.1-PoIFN-λ1-SynORF5 plasmid were shown in Figure 1.

2.5. Restriction Enzyme Digestion of the Plasmid DNA. The recombinant plasmids were purified by AxyPrep Plasmid Miniprep Kit (Axygen Biosciences, Zhejiang, China). Then the obtained plasmids were, respectively, digested with three pairs of restriction enzyme which included Nhe I/Kpn I, Nhe I/Xho I, and Kpn I/Xho I.

2.6. Transfection and Western Blotting. Hela cells were seeded at a concentration of 2.5 × 10⁴ cells/well into 6-well tissue culture plate until the cells reached approximately 70–80% confluence. Transfection was performed with LipofectAMINE 2000 reagent (Invitrogen) as specified by the manufacturer. The transfected cells were collected at 48 h after transfection and lyzed in a buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, and protease inhibitors cocktail (Roche). Protein quantification was carried out using a BCA T 223 protein assay kit (Pierce). Equal amounts of proteins were separated using 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in phosphate-buffered saline (PBS) and incubated with GP5-specific monoclonal antibodies (kept in our lab) and, subsequently, with HRP-conjugated goat anti-mouse IgG (Sigma). Signals were developed using SuperSignal West Pio Luminol kit (Pierce).

2.7. Immunization of BALB/c Mice with Plasmid DNA. Large-scale preparations of plasmid DNA, including pcDNA3.1, pcDNA3.1-SynORF5, and pcDNA-PoIFN-λ1-SynORF5, were purified by EndoFree Maxi Plasmid Kit (TIANGEN, Beijing, ON, China), as instructed by the manufacturer. The plasmids, respectively, adjust to a final concentration of 1 μg/μL.

Six-week-old BALB/c mice were purchased from Yang Zhou University. Twenty-one mice were randomly divided into groups and mice were vaccinated intramuscularly twice at 2-week intervals with pcDNA3.1-PoIFN-λ1-SynORF5, pcDNA3.1-SynORF5, and the empty vector pcDNA3.1 (+), respectively. Serum samples were collected 2 and 4 weeks after primary inoculation for serological tests. Six weeks after primary immunization, mice were euthanized and the sera were harvested for the detection of antibodies against PRRSV and splenocytes were isolated as described previously [25] for IFN-γ assay and lymphocyte-proliferation assay.

2.8. Serological Tests. GP5-specific antibodies were determined with an endpoint ELISA using the purified recombinant GP5 as antigen as described previously [26]. The
titers were expressed as the reciprocal of the highest dilution of sera producing ratio values of 2.1. Serum neutralization assays were essentially performed as described by Ostrowski et al. [27]. The neutralization titers were expressed as the reciprocal of the highest serum dilution resulting in complete neutralization. Each sample was run in triplicate.

2.9. Lymphocytes Proliferation Assay. Lymphocyte proliferation assay was performed using the splenocytes of immunized mice. Six weeks after the primary inoculation, splenocytes were collected, respectively. Lymphocyte proliferation assays were performed as described previously [25]. The stimulation index (SI) was calculated as the ratio of the average OD value of wells containing antigen-stimulated cells to the average OD value of wells containing only cells with medium.

2.10. IFN-γ Release Assay. The isolated splenocytes (1 × 10^6 cells/mL) were cultured in 24-well plates at 37°C in the presence of 5% CO₂ with or without the PRRSV inactivated by UV. After 72 h incubation, culture supernatant was harvested and the presence of IFN-γ was tested with commercial mouse IFN-γ immunoassay ELISA kits (Boster Biological Technology, LTD., Wuhan, China) according to manufacturer’s instructions. The concentrations of IFN-γ in the samples were determined based on the standard curves.

