Porcine Reproductive and Respiratory Syndrome Virus Inhibits Type I IFN Signaling by Blocking STAT1/STAT2 Nuclear Translocation

Deendayal Patel1†‡, Yuchen Nan1‡, Meiyan Shen1§, Krit Ritthipichai1, Xiaoping Zhu2, and Yan-Jin Zhang1*†

1Molecular Virology Laboratory, and 2Immunology Laboratory, VA-MD Regional College of Veterinary Medicine and Maryland Pathogen Research Institute, University of Maryland, College Park, MD, USA

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§Co-first authors.
†Present address: Hauptman-Woodward Medical Research Institute, 700 Ellicott Street, Buffalo, NY
§Present address: Shandong Vocational College of Veterinary Medicine and Animal Science, Weifang, Shandong, China.

*Corresponding author: Molecular Virology Laboratory, VA-MD Regional College of Veterinary Medicine, University of Maryland, 8075 Greenmead Drive, College Park, MD 20742. Phone: +1 301 314-6596. Fax: +1 301 314-6855. E-mail address: zhangyj@umd.edu
Abstract

Type I interferons (IFNs), IFN-α/β, play an important role in innate immunity against viral infections by inducing antiviral responses. Porcine reproductive and respiratory syndrome virus (PRRSV) inhibits the synthesis of type I IFNs. However, whether PRRSV can inhibit the IFN signaling is less understood. In the present study, we found that PRRSV interferes with IFN signaling pathway. The transcripts of IFN-stimulated genes, ISG15 and ISG56, and the protein of the signal transducer and activator of transcription (STAT) 2 in PRRSV VR2385-infected MARC-145 cells were significantly lower than those in mock-infected cells after IFN-α treatment. IFN-induced phosphorylation of both STAT1 and STAT2, and their heterodimer formation in the PRRSV-infected cells were not affected. However, majority of the STAT1/STAT2/IRF9 heterotrimer remained in the cytoplasm of PRRSV-infected cells, which indicates that the nuclear translocation of the heterotrimer was blocked. Overexpression of NSP1β of PRRSV VR2385 inhibited expression of ISG15 and ISG56 and blocked nuclear translocation of STAT1, which suggests that NSP1β might be the viral protein responsible for the inhibition of IFN signaling. PRRSV infection of primary porcine pulmonary alveolar macrophages (PAMs) also inhibited IFN-α stimulated expression of the ISG genes and STAT2 protein. In contrast, a licensed low virulent vaccine strain, Ingelvac PRRS MLV, activated expression of IFN-inducible genes, including chemokines and antivirals, in PAMs without the addition of external IFN, and had no detectable effect on IFN signaling. These findings suggest that PRRSV interferes with the
activation and signaling pathway of type I IFNs by blocking the ISGF3 nuclear translocation.

Key words: inhibition of type I IFN signaling, porcine reproductive and respiratory syndrome virus, PRRSV, IFN-α, IFN signaling, MLV.
Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease, causing an estimated $560 million loss per year to the swine industry in the United States (24). The causative agent, PRRS virus (PRRSV), is a positive-sense single-stranded RNA virus belonging to the family *Arteriviridae* (20). The genome of PRRSV is about 15 kb in length with nine open reading frames (ORFs) (7, 22). ORFs 1a and 1b comprise 80% of the viral genome and are predicted to encode viral enzymes for RNA synthesis. ORFs 2, 2a, 3, and 4 of PRRSV encode minor membrane-associated proteins GP2, E, GP3, and GP4, respectively. ORFs 5, 6, and 7 encode major structural proteins: a major envelope glycoprotein (GP5), a membrane protein (M) and a nucleocapsid protein (N), respectively (18, 21). PRRSV can be propagated in vitro in the epithelial-derived monkey kidney cells MARC-145 (12) and in primary culture of porcine pulmonary alveolar macrophages (PAMs). PAMs are main target cells for PRRSV during its acute infection of pigs (31).

PRRSV-infected pigs develop delayed appearance of neutralizing antibodies (15) and weak cell-mediated immune response (39). PRRSV inhibits synthesis of type I interferons (IFNs) in infected pigs (1, 5, 17). IFNs could not be detected in the lung of pigs in which PRRSV actively replicated. PRRSV infection of PAMs and MARC-145 in vitro leads to very low interferon-α expression (1, 23). Suppression of innate immunity is believed to be an important contributing factor to the PRRSV modulation of host immune responses.
Type I IFNs, such as IFN-α and -β, acting in concert with IFN-γ, are critical to innate immunity against viruses and play an important role in the modulation of adaptive immunity (34). Activation of IFN signaling leads to induction of antiviral responses. The signaling of Type I IFNs is initiated after IFN-α and -β bind to their receptors on cell surface (8, 32, 33). The receptor binding activates Janus kinase (JAK) and Tyk2 to phosphorylate the signal transducers and activators of transcription (STATs), STAT1 and STAT2. The phosphorylated STAT1 and STAT2 form heterotrimers with IRF9 and translocate into the nucleus to induce expression of IFN-stimulated genes (ISGs), which result in the establishment of an antiviral state (8, 32, 33).

It was found that PRRSV suppresses IFN-β production in MARC-145 cells by interfering with RIG-I signaling pathway (17) and PRRSV NSP1β inhibits interferon production (3, 6, 13). Overexpression of PRRSV NSP1β in HEK293T cells interferes with the nuclear translocation of STAT1-GFP observed under fluorescence microscopy (6). However, whether PRRSV can inhibit type I IFN signaling and induction of IFN-stimulated genes, especially in primary PAMs, is not known.

