RESEARCH REPORT
SWINE HEALTH

Title: **Mechanism of PRRSV inhibition of interferon-mediated antiviral response – NPB #10-118**

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Industry Summary: The objective of this project was to study the effect of PRRSV infection on interferon (IFN) signaling and determine the mechanism of the effect. Type I IFNs, such as IFN-α and -β, are critical to innate immunity against viruses and play important roles in the modulation of adaptive immunity. In this study, different PRRSV strains were compared for their effects in blocking the activity of type I IFNs, the PRRSV proteins were screened for their roles in the inhibition, and cellular proteins that PRRSV targets to dampen the host antiviral response were determined. We found that different PRRSV strains inhibit IFN-activated antiviral response at variable levels. Almost all strains tested inhibited the IFN signaling in MARC-145 cells, but two strains including MLV had much less effect in primary porcine alveolar macrophages (PAMs) than the other strains. Screening of the PRRSV structural and non-structural proteins identified several of them playing a role in the blocking of IFN signaling. A cellular protein, importin-α5 transporting proteins from cytoplasm into nucleus, was found to be the PRRSV target in inhibiting the IFN-mediated signal transduction. These results indicate that PRRSV has complex mechanisms in antagonizing the IFN-mediated antiviral response and different strains may use a whole or partial set of the means to accomplish the goal. This information will be helpful in designing new or improving current vaccines to combat PRRS. For further information, please contact Dr. Zhang at zhangyj@umd.edu.

Keywords: PRRSV, interferons (IFNs), innate immunity, IFN signaling. IFN-activated JAK/STAT signaling, karyopherin-α1 (KPNA1), importin-α5, KPNA1

Scientific Abstract: This should be a scientific description limited to one page in length to describe your project and its results.

Porcine reproductive and respiratory syndrome virus (PRRSV) interferes with interferon (IFN)-activated antiviral response. In this study, different PRRSV strains were compared for their effects on IFN signal transduction pathway. One strain of genotype 1 PRRSV (LeLystad) and six strains of genotype 2 (VR-2385, Ingelvac PRRS MLV, VR-2332, NVSL, A2MC2 and MN-184) were used in this experiment. Compared to

uninfected MARC-145 cells, all of the strains tested except A2MC2 and MN-184 had much lower protein level of IFN-stimulated gene 56 (ISG56) and signal transducer and activator of transcription 2 (STAT2) post-IFN treatment. Infection of the cells with VR-2385, MLV, VR-2332 and NVSL led to significant reduction of the transcript level of ISG15 and ISG56. In primary porcine alveolar macrophages (PAMs), all the strains except MLV and NVSL inhibited IFN-induced elevation of STAT2 protein. To determine which PRRSV protein(s) are responsible for the inhibition of the IFN signaling, individual proteins of VR-2385 were cloned for overexpression. IFN signaling luciferase reporter assay showed that in addition to nsp1β, GP3, GP4 and N proteins inhibited the IFN-activated reporter expression. Nsp1β protein was selected for further analysis and comparison between the different PRRSV strains for their effects on the IFN-activated JAK/STAT signaling. Nsp1β from these strains were cloned into an expression vector and their effect on IFN signaling was compared. Overexpression of all the nsp1β plasmids except MLV led to inhibition of the IFN reporter expression. This indicates that nsp1β from all the strains except MLV has inhibitory effect on IFN signaling. Our findings delineate the different effects of these strains on IFN-activated signal transduction.

Type I IFNs induce the expression of IFN-stimulated genes by activating phosphorylation of both the STAT1 and STAT2, which form heterodimers, interact with IRF9 and translocate to the nucleus in heterotrimers (ISGF3). PRRSV nsp1β was found to block the nuclear translocation of ISGF3 complex. We further discovered that nsp1β induced degradation of karyopherin-α1 (KPNA1, also known as importin-α5). KPNA1 is known to mediate import of ISGF3 from cytoplasm to the nucleus. Overexpression of nsp1β led to a reduction of KPNA1 but had little effect on its transcript level. Treatment of the nsp1β-transfected cells with proteasome inhibitor MG132 restored KPNA1 protein level. Presence of nsp1β led to elevation of KPNA1 ubiquitination and a shortening of its half-life. Analysis of nsp1β deletion constructs showed that the N-terminal domain of nsp1β was involved in the ubiquitin-proteasomal degradation of KPNA1. Infection of MARC-145 cells by PRRSV strain VR-2332 and VR-2385 led to a reduction of KPNA1, while a low virulent strain MLV had little effect. These results indicate that nsp1β blocks ISGF3 nuclear translocation by way of stimulating KPNA1 degradation.

Introduction: An overview of the researchable question and its importance to producers.