2.11. Real-Time PCR Analysis of IFN-γ mRNA Expression. Splenocytes (1 × 10^6 cells/mL) were cultured in 24-well plates for 18 h at 37°C in the presence of 5% CO₂. Total RNA was extracted and 0.4 μg of RNA was reverse transcribed in a 20 μL reaction mixture. The cDNA product (0.5 μL) was amplified in a 25 μL reaction mixture containing SYBR Green Real-time PCR Master Mix (ToYoBo) and 0.2 μM of each of the forward and reverse gene-specific primers (Mouse-IFN-γ: TCAAGTGGGATAGATGTGGAAGAA/TGGCTCCTGAGGATTTTCATG; Mouse-β-actin: CACTGCCGCTTCCCTCTC/CAATAGTGATGACCTGGCCGT). Each cDNA sample was performed in triplicate. PCR amplifications were performed using an Applied Biosystems 7500 Real-Time PCR System (ABI). Thermal cycling conditions were 2 min at 50°C, 10 min at 94°C, and 40 cycles of 15 s at 94°C and 1 min at 60°C. Gene expression was measured by relative quantity as described previously [28].

2.12. Statistical Analysis. Student’s t-test was used to compare the level of immune responses among the different groups. P values of <0.05 were considered statistically significant.

3. Results

3.1. Cloning and Sequencing of the PoIFN-λ1 Gene Fragment. A single PCR product of an estimated 576 bp of the PoIFN-λ1 gene (Figure 2) was amplified using the cDNAs, which were obtained by RT-PCR with mRNAs of porcine PBMC, as template. The fragment was cloned into the pMD18-T vector.
vector and sequenced. The nucleotide sequences for PoIFN-λ1 were 99% identical to published PoIFN-λ1 (Acc no. FJ853390) sequences. The predicted protein sequences for PoIFN-λ1 were 100% identical to published PoIFN-λ1 (Acc no. NP_001136309) sequences, as determined by BLAST analysis.

3.2. Construction of Plasmids. The gene fragment encoding PoIFN-λ1 (Figure 2(a)) was cloned into the cloning plasmid vector pMD18-T. PoIFN-λ1 was analyzed by restriction endonuclease double digestion with Nhe I and Kpn I. The size of the digested fragments was 576 bp and an estimated 2692 bp pMD18-T vector band (Figure 2(b)). Eukaryotic expression plasmids pcDNA3.1-PoIFN-λ1-SynORF5 were also constructed as described (Figure 1) and analyzed by three pairs of restriction endonuclease double digestion with Nhe I/Kpn I, Kpn I/Xho I, and Nhe I/Xho I, respectively. The size of the digested fragments containing the inserted fragments was 576, 663, and 1239 bp, respectively, and an estimated 5428 bp pcDNA3.1 vector band (Figure 2(c)).

3.3. Western Blotting Detection of Recombinant Proteins. To investigate whether the inserted gene fragment PoIFN-λ1 influences the in vitro expression and authenticity of the SynORF5 gene, Hela cells were transiently transfected with pcDNA3.1-PoIFN-λ1-SynORF5, and Western blot was performed at 48 h after transfection. The DNA construct pcDNA3.1-SynORF5, only expressing the SynORF5 gene of PRRSV strain NJGC, was used as a control. As shown in Figure 3, the fusion protein bands with expected molecular sizes could be detected in lysates of cells transfected with pcDNA3.1-PoIFN-λ1-SynORF5 (48 KDa), and just GP5-specific protein bands with expected molecular sizes could be detected in lysates of cells transfected with pcDNA3.1-SynORF5 (25 KDa), but there are no protein bands in lysates of cells transfected with the empty vector. So, the results showed that the inserted gene fragment PoIFN-λ1 did not influence the in vitro expression of SynORF5 gene.

3.4. Humoral Immune Responses Induced in Mice Immunized with Different DNA Constructs. To further compare the ability of pcDNA3.1-SynORF5 and pcDNA3.1-PoIFN-λ1-SynORF5 to induce specific immune responses in vivo, three groups of 6-week-old BALB/c mice (seven mice per group) were injected twice, at 2-week intervals, into the quadriceps muscle with 100 μg of pcDNA3.1-PoIFN-λ1-SynORF5, pcDNA3.1-SynORF5, and the empty vector pcDNA3.1, respectively. Serum samples were collected at 2, 4, and 6 weeks after the primary immunization. GP5-specific ELISA antibody was determined using the purified GP5 protein as the antigen. As shown in Figure 4, 2 weeks after primary immunization, the antibody titer reached a detectable level only in the group immunized with pcDNA3.1-PoIFN-λ1-SynORF5, and a further increase in antibody levels
Figure 3: Expression of the fusion protein which encoded by the recombinant plasmid pcDNA3.1-PoIFN-λ1-SynORF5 in the transfected cells. Approximately 70–80% confluent Hela cells were transfected with 2 μg of pcDNA3.1-PoIFN-λ1-SynORF5 (lane 3), pcDNA3.1-SynORF5 (lane 2) and control vector pcDNA3.1(+) (lane 1), respectively. At 48 h after transfection, the cells were collected and subjected to Western blot as described in Section 2. Protein standards are indicated on left side of panel.