To further define the mechanisms of PRRSV-induced inhibition of innate immunity, we examined the effects of PRRSV infection on type I IFN signaling. In the present study, we found that PRRSV inhibited type I IFN signaling and downstream gene expression. The nuclear translocation of STAT1/STAT2/IRF9 heterotrimers was blocked, while the IFN-induced phosphorylation of STAT1 and STAT2 was not affected. PRRSV NSP1β is the possible viral protein that is responsible for the inhibition of IFN signaling. The interference of the IFN signaling was also demonstrated in primary culture of PAM cells.
Materials and methods

Cells and viruses. MARC-145 (12), HEK293, and HeLa cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The PRRSV VR2385 (19) was used to inoculate MARC-145 cells at 0.5 to 1 multiplicity of infection (MOI). A licensed modified live vaccine strain, Ingelvac PRRS MLV, was kindly provided by Dr. Kay S. Faaberg (National Animal Disease Center, Ames, IA). Virus titers were determined in MARC-145 cells for the median tissue culture infectious dose (TCID$_{50}$) as described previously (40).

Primary PAM cells were prepared from broncho-alveolar lavage of 8-week-old PRRSV-negative piglets. The preparation and subsequent culture of PAMs in RPMI 1640 culture medium were conducted as previously described (25). For virus inactivation, supernatant containing virus was treated in a UV cross-linker for two 10-min pulses at 1 min interval. Virus inactivation was confirmed by inoculation of MARC-145 cells and the absence of cytopathic effect development 72 h post-infection (hpi).

For interferon stimulation, recombinant human IFN-α A/D (Sigma-Aldrich, St. Louis, MO) was added to the cell culture at a final concentration of 1000 u/ml, unless stated differently in the Results section and the figure legends. The cells were harvested at 30 min to 1 h or later depending on experiments for further analysis.

Plasmids. pEGFP-C1-STAT1 for STAT1-eGFP expression was obtained from Addgene (36). The sequences of NSP1α were amplified from cDNA of VR2385 and MLV, respectively, with primers 85NSP1F1 and 85NSP1R1, and NSP1β with primers...
85NSP1F2 and 85NSP1R2 (Table 1), which contain restriction sites for EcoRI or XhoI to facilitate directional cloning. The two fragments were cloned into pCMVTag2B vector, separately. The resulting recombinant plasmids produce FLAG-tagged NSP1α and NSP1β. In each plasmid, cloning was confirmed by restriction enzyme digestion and DNA sequencing. NSP1β was also cloned into an RFP reporter vector to express NSP1β-RFP.

Confocal fluorescence microscopy. MARC-145 cells were seeded directly onto Lab-Tek chamber slide, cultured overnight, and transfected with STAT1-eGFP plasmid. At 4 h after transfection, the cells were infected with PRRSV VR2385 at 1 MOI. IFN-α was added to the cells in a final concentration of 1000 u/ml at 16-24 hpi. The cells were fixed 1 h after IFN-α treatment with 2% paraformaldehyde and mounted onto slides using SlowFade Gold anti-fade reagent containing 4′6′-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA). The STAT1-eGFP distribution in the cells was visualized by confocal fluorescence microscopy.

To determine STAT1-eGFP nuclear translocation in HeLa cells after IFN treatment, the cells were co-transfected with plasmids of STAT1-eGFP and NSP1β-RFP. At 24 h after transfection, the cells were treated with IFN-α at 300 u/ml for 1 h and fixed for confocal microscopy as described above.

Western blot analysis. Cells were lysed in Laemmli sample buffer. The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis as described previously (41). Briefly, the cell lysates were resolved in 12% polyacrylamide gel. The separated proteins were then transferred onto a nitrocellulose membrane and probed with rabbit anti-STAT1 antibody (Santa Cruz
Biotechnology, Santa Cruz, CA). Specific reactions were detected using goat anti-rabbit 
IgG conjugated with horseradish peroxidase (Sigma) and revealed using a 
chemiluminescence substrate. The chemiluminescence signal was recorded digitally by a 
ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA). β-tubulin was 
detected on the same blot membrane to normalize protein loading. Digital signal 
acquisition and analysis were conducted using the Quantity One Program, Version 4.6 
(Bio-Rad). The expression of other proteins was detected through corresponding 
antibodies to STAT2, histone H1, phospho-STAT2 (tyr690, hereinafter named STAT2-
Y690) (Santa Cruz Biotechnology), and phospho-STAT1 (tyr701, hereinafter named 
STAT1-Y701) (Millipore, Billerica, MA). Antibodies against STAT1-Y701 and STAT2-
Y690 were used to detect IFN-activated phosphorylation of STAT1 and STAT2 after 
IFN-α stimulation. Convalescent antiserum from a pig inoculated with PRRSV VR2385 
was used to detect PRRSV proteins in the lysate of the virus-infected cells.

**Subcellular fractionation.** Nuclear fraction was extracted from MARC-145 cells using 
the CelLytic™ NuCLEAR™ Extraction Kit (Sigma). The cells were inoculated with 
VR2385 at 1 MOI, and at 16-24 hpi, treated with IFN-α at 1000 u/ml for 1 h. Cell 
collection, lysis, and subcellular fractionation were done following the manufacturer’s 
instructions. The nuclear and cytoplasmic fractions were subjected to Western blotting 
analysis. Antibodies against β-tubulin and histone H1 were used to assess the 
fractionation. Separation of cytoplasmic and nuclear fractions of HEK293 cells were 
done similarly.

**Immunoprecipitation (IP).** MARC-145 cells were infected with VR2385 and treated 
with IFN-α as described above. The cells were lysed with lysis buffer (50 mM Tris pH
7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with a protease inhibitor cocktail (Sigma). The lysate was clarified by centrifugation at 14000 xg for 5 min at 4°C. Antibodies against STAT1 or STAT2 (Santa Cruz) were added to the supernatant. IP with protein G agarose (KPL Inc, Gaithersburg, MD) was done following the manufacturer’s instructions. The IP samples with antibody against STAT1 were subjected to Western blotting analysis with STAT2-Y690 antibody. The IP samples with antibody against STAT2 were subjected to Western blotting analysis with STAT1-Y701 antibody.