PRRS causes an estimated $560 million in losses per year to the swine industry in the USA alone (22). The causative agent of this disease is PRRSV, an enveloped, single-stranded and positive-sense RNA virus (2, 18). Current management strategies and commercial live or autogenously killed vaccines have generally proven inadequate for long-term control of PRRS. Modified live virus vaccine has been used widely to prevent the virus infection; however, outbreaks of PRRS resulting from virus strains nearly identical in sequence to the vaccine strain were reported (5, 23, 30). Outbreaks of atypical or acute PRRS in vaccinated pigs have raised serious concern about the efficacy and safety of the current vaccines (15, 17), which demonstrates the need to define PRRSV protective immune response and develop novel vaccines.

PRRSV seems to inhibit synthesis of type I interferons (IFNs) in infected pigs (1, 6). IFNs could not be detected in the lungs of pigs in which PRRSV actively replicated. PRRSV infection of PAMs and MARC-145 cells *in vitro* leads to very low IFN-α expression (1, 21). The suppression of innate immunity can be an important contributing factor to the PRRSV modulation of host immune responses. PRRSV infection of pigs leads to delayed production and low titer of neutralizing antibodies (13), as well as weak cell-mediated immune response (34).

Type I IFNs, including IFN-α, -β and -λ, are critical to innate immunity against viral infections and have an important role in the stimulation of adaptive immune response (10, 33). The activation of IFN signaling leads to the induction of antiviral responses. The signaling of type I IFNs is initiated after they bind to their receptors on the cell surface (9, 29, 31). The receptor binding activates Janus kinase (JAK), which then phosphorylates both the STAT1 and STAT2. The phosphorylated STAT1 and STAT2 form heterodimers, which interact with

interferon regulatory factor 9 (IRF9) and form heterotrimers, also known as interferon-stimulated gene factor 3 (ISGF3). Translocation of ISGF3 into the nucleus followed by binding to consensus DNA sequence leads to expression of IFN-stimulated genes (ISGs). PRRSV inhibits the IFN-activated JAK/STAT signal transduction and expression of ISGs in both MARC-145 and PAM cells (24). The nuclear translocation of ISGF3 is blocked in the PRRSV-infected cells (24).

The genome of PRRSV is about 15 kb in length with nine open reading frames (ORFs) (8, 20). ORFs 1a and 1b comprise 80% of the viral genome and are predicted to encode viral enzymes for RNA synthesis, including polymerase, protease, helicase and so on. The polypeptides from the two ORFs are processed into 12 non-structural proteins (nsps) in infected cells. 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 (16, 19). PRRSV can be propagated *in vitro* in the epithelial-derived monkey kidney cells MARC-145 (11) and in primary culture of porcine pulmonary alveolar macrophages (PAMs). PAMs are main target cells for PRRSV during its acute infection of pigs (28).

PRRSV can evade IFN-activated antiviral response by suppressing IFN-signaling, in addition to inhibiting production of type I IFNs. The effect of different strains on the activity of IFNs was speculated to be variable. In addition to nsp1β, the viral proteins that play a role in the inhibition were not known. In this project, we defined the effect of different PRRSV strains on IFN signaling and examined the mechanisms of the effect. Our findings showed that different PRRSV strains have variable effects in PAM and MARC-145 cells; several viral proteins are able to play a role in the inhibition; and nsp1β blocks the IFN signaling by reducing importin-α5. This study provides useful information that will be helpful in vaccine improvement and PRRS control.

Objectives: From your research proposal.

1. *To examine the effect of different PRRSV strains on IFN signaling*

   Research questions to be answered: Is there any difference between PRRSV strains in their effects on IFN signaling? Does low virulent strain inhibit the IFN-activated antiviral response?

2. *To identify the structural components of PRRSV that cause inhibition of IFN signaling*

   Research questions to be answered: Which PRRSV gene(s) is responsible for inhibition of IFN signaling? Is there any difference between PRRSV strains?

3. *To determine the mechanism of PRRSV-mediated inhibition of IFN signaling*

   Research questions to be answered: What is the cellular protein(s) that PRRSV interacts and causes the inhibition of IFN signaling? What is the mechanism for the inhibition?

Materials & Methods: This section should include experimental design, methods and procedures used, number of animals, etc.

Cells and virus: MARC-145, HEK293, and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum. Porcine alveolar macrophages (PAMs) were isolated from 3-4-week-old healthy piglets and were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum. PRRSV strain VR2385, VR-2332, Ingelvac PRRS MLV, NVSL, Lelystad, A2MC2, and MN184 were used in this study. For virus titration, 10-fold series of dilutions were made and tested on the monolayer MARC-145 cells. Indirect immunofluorescence assay with PRRSV monoclonal antibody was conducted 36 h post inoculation. Median tissue culture infectious dose ($TCID_{50}$) was calculated according to the method of Reed and Munch.