was observed at 4 and 6 weeks after primary immunization. Although a continuous increase in antibody levels was observed at 4 and 6 weeks after primary immunization in the group immunized with pcDNA3.1-SynORF5, the whole increasing trend observed in the group immunized with pcDNA3.1-SynORF5 was not significant compared with the group immunized with pcDNA3.1-PoIFN-λ1-SynORF5.

Serum samples were further evaluated for the ability to neutralize PRRSV strain NJGC in vitro using serum neutralization assays. As shown in Figure 5, mice immunized with pcDNA3.1-PoIFN-λ1-SynORF5 developed higher PRRSV-specific neutralizing antibody titer (1: 5.14) than that of mice received pcDNA3.1-SynORF5 (1: 4.67) at 2 weeks after primary immunization (P < 0.05). After boost immunization, the neutralizing antibody levels went increasingly higher and reached up to 1: 16 in group immunized with pcDNA3.1-PoIFN-λ1-SynORF5 at 6 weeks after primary immunization, in comparison to 1: 9.71 in mice immunized with pcDNA3.1-SynORF5. No detectable neutralizing antibodies (<1: 4) were observed in the sera from mice immunized with the empty vector during the experimental period.

3.5. Cellular Immune Responses Induced in Mice Immunized with Different DNA Constructs. The results presented above clearly demonstrated that PoIFN-λ1 could effectively enhance humoral immune responses elicited by DNA vaccine. To investigate whether PoIFN-λ1 could also enhance cellular immune responses elicited by DNA vaccine, the lymphocyte-proliferative responses were analyzed at 6 weeks after primary immunization. As shown in Figure 6, the SI was higher (P < 0.05) in mice immunized with pcDNA3.1-PoIFN-λ1-SynORF5 than that in those immunized with pcDNA3.1-SynORF5. These results indicated that PoIFN-λ1 can also enhance Th1-type immune response.

To further characterize the cellular immune responses in mice immunized with pcDNA3.1-PoIFN-λ1-SynORF5, IFN-γ secretion in splenocytes restimulated with PRRSV protein was measured by ELISA. As shown in Figure 7, the mean IFN-γ production of 395.8 pg/mL was detected in mice immunized with pcDNA3.1-PoIFN-λ1-SynORF5 and was
significantly higher \( (P < 0.05) \) than that in mice immunized with pcDNA3.1-ORF5 \( (297.8 \text{pg/mL}) \). Quantitative real-time RT-PCR was also performed to analyze the level of IFN-\( \gamma \) mRNA expression in the restimulated splenocytes. Similarly to the results of IFN-\( \gamma \) ELISA assay, the highest IFN-\( \gamma \) mRNA expression was found in restimulated splenocytes from mice immunized with pcDNA3.1-PoIFN-\( \lambda 1 \)-SynORF5 (Figure 8). The mean relative IFN-\( \gamma \) mRNA expression in this group was 3.42-fold higher than that in group empty vector and 1.99-fold higher than that in group pcDNA3.1-SynORF5, respectively.