**RNA isolation and real-time RT-PCR.** Total RNA was isolated from HEK293, MARC-145 and PAM cells with TRIzol® Reagent (Invitrogen) following the manufacturer’s instructions. Real-time PCR primers used in this study are listed in Table 1. Reverse transcription of RNA and real-time quantitative PCR were conducted as previously described (25, 26). Transcripts of ribosomal protein L32 (RPL32) or β-actin were also amplified from the samples of PAM and CRL or HEK293 cells, respectively, and used to normalize the total amount of input RNA. Relative transcript levels were quantified by the $2^{-\Delta\Delta CT}$ method (16) and shown as relative fold of change in comparison with mock-treated control.

**Statistical analysis.** The significant differences of cellular RNA level between the groups of IFN-treated cells in the presence or absence of virus infection were assessed by Student $t$-test. A two tailed $P$-value of less than 0.05 was considered significant.
Results

**PRRSV interferes with IFN-α induction of ISG gene expression.** Type I IFN signaling leads to an elevated gene expression of a variety of cellular genes, including ISG15 and ISG56 (8, 32, 33). To examine the effect of PRRSV replication on type I IFN signaling, we inoculated MARC-145 cells with PRRSV VR2385 and treated the cells with IFN-α at 16-24 hpi. Quantitative RT-PCR was conducted to assess the transcript levels of ISG15 and ISG56 1 h after IFN-α treatment. The transcript levels of ISG15 and ISG56 in IFN-treated cells were increased by 5.5- and 43.2-fold, respectively, in comparison with mock-treated cells (Fig. 1A). In PRRSV-infected MARC-145 cells after IFN stimulation, the transcripts of ISG15 and ISG56 were 3- and 2.6-fold, respectively, lower than those in the mock-infected cells (Fig. 1A). The expression of the two genes in PRRSV-infected cells without IFN-α treatment was similar to that of mock-inoculated cells, indicating that PRRSV infection did not affect the basal level expression of these genes.

To test whether the viral replication is needed for the interference effect, we inactivated the PRRSV virions by UV illumination and verified the viral inactivation by the lack of viral replication 72 h after inoculating the cells. When the UV-inactivated virus was used to inoculate the cells, the gene expression of the ISG genes after IFN-α treatment was similar to that of the mock-inoculated cells. No difference in the transcript levels of the two ISG genes was detected between the cells receiving mock inoculum or UV-inactivated PRRSV after the IFN-α treatment (Fig. 1B). The result indicates that active PRRSV replication was needed for the transcript reduction of the two ISG genes after IFN stimulation.
When incubation of the cells was extended to 15 h after IFN-α treatment, the transcript levels of ISG15 and ISG56 were increased to 234- and 290-fold, respectively, in comparison with mock-treated cells (Fig.1C). In comparison with IFN treatment of mock-infected cells, PRRSV-infected cells had a significantly lower expression of ISG15 and ISG56 after IFN treatment by 10- and 14-fold, respectively. The transcript levels of STAT1 and STAT2 were also assessed and no significant difference was noticed between cells with or without the IFN treatment (data not shown). Together, the data demonstrate that PRRSV infection of MARC-145 cells interferes with IFN stimulation of ISG15 and ISG56 gene expression.

Type I IFN signaling leads to an elevation of protein expression of a variety of IFN-responsive genes, including STAT2 (8, 32, 33). The MARC-145 cells in the presence or absence of PRRSV infection were treated with IFN-α for 8 h and harvested for Western blotting analysis. After IFN-α stimulation, STAT2 protein level in the IFN-treated cells increased considerably (Fig. 1D), which is consistent with STAT2 function as an ISG. However, STAT2 protein in VR2385-infected cells after IFN-α treatment remained at low basal level similar to mock-treated MARC-145 cells. The levels of STAT1 in all samples were similar, possibly because of high basal level STAT1 protein in the cytoplasm. When the IFN-treated cells were incubated for 24 h, the STAT2 protein level in mock-infected cells was much higher than that in the VR2385-infected cells (Fig. 1E). The STAT2 protein level in VR2385-infected cells was similar to that of mock-infected cells in the absence of IFN-α treatment. The STAT1 level in mock-infected cells was slightly increased at 24 h after IFN-α stimulation. A slight increase was also noticed in VR2385-infected cells without addition of IFN (Fig. 1E), which might be caused by
sample loading shown by a higher intensity of β-tubulin band in the last lane. These results indicate that PRRSV infection interfered with IFN-α signaling and thus, resulted in the reduction of downstream gene expression. Immunofluorescence assay with PRRSV N-specific monoclonal antibody was conducted to examine the rate of PRRSV-infected cells at 24 hpi. Positive staining was observed in over 95% cells by confocal microscopy (data not shown). The high percentage of PRRSV-infected cells indicated that the interference of IFN-induced STAT2 protein expression was due to PRRSV infection.

**PRRSV does not alter the IFN-induced phosphorylation of STAT1 and STAT2.**

STAT1 and STAT2 are key players in IFN-α-activated JAK/STAT signaling pathway (8, 32, 33). Phosphorylation of STAT1 and STAT2 is an early step in the pathway after type I IFNs bind to their receptors. To determine if PRRSV interferes with the IFN-induced activation of these two proteins, we tested the phosphorylation status of STAT1 and STAT2 in MARC-145 cells 1h after IFN-α treatment. The levels of phosphorylated STAT1 at tyrosine 701 (STAT1-Y701) and STAT2 at tyrosine 690 (STAT2-Y690) were greatly increased after IFN-α treatment (Fig. 2A). No difference was observed between the IFN-treated cells with or without PRRSV infection. In mock-treated cells, the phosphorylation of these two proteins was below detection level. The result showed that PRRSV replication did not change STAT1 and STAT2 phosphorylation status after IFN-α stimulation in comparison with mock-infected cells. To make sure that the PRRSV proteins were similarly expressed in the PRRSV-infected cells with or without IFN-α treatment, the cell lysates were subjected to Western blotting analysis with convalescent
pig antiserum. Both lanes showed similar band pattern and intensity of the PRRSV proteins (Fig. 2A).