MG132 (Sigma), a proteosome inhibitor, was used to treat cells at 10 μM final concentration for 6 h before harvested for further analysis. In order to determine the half-life of KPNA1, cycloheximide (Sigma) was added to cultured cells at a final concentration of 100 μg/ml to block protein translation. Next, the cells were harvested at the indicated time points for Western blotting.

Virus Infection and IFN treatment: MARC-145 cells were plated in 12-well cell culture plates with a density of 500,000 cells per well and grown overnight. The cells were inoculated with PRRSV at 1 multiplicity of infection (MOI). At 24 h post infection, the cells were treated with IFN-α at 1000 U/mL. At 12 h post IFN treatment, the cells were harvested by lysis in TRIzol for RNA isolation or Laemmli sample buffer for Western blot analysis. For PAM cells, plating was done in 12-well cell culture plates with a density of one million cells per well, followed by overnight preincubation. The cells were inoculated with PRRSV at 0.05 MOI and, at 12 h post infection, treated with IFN for 8 h before harvested for further analysis.

Plasmids: pEGFP-C1-STAT1, for STAT1-eGFP expression, was obtained from Addgene. The PRRSV nsp1α, nsp1β, nsp2, nsp3, nsp4, nsp5, nsp7, nsp9, nsp10, nsp11, nsp12, ORF2a, ORF2, ORF3, ORF4, ORF5a, ORF5b, ORF6 and ORF7 of VR-2385 were cloned into pCAGEN vector, separately. The nsp1βs of VR2385, NVSL, VR2332, MLV, MN184, Jiangxi-2 and Lelystad were cloned into VenusC1 vector, respectively. The resulting recombinant plasmids were confirmed by restriction enzyme digestion and DNA sequencing.

Confocal Fluorescence Microscopy: To determine STAT1-eGFP nuclear translocation in HeLa cells after IFN treatment, the cells were co-transfected with STAT1-eGFP and NSP1β-RFP plasmids. At 24 h after transfection, the cells were treated with IFN at 300 U/ml for 1 h and fixed by 1% paraformaldehyde for 15 min at room temperature for confocal microscopy.

RNA extraction, Reverse Transcription and Real-time PCR: Cells were lyzed in TRIzol Reagent (Invitrogen) and RNA extraction was performed by following manufacturer's instructions. 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, MARC-145, 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 (14) and shown as relative fold of change in comparison with mock-treated control.

Western blot analysis: Cells were lyzed in Laemmli sample buffer. The cell lysates were resolved in a 12% polyacrylamide gel. The separated proteins were then transferred onto a nitrocellulose membrane and probed with rabbit anti-STAT2 or -ISG56 antibodies. Specific reaction was 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). β-tubulin was detected on the same blot membrane to normalize protein loading. The expression of other proteins was detected through corresponding antibodies against FLAG, phospho-STAT2 (Tyr690), and phospho-STAT1 (Tyr701).

Immunoprecipitation (IP). IP was conducted as previously described (24, 27) with modifications. HEK293 cells were lysed with a lysis buffer (50mM Tris pH 7.4, 150 mM NaCl, 0.2 mM EDTA, 2 mM EGTA, 0.5% IGEPAL CA-630, 10% glycerol, 1 mM sodium vanadate) supplemented with a protease inhibitor cocktail (Sigma). The lysate was clarified by centrifugation at 14,000 xg for 5 min at 4ºC. The supernatant was transferred to a new tube and incubated with FLAG antibody (Sigma), then followed by incubation with protein G agarose (KPL Inc, Gaithersburg, MD). The IP samples were subjected to Western blotting with antibodies against pSTAT1, ubiquitin, and HA. IP with HA antibody and then Western blotting with FLAG antibody was similarly conducted. To detect ubiquitinated KPNA1, ubiquitin aldehyde (Boston Biochem Inc, Cambridge, MA), a specific inhibitor of ubiquitin C-terminal hydrolases, was included in the lysis buffer at a final concentration of 2.53 μM.

Statistical analysis. Differences in indicators between treatment groups, such as cellular RNA levels between infections of different virus strains, were assessed by Student *t*-test. A two tailed *P*-value of less than 0.05 was considered significant.

VIII. Results: Report your research results by objective.

Objective 1. To examine the effect of different PRRSV strains on IFN signaling.

Suppression of IFN signaling by different PRRSV strains.