**4. Discussion**

At present, PRRS continues to be one of the most economically significant viral diseases in the swine industry worldwide. Though there are many commercial vaccination strategies, they can provide only a limited protection. Thus PRRSV genetic engineered vaccines have recently been reported, including pseudorabies virus expressing GP5 [16], recombinant fowlpox virus coexpressing GP5/GP3 and swine IL-18 [20], recombinant adenoviruses expressing GP5/GP4/GP3 [29], and mycobacterium bovis BCG expressing GP5 and M [18, 30]. In order to increase the efficiency of the vaccine, an alternative approach is to codeliver cytokines to upregulate the immune response of PRRSV, including HSP70 [31], IL-18 [30], GM-CSF [32], C3d-p28 [33], and interferon \( \alpha/\gamma \) [34]. In this study, porcine IFN-\( \lambda 1 \) was amplified and recombinant plasmid encoding PoIFN-\( \lambda 1 \) and the modified GP5 of PRRSV were constructed. It was found that the porcine IFN-\( \lambda 1 \) can effectively increase the humoral and cellular immune responses of GP5 of PRRSV in mice. GP5 protein is a structural PRRSV protein with the size of 25 KDa. GP5 is the most important glycosylation of PRRSV involved in the generation of PRRSV-neutralizing antibodies and protective immunity [12, 35–38]. So most vaccine research PRRSV is focused on the GP5. In our previous research, the DNA vaccine encoding the modified GP5 induced significantly
enhanced GP5-specific ELISA antibody, PRRSV-specific neutralizing antibody, IFN-γ level, and lymphocyte proliferation response, compared to native GP5 in the vaccinated mice and piglets, indicating that these modifications could enhance the immunogenicity of GP5. And in another research, the purified recombinant PoIFN-λ1 exhibited significant antiviral effects against porcine reproductive and respiratory syndrome virus (PRRSV) and pseudorabies virus (PRV), suggesting that PoIFN-λ1 is a potential antiviral agent against swine infectious diseases [39]. So in this study, the recombinant DNA units pcDNA3.1-PoIFN-λ1-SynORF5 were constructed and the immune responses were detected in mice, in order to identify whether PoIFN-λ1 can further improve the efficacy of the immune responses induced by pcDNA3.1-SynORF5 or not.

Figures 3 and 4 showed that PoIFN-λ1 can effectively enhance humoral immune responses elicited by DNA vaccine. Although the mechanisms of adaptive immune response that are responsible for mediating the vaccine induced protective immunity have not been fully understood, it is widely accepted that neutralizing antibodies could possibly represent a valuable parameter to evaluate the efficacy of a vaccine against PRRSV [40–42]. Likewise, cell-mediated immunity, particularly the level of virus-specific IFN-γ, has been another potential correlate of protective immunity against PRRSV [43–46]. In previous study, Jiang et al. found that mice immunized with recombinant adenoviruses expressing GP5 with mutation in different glycosylation sites developed significantly enhanced neutralizing antibodies, but not in lymphocyte proliferation response [47]. In Figures 5 and 6, enhanced IFN-γ level, as well as lymphocyte proliferation response, could be observed in pcDNA3.1-PoIFN-λ1-SynORF5-immunized mice. It is indicated that the enhanced cellular immune responses might be enhanced by the adjuvant effect of the PoIFN-λ1 in mice. In a word, the recombinant construct containing PoIFN-λ1 and SynORF5 were successfully constructed, then the grouped mice were vaccinated with different plasmids. Results showed that significantly enhanced GP5-specific ELISA antibody, PRRSV-specific neutralizing antibody, IFN-γ level, and lymphocyte proliferation response could be induced in mice immunized with DNA vaccine co-expressing the modified GP5 and PoIFN-λ1 more than those which received DNA vaccine only expressing the modified GP5. The results demonstrate that PoIFN-λ1 could significantly enhance the humoral and cellular immune responses and may provide protection which was induced by pcDNA3.1-PoIFN-λ1-SynORF5 against PRRSV challenge in piglets.

To our knowledge, this study is the first demonstration that porcine IFN-λ1 fused the modified GP5 of PRRSV could markedly enhance the immune responses. PoIFN-λ1 might be a useful molecular adjuvant in improving PRRSV immune response and maybe it will be further used in PRRSV vaccine.

Conflict of Interests

The authors declare no potential conflict of interests with respect to the research, authorship, and/or publication of this paper.

Authors’ Contribution

Luping Du and Bin Li contributed equally to this work.

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