To further confirm that the PRRSV replication has no effect on the IFN-α-activated phosphorylation of these two proteins, we harvested cells at 0.5, 2, and 8 h after IFN-α addition. The levels of STAT1-Y701 and STAT2-Y690 were highest 0.5 h after IFN addition (Fig. 2B). The level of STAT1-Y701 in cells 8 h after IFN-α addition decreased substantially to below detection level. In all time points, the levels of STAT1-Y701 and STAT2-Y690 in the PRRSV-infected MARC-145 cells were similar to those of mock-infected cells after IFN-α treatment (Fig. 2B). In PRRSV-infected and mock-treated cells, the STAT1-Y701 and STAT2-Y690 were below detection level. Therefore, we conclude that PRRSV infection does not affect the IFN-α-induced phosphorylation of STAT1 and STAT2.

The STAT1/STAT2/IRF9 heterotrimer formation is not altered. The IFN-induced activation of STAT1 and STAT2 results in the formation of STAT1/STAT2 heterodimers that further associate with IRF9 to form the mature IFN-stimulated-gene factor 3 (ISGF3) (8, 32, 33). Since the IFN-induced phosphorylation of STAT1 and STAT2 was not significantly changed in PRRSV-infected cells, we further analyzed the ISGF3 complex in MARC-145 cells by an experiment of immunoprecipitation followed by Western blotting analysis. IP with STAT1 antibody and then blotting with antibody against STAT2-Y690 showed the presence of the phosphorylated STAT2 in the samples from the IFN-treated cells regardless of PRRSV infection (Fig. 3). Similarly, IP with STAT2 antibody and then blotting with antibody against STAT1-Y701 showed the presence of the phosphorylated STAT1 in the two IFN-treated samples. In contrast, no specific
signal was detected in samples from cells without IFN stimulation. The result indicated that the ISGF3 heterotrimer formation after IFN treatment in VR2385-infected cells is not significantly affected.

**PRRSV interferes with ISGF3 nuclear translocation.** The IFN-induced ISGF3 complex translocates to the nucleus to initiate gene transcription by binding to interferon stimulated response elements (ISREs). To examine the translocation step of JAK/STAT signaling pathway in PRRSV-infected cells, we transfected MARC-145 cells with pEGFP-STAT1 and subsequently infected the cells with VR2385. At 24 hpi, the cells were treated with IFN-α for 1 h, fixed, and mounted for confocal microscopy. In the IFN-treated cells without PRRSV infection, the major portion of STAT1-eGFP was translocated to the nucleus (Fig. 4A). However, the majority of STAT1-eGFP protein remained in the cytoplasm of PRRSV-infected cells after IFN-α treatment. This result indicated that VR2385 inhibits STAT1 nuclear translocation.

To confirm the observation, we conducted nuclear and cytoplasmic fractionation of the cells after IFN treatment. Antibodies against STAT1-Y701 and STAT2-Y690 were used to detect the presence of the phosphorylated proteins in the two fractions. After IFN-α treatment of mock-infected cells, more STAT1-Y701 and STAT2-Y690 were found in the nuclear than in the cytoplasmic fraction (Fig. 4B), as expected. In contrast, in PRRSV-infected cells after IFN stimulation, more STAT1-Y701 and STAT2-Y690 were detected in the cytoplasmic than in the nuclear fraction. The absence of either β-tubulin in nuclear fraction or histone H1 in cytoplasmic fraction verified a successful subcellular fractionation. Densitometry analysis of the digital images of the blotting results showed that 76% of STAT1-Y701 and 78% STAT2-Y690 were detected in the nuclear fraction of
mock-infected cells, while only 14% and 30% of the two proteins, respectively, were detected in the nuclear fraction of VR2385-infected cells (Fig. 4C). The remaining portions of the phosphorylated proteins remained in the cytoplasmic fractions. The fractionation result was consistent with the observation from confocal microscopy and indicated that PRRSV infection strongly blocks nuclear translocation of ISGF3 (STAT1/STAT2/IRF9) complex.

**NSP1β inhibits the IFN-induced expression of ISG genes by blocking nuclear translocation of STAT1.** Since VR2385 inhibits the type I IFN signaling, we wished to determine which PRRSV protein is responsible for the effect. NSP1α and NSP1β were selected for the analysis as recent studies indicate their roles in the IFN pathway (3, 6). To determine if NSP1α or NSP1β of VR2385 can inhibit IFN signaling, we cloned NSP1α and NSP1β into a pCMVTag vector, separately, and transfected HEK293 cells. The gene expression of ISG15 and ISG56 in HEK293 cells 12 h after IFN-α stimulation was determined by real-time PCR. Results showed that the cells with NSP1β expression had 4- and 7-fold lower ISG15 and ISG56 transcripts, respectively, than cells transfected with an empty vector (Fig. 5A). The cells with NSP1α expression had similar levels of ISG15 and ISG56 transcripts to the cells transfected with an empty vector. The results indicate that NSP1β inhibited IFN-stimulated ISG gene expression in the cells.

To determine the mechanism of the NSP1β inhibition of the IFN signaling, we analyzed the phosphorylation of STAT1 and STAT2. After IFN-α stimulation, the HEK293 cells with NSP1α or NSP1β expression had similar levels of STAT1-Y701 and STAT2-Y690 to those in cells transfected with an empty vector (Fig. 5A), which indicated that both proteins have no effect on IFN-activated phosphorylation of STAT1.
Since NSP1β does not affect the IFN-stimulated phosphorylation of STAT1, we speculated that it might interfere with the STAT1 nuclear translocation, as VR2385 in MARC-145 cells. To test this speculation, we transfected HeLa cells with STAT1-eGFP and NSP1β-RFP plasmids. HeLa cells were used in this experiment as they attach cover glass better than HEK293 cells. At 24 h after transfection, the cells were treated with IFN-α for 1 h and observed under confocal microscopy. In cells expressing both STAT1-eGFP and NSP1β-RFP, majority of STAT1 remained in cytoplasm (Fig. 5C), indicating that NSP1β inhibits the STAT1 nuclear translocation.

To confirm the observation, we conducted subcellular fractionation of HEK293 cells to determine the distribution of phosphorylated STAT1. In HEK293 cells with NSP1β expression, majority of STAT1-Y701 remained in the cytoplasm 1h after IFN stimulation, while cells transfected with the empty vector had the majority of STAT1-Y701 in the nucleus (Fig. 5D). NSP1β was detected in both cytoplasmic and nuclear fractions. The results indicated that NSP1β blocks the IFN-stimulated nuclear translocation of ISGF3.

In PAM cells, VR2385 interferes with IFN-α signaling similarly to that observed in MARC-145 cells. Since PAM cells are the major targets for PRRSV infection in pigs, PRRSV infection of PAMs was conducted for greater physiological relevance to the viral infection in its natural host. We tested whether VR2385 can have a negative effect on IFN signaling in PAMs as observed in MARC-145 cells. Primary PAM cells were infected with VR2385 at 0.05 MOI for 15 h and then treated with IFN-α for 8 h. Quantitative RT-PCR showed that the IFN treatment of mock-infected cells increased transcript levels of ISG15 and IFI56 (the porcine gene equivalent to ISG56) by 223- and
637-fold, respectively (Fig. 6A). Upon IFN-α stimulation, the VR2385-infected PAMs had a significantly lower levels of the transcripts of ISG15 and IFI56 by 5.1- and 4.6-fold, respectively, than those in mock-infected cells. We concluded that the PRRSV VR2385 interferes with the expression of IFN-induced genes in PAM cells after IFN-α stimulation.

STAT2 protein level in the PAMs after PRRSV infection and IFN treatment was also assessed. Similar to MARC-145 cells, the STAT2 protein level in mock-infected PAMs after IFN-α stimulation increased significantly (Fig. 6B). The STAT2 protein in VR2385-infected PAMs after IFN treatment remained at basal level, similar to that of VR2385-infected cells without addition of IFN. The result indicated that VR2385 blocks the IFN-induced STAT2 elevation in PAMs. VR2385 infection had no detectable effect on the basal level of STAT2 in PAM cells without external IFN.

To assess the activation of JAK/STAT signaling pathway in PAMs after IFN treatment, we detected phosphorylated STAT1 and STAT2. The levels of STAT1-Y701 and STAT2-Y690 in PAMs with VR2385 infection were similar to those in mock-infected PAMs (Fig. 6C). The result suggests that the VR2385 does not alter the phosphorylation of both STAT1 and STAT2 in PAM cells receiving IFN treatment.

Effect of the low virulent PRRSV vaccine strain on type I IFN signaling. Ingelvac PRRS MLV is a licensed low virulent vaccine strain. We speculated that MLV had lower inhibitory effect on the IFN signaling than the virulent VR2385 strain. PAM cells were infected with MLV and then treated with IFN-α similarly as the experiment described above for VR2385. The transcript levels of ISG15 and IFI56 in the MLV-infected PAMs receiving IFN treatment were increased by 50- and 234-fold, respectively (Fig. 7A),
which were slightly lower than, but had no significant difference from, mock-infected PAMs after IFN stimulation. Interestingly, the MLV infection of PAMs receiving no external IFN resulted in an increase of ISG15 and IFI56 transcripts by 49- and 211-fold, respectively, which were similar to those of MLV-infected PAMs receiving external IFN stimulation. The result indicates that MLV had no effect on IFN signaling in PAMs.

The viral yields of MLV and VR2385 in the PAMs were also determined by real-time RT-PCR to ensure viral infection of the cells. The MLV and VR2385 had viral genomic RNA copy numbers of 6 and 7 Log_{10}/ml in cell culture supernatant, respectively, which suggested that VR2385 had higher viral replication than MLV. The result was consistent with the data of ISG gene expression, which showed high level ISG15 and IFI56 transcripts in MLV-infected PAMs. The ISG gene expression might correlate with antiviral responses and result in reduction of MLV replication.

To determine the effect of MLV on IFN-activated STAT2 expression, we detected STAT2 protein level by Western blotting. Similar to mock-infected cells receiving IFN treatment, the MLV-infected PAMs receiving IFN treatment had an increased level of STAT2 protein (Fig. 7B). A sample of VR2385-infected PAMs was included as a control and had low STAT2 level. It is interesting to note that the addition of the external IFN to MLV-infected PAMs did not lead to change in STAT2 protein level. The result was consistent with the real-time PCR data showing increased transcripts of ISG15 and IFI56 in the MLV-infected PAMs without the addition of IFN. These results indicated that the MLV infection activates the IFN signaling in PAMs in the absence of external IFN and that the addition of IFN has no additional effect on the expression of the IFN-induced genes in MLV-infected PAMs.
To further examine the difference in IFN signaling in the PAMs receiving MLV or VR2385, we determined transcripts of IFN-induced chemokine ligand 10 (CXCL10/IP10) (35), chemokine ligand 2 (CCL2, also known as monocyte chemotactic protein-1 (MCP-1)) (30), and three antiviral genes: myxovirus (influenza virus) resistance 1 (MX1) (11), 2′-5′-oligoadenylate synthetase 2 (OAS2) (42) and ribonuclease L (RNase L) (4). Results showed that, after IFN stimulation, PAMs with VR2385 infection had a significant 5-fold decrease in CXCL10 expression compared to mock-infected cells, while cells with MLV infection had similar level of CXCL10 to mock-infected cells (Fig. 7C). The transcript levels of CCL2, MX1, OAS2 and RNase L in PAMs in the presence of VR2385 infection were 3.9-, 7.3-, 2.4-, and 3.4-fold, respectively, lower than mock-infected cells (Fig. 7D). MLV-infected PAMs had similar levels of these transcripts to those in mock-infected cells. These results are consistent with our data above, indicating that the virulent strain VR2385 can evade IFN-activated antiviral responses.

As VR2385 NSP1β inhibits IFN signaling, we wondered if MLV NSP1β has any effect. The NSP1α and NSP1β of MLV were cloned into pCMVTag vector and expression of these two proteins from the recombinant plasmids were confirmed by Western blotting. HEK293 cells were transfected with the recombinant plasmids and treated with IFN-α. The transcript level of ISG56 in the cells expressing either NSP1α or NSP1β of MLV was similar to the cells transfected with empty vector (Fig. 7E). The result indicated that the MLV NSP1α and NSP1β has no effect on IFN signaling and was consistent with the data above showing that MLV infection of PAM cells has no effect on IFN signaling.
Discussion

Dual infection of pigs with PRRSV and porcine respiratory coronavirus (PRCV) or swine influenza virus (SIV) causes a more severe respiratory disease and growth retardation than with PRRSV infection alone (38). Both PRCV and SIV infections can induce a high level of bioactive IFN-\(\alpha\) (37). How can PRRSV that is sensitive to pre-treatment with IFNs replicate in the pigs co-infected with PRCV or SIV? Our study provides a clue that PRRSV can interfere with type I IFN signaling. We found that PRRSV interferes with IFN-\(\alpha\) signaling in MARC-145 cells and PAMs via blocking nuclear translocation of the ISGF3 heterotrimers.

To determine the mechanism of the PRRSV interference of IFN signaling, several experiments were conducted to analyze the JAK/STAT signaling pathway. First, IFN-induced phosphorylation status of STAT1 and STAT2 was analyzed. We found that PRRSV does not affect the IFN-induced phosphorylation status of STAT1 and STAT2. After IFN treatment, both STAT1 and STAT2 undergo phosphorylation, form heterotrimers with IRF9, translocate to the nucleus, and then undertake dephosphorylation before being redistributed back to the cytoplasm (2, 32). The dephosphorylation rate of STAT2 in MARC-145 cells was lower than STAT1, as shown in Fig. 2B. Second, ISGF3 heterotrimers were assessed by IP and Western blotting analysis. We concluded that PRRSV infection does not alter the ISGF3 heterotrimer formation in MARC-145 cells receiving IFN treatment. The band in the lane of PRRSV-infected cells receiving IFN stimulation in Fig. 3 was weaker than that of mock-infected cells after IFN treatment. It may indicate there were more proteins in the latter cell lysate than the former.
one. This assay was designed to show the presence of the ISGF3 complex, but not serve as a quantitative assessment. We were unable to identify IRF9 in the IP pellets perhaps because IRF9 is a 48-kDa protein, which is located very close to the heavy chain of immunoglobulin G band in our Western blotting analysis, possibly masking the view of weak bands nearby. However, our data provide further evidence that VR2385 replication interferes with ISGF3 nuclear translocation.

Lastly, ISGF3 heterotrimer nuclear translocation was analyzed. We found that VR2385 infection blocks nuclear translocation of ISGF3 heterotrimers. The nuclear translocation of STAT1-eGFP after IFN treatment was blocked in VR2385-infected cells. The results of our subcellular fractionation assay suggest that interference of ISGF3 nuclear translocation leads to the inhibition of IFN-α signaling in the PRRSV-infected cells. The data in Fig. 4 showed a small portion of phosphorylated STAT1 and STAT2 present in the nuclei of VR2385-infected cells, which is consistent with the small scale elevation of ISG15 and ISG56 transcripts in the cells after IFN-α stimulation.

To identify which of the PRRSV protein(s) is responsible for blocking the ISGF3 nuclear translocation, we cloned NSP1α and NSP1β and expressed them in HeLa and HEK293 cells. Results from confocal microscopy and the subcellular fractionation assay clearly showed that NSP1β blocks STAT1 nuclear translocation, which is consistent with a recent publication (6) showing overexpression of NSP1β in HEK293T cells blocks STAT1-GFP nuclear translocation. It was also shown that NSP1β inhibits IFN-activated phosphorylation of STAT1. In contrast, our results demonstrated that NSP1β does not affect IFN-induced phosphorylation of STAT1 and STAT2, which is consistent with our data that PRRSV infection of MARC-145 and PAM cells does not affect the IFN-
activated phosphorylation of these two proteins. This discrepancy might be caused by
different virus strain and IFN subtype used, but still needs further investigation. The
exact mechanism of NSP1β inhibition of STAT1 nuclear translocation is being studied.

After finding that PRRSV infection of MARC-145 cells interfered with IFN-α
signaling, we showed that VR2385 inhibited the IFN signaling in primary PAM cells.
PAMs are key sentinel cells in the respiratory system, and the primary target cells for
PRRSV infection in vivo. Our finding that VR2385 inhibited the type I IFN signaling in
PAMs has physiological relevance to PRRSV infection of pigs.

We further investigated PRRSV interference of IFN signaling in PAMs. IFN
treatment of MLV-infected PAM cells does not affect the expression of ISG15 and IFI56
transcripts and STAT2 protein. The possible reasons are that MLV has no effect on IFN
signaling or that the cells were less responsive to an external IFN due to priming from
MLV-induced endogenous IFN. Another interesting observation was that in the absence
of external IFN addition, the transcript levels of ISG15 and IFI56 and the level of STAT2
protein in the MLV-infected PAMs increased significantly in comparison with mock-
infected cells. We postulate that, 1) MLV has no interference in type I IFN production, or
2) MLV does not inhibit the positive feedback loop for induction of IFN. Our results are
more consistent with the latter speculation since slight increase in ISG gene expression
was detected in VR2385-infected cells. The MLV-mediated up-regulation of IFN-
stimulated genes is consistent with the low virulence nature of this strain. Real-time PCR
data further demonstrated that VR2385 inhibited the expression of IFN-inducible genes
in PAMs, including CXCL10, CCL2, MX1, OAS2, and RNase L, while MLV-infected
PAMs had similar levels to mock-infected cells. Reduction of expression of the
chemokines and antiviral genes in VR2385-infected cells is consistent with the virulent nature of this strain. The NSP1β of these two PRRSV strains function differently. The MLV NSP1β has no effect on IFN signaling, while VR2385 NSP1β inhibits IFN-activated antiviral responses.

The viral interference of type I IFN signaling can be beneficial for the viral replication and serves as an important mechanism for the virus to evade host innate immune response. Severe acute respiratory syndrome (SARS) coronavirus, which belongs to the same order (Nidovirales) as PRRSV, infects macrophages and interferes with the host innate immune response. Both of the ORF3b and ORF6 proteins of SARS virus inhibit the synthesis and signaling of type I IFNs (14), while ORF6 protein alone blocks STAT1 nuclear translocation via sequestering nuclear import factor karyopherin-α2 (KPNA2) (9). We transfected HeLa and HEK293 cells with NSP1β and FLAG-tagged KPNA1, KPNA2, KPNA3 or KPNA4 plasmids. No change in the nuclear localization of the KPNA was observed and no interaction between NSP1β and any of the KPNA was detected. It seems that PRRSV employs a different mechanism to block the ISGF3 nuclear translocation. It is known that viruses use multiple independent mechanisms to inhibit the IFN response. For example, paramyxovirus V proteins bind to STAT2 and block ISGF3 nuclear accumulation (27, 29), Ebola virus VP24 binds to a nuclear localization signal receptor for phosphorylated STAT1 and blocks STAT1 nuclear accumulation (28), and rotavirus antagonizes IFN response by inhibiting nuclear translocation of STAT1 and STAT2 (10).

In summary, PRRSV VR2385 inhibits IFN-α signaling in MARC-145 and primary PAM cells by interfering with ISGF3 nuclear translocation. MLV infection of
PAMs can activate IFN signaling without addition of external IFN. The variable effect on IFN induction might be a contributing factor to the varying viral pathogenesis between the two PRRSV strains and have a biological relevance on PRRS vaccine design or improvement.
Acknowledgements

We are grateful to Dr. Joseph F. Urban at Human Nutrition Research Center, USDA, Beltsville, MD for his gift of the lung lavage of piglets. We thank Dr. Kay S. Faaberg at National Animal Disease Center, Ames, IA for her gift of Ingelvac PRRS MLV. M. Shen was partially supported by Shandong Bureau of Education. This project was supported by institutional funds from the University of Maryland.
References


Table 1. List of primers for real-time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5’ to 3’)</th>
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<tbody>
<tr>
<td>ISG15-F1</td>
<td>CACCGTGTTCATGAATCTGC</td>
</tr>
<tr>
<td>ISG15-R1</td>
<td>CTTTATTTCCGGCCTTGAT</td>
</tr>
<tr>
<td>ISG56-F1</td>
<td>CCTCCTTGGGTTCGTCTACA</td>
</tr>
<tr>
<td>ISG56-R1</td>
<td>GGCTGATATCTGGGTGCTTA</td>
</tr>
<tr>
<td>Actin-F1</td>
<td>ATCGTGCGTGACATTAAG</td>
</tr>
<tr>
<td>Actin-R1</td>
<td>ATTGCCAATGGTGATGAC</td>
</tr>
<tr>
<td>sISG15-F1</td>
<td>GGTGCAAAGCTTCAGAGACC</td>
</tr>
<tr>
<td>sISG15-R1</td>
<td>GTAGCCAGACCTCATAGGC</td>
</tr>
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<td>sIFI56-F1</td>
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<td>sCCL2-R1</td>
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</tr>
<tr>
<td>85NSP1R2</td>
<td>CCGCTCGAGTTAGCCGTCACCCTGTCG</td>
</tr>
</tbody>
</table>

a. F1: forward primer, R1: reverse primer
b. The “s” before a primer indicates the primer is for a porcine gene.
c. The “85” before a primer indicates the primer is based on sequences of PRRSV VR2385.
Figure legends

Fig. 1. PRRSV inhibits expression of IFN-stimulated genes in MARC-145 cells. A. Reduction of IFN-stimulated ISG15 and ISG56 transcripts detected by real-time RT-PCR. The cells were inoculated with PRRSV VR2385, incubated for 24 h, and then treated with IFN-α for 1 h. Significant differences between the two groups for each transcript are denoted by "●" and "●●●", which indicate $P < 0.05$ and $P < 0.01$, respectively. B. UV-inactivated PRRSV has no effect on IFN-stimulated expression of ISG15 and ISG56 detected by real-time RT-PCR. The cells were inoculated with PRRSV VR2385 or UV-inactivated VR2385, incubated for 24 h, and then treated with IFN-α for 1 h. C. Reduction of IFN-stimulated ISG15 and ISG56 transcripts in PRRSV-infected cells 15 h after IFN treatment. The cells were inoculated with PRRSV VR2385, incubated for 24 h, and then treated with IFN-α for 15 h. D. Inhibition of IFN-induced STAT2 protein expression by Western blotting analysis. The cells were infected with PRRSV VR2385 or mock-infected for 24 h, and then treated with IFN-α for 8 h. The same blot was incubated with β-tubulin antibody as a protein loading control. E. Western blotting analysis of STAT1 and STAT2 from the cells 24 h after IFN-α treatment in the presence or absence of PRRSV infection.

Fig. 2. Phosphorylation status of STAT1 and STAT2 in PRRSV-infected MARC-145 cells after IFN-α stimulation. A. Western blotting analysis with antibodies against STAT1-Y701 and STAT2-Y690. The cells were infected with PRRSV VR2385 or mock-infected and at 24 hpi treated with IFN-α for 1 h. The same blot was incubated with β-
tubulin antibody as a protein loading control. Convalescent pig antiserum was used to
blot the membrane to show the PRRSV proteins in the lysate of PRRSV-infected cells.
Positions of pre-stained molecular weight markers are shown on the left. B. Western
blotting analysis with antibodies against STAT1-Y701 and STAT2-Y690 in the VR2385-
infected or mock-infected cells 0.5, 2, and 8 h after IFN-α treatment.

Fig. 3. IP detection of STAT1/STAT2 heterodimer formation in MARC-145 cells after
IFN-α treatment. The cells were infected with PRRSV VR2385 or mock-infected and at
24 hpi treated with IFN-α for 1 h. The upper image is IP with STAT1 antibody and
Western blot with antibody against STAT2-Y690. The lower image is IP with STAT2
and Western blot with antibody against STAT1-Y701.

Fig. 4. Blockage of nuclear translocation of ISGF3 hetrotrimers in PRRSV-infected
MARC-145 cells. A. PRRSV inhibits nuclear translocation of STAT1-eGFP observed by
confocal microscopy. The cells were transiently transfected with STAT1-eGFP plasmid
and inoculated with VR2385 4 h later. At 24 hpi, the cells were treated with IFN-α and
fixed 1 h later. B. Phosphorylated STAT1 and STAT2 in nuclear and cytoplasmic
fractions. Subcellular fractionation of the cells 1 h after IFN treatment and Western
blotting analysis were conducted. The same blot was incubated with antibodies against β-
tubulin and histone H1 as controls for loading and fractionation. C. Densitometry
analysis of the digital image shown in “B”. The band intensity of each fraction is shown
as relative percentage of sum density of corresponding cytoplasmic and nuclear fractions
of the same treatment. Normalization for cytoplasmic and nuclear fractions was done
with tubulin and histone H1, respectively.

Fig. 5. PRRSV NSP1β protein inhibits IFN signaling. A. NSP1β inhibits IFN-stimulated
expression of ISG15 and ISG56 in HEK293 cells. The cells were transiently transfected
with NSP1α and NSP1β plasmids or an empty vector (EV) and, 48 h after transfection,
treated with IFN-α at 300 u/ml. The cells were harvested 12 h after IFN treatment.
Significant differences in ISG15 and ISG56 transcripts between the two groups of NSP1β
and empty vector are denoted by ‘*’, which indicates P < 0.05. B. NSP1α and NSP1β
have no effect on IFN-induced phosphorylation of STAT1 in HEK293 cells. The cells
were harvested for STAT1-Y701 detection 1 h after IFN treatment. C. NSP1β inhibits
nuclear translocation of STAT1-eGFP in HeLa cells observed by confocal microscopy.
The cells were transiently transfected with STAT1-eGFP and NSP1β-RFP plasmids. At
24 h after transfection, the cells were treated with IFN-α at 300 u/ml for 1 h. D. NSP1β
inhibits STAT1 nuclear translocation. HEK293 cells were transiently transfected with
NSP1β plasmid or an empty vector (EV) and, 48 h after transfection, treated with IFN-α
at 300 u/ml for 1 h. Subcellular fractionation of the cells and Western blotting analysis
were conducted to detect phosphorylated STAT1 in nuclear and cytoplasmic fractions.
The same blot was incubated with antibodies against NSP1β, β-tubulin, and histone H1 as
controls for loading and fractionation. EV: empty vector; 1α: NSP1α; 1β: NSP1β.

Fig. 6. VR2385 interferes with IFN signaling in PAMs. A. Real-time RT-PCR detection
of ISG15 and IFI56 from PAMs 8 h after IFN-α treatment in the presence or absence of
VR2385 infection. Significant differences between the two IFN-treated groups are denoted by "*", which indicates \( P < 0.05 \). B. Western blot detection of STAT2 from PAMs 8 h after IFN-\( \alpha \) treatment in the presence or absence of VR2385 infection. C. Western blotting analysis with the antibodies against phosphorylated STAT1 and STAT2 from PAM cells 1 h after IFN-\( \alpha \) treatment in the presence or absence of VR2385 infection.

Fig. 7. Effect of PRRSV MLV on IFN signaling in PAMs. A. Real-time RT-PCR detection of ISG15 and IFI56 from PAMs 8 h after IFN-\( \alpha \) treatment in the presence or absence of PRRSV infection. Significant differences in the transcript levels between the IFN-treated groups or between the no-IFN groups are denoted by "*" and "**", which indicate \( P < 0.05 \) and \( P < 0.01 \), respectively. B. Western blotting analysis of STAT2 in PAMs 8 h after IFN-\( \alpha \) treatment in the presence or absence of PRRSV infection. Samples of mock-treated PAM cells were included as controls. VR: VR2385. C. Real-time RT-PCR detection of CXCL10 transcript in PAMs 8 h after IFN-\( \alpha \) treatment in the presence or absence of PRRSV infection. Significant difference between VR2385-infected and mock-infected cells is denoted by "*", which indicates \( P <0.05 \). D. Real-time RT-PCR detection of CCL2, MX1, OAS2, and RNase L transcripts in PAMs 8 h after IFN-\( \alpha \) treatment in the presence or absence of PRRSV infection. Significant differences between virus-infected and mock-infected cells are denoted by "*" and "**", which indicate \( P <0.05 \) and \( P < 0.01 \), respectively. E. Real-time RT-PCR detection of ISG56 from HEK293 cells transfected with MLV NSP1\( \alpha \) and NSP1\( \beta \) plasmids, or empty vector pCMVTag. At 48 h after the transfection, the cells were treated with IFN-\( \alpha \) at 300 u/ml.
for 12 h. No significant difference in ISG56 transcript level between the samples was detected.
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Porcine Reproductive and Respiratory Syndrome Virus Inhibits Type I Interferon Signaling by Blocking STAT1/STAT2 Nuclear Translocation

Deendayal Patel, Yuchen Nan, Meiyan Shen, Krit Ritthipichai, Xiaoping Zhu, and Yan-Jin Zhang

Molecular Virology Laboratory and Immunology Laboratory, VA-MD Regional College of Veterinary Medicine and Maryland Pathogen Research Institute, University of Maryland, College Park, Maryland

Volume 84, no. 21, p. 11045–11055, 2010. Page 11052, Fig. 7A: The series label “ISG56” should read “ISG15.”