We first examined whether there is difference between PRRSV strains in their capability in inhibiting IFN signaling in MARC-145 cells. PRRSV strain VR-2385, MLV, Lelystad, VR-2332, MN-184, NVSL and A2MC2 were used to infect MARC-145 cells. The cells were treated with IFN-α next day. Real time RT-PCR was performed. Results showed that VR-2385, MLV, and VR-2332 significantly reduced ISG15 transcript and that VR-2385, MLV, VR-2332 and NVSL significantly reduced ISG56 transcript (Fig. 1). A2MC2 and MN184 had minimal effect on the IFN-stimulated gene expression.

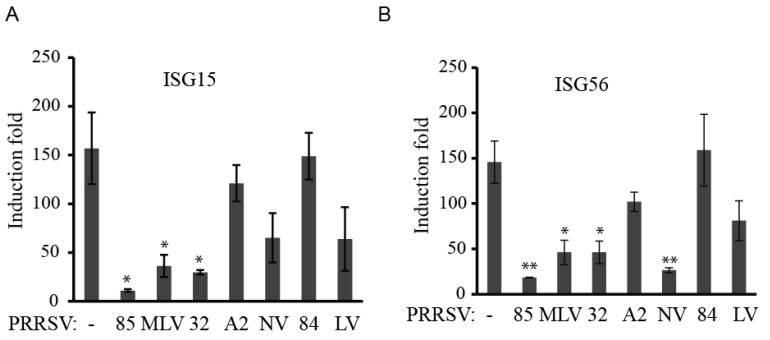

Fig.1. PRRSV inhibits expression of IFN-stimulated genes in MARC-145 cells. The cells were infected with different strains of PRRSV at one MOI. At 48 hpi, the cells were treated with IFN-α and, 12 h later, harvested for further analysis. A. Level of ISG15 transcripts detected by RT-qPCR. Induction folds are shown in comparison with mock-treated cells. Error bars represent variation of repeated experiments. Significant differences from uninfected cells are denoted by "*" , which indicates $P < 0.05$. 85: VR-2385; MLV: Ingelvac PRRS MLV; 32: VR-2332; A2: A2MC2; NV: NVSL; 84: MN-184; and LV: LeLystad. B. Level of ISG56 transcripts detected by RT-qPCR. Significant differences from uninfected cells are denoted by "*" and "**", which indicates $P < 0.05$ and $P < 0.01$, respectively.

To determine protein levels of STAT2 and ISG56 in the MARC-145 cells after IFN treatment, we conducted Western blotting. STAT2 and ISG56 levels were reduced in MARC-145 cells infected with MLV, LeLystad, VR-2332, VR-2385, and NVSL, while less change was observed for cells infected with MN184 and A2MC2 (Fig. 2). The result indicates that all the PRRSV strains tested except MN184 and A2MC2 inhibit the activity of IFN-α.

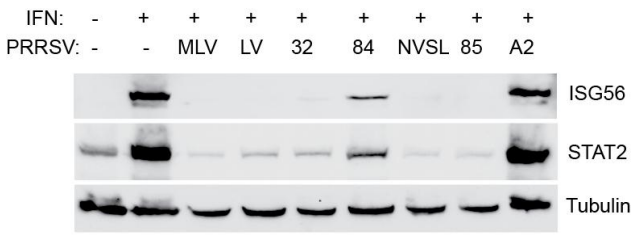

Fig. 2. Inhibition of IFN signaling in MARC-145 cells by PRRSV. MARC-145 cells were infected with different PRRSV strains for 24 h, and then treated with IFN-α for 12 h. Protein levels of STAT2 and ISG56 were detected by Western blotting with respective antibodies. Non-infected and non-treated MARC-145 cells were included as controls.

We also tested the effect of PRRSV replication on IFN signaling in primary PAM cells. PAMs were infected with PRRSV VR2385 and MLV at 0.05 MOI and incubated for 17 h. The cells were treated with IFN-α for 8 h before harvested for Western blotting of STAT2. All strains except MLV and NVSL reduced protein level of STAT2 in PAMs

after IFN-α treatment (Fig. 3A). Infection of PAMs with MLV and NVSL led to minimal limited effect on STAT2 level. The viral RNA levels of MLV, VR-2332 and MN184 VR2385 and MN184 in PAMs were significantly lower than VR-2385 and NVSL (Fig. 3B).

In summary, VR2385, VR-2332, and Lelystad inhibit IFN signaling in both cell types. MLV inhibits the IFN signaling in MARC-145 cells but not in PAMs, while MN184 inhibits IFN signaling in PAMs but minimal in MARC-145 cells. It appears that the PRRSV inhibition of IFN signaling is cell-type specific and there are strain differences. These results imply that PRRSV inhibitory effect on IFN signaling might be related with the PRRSV virulence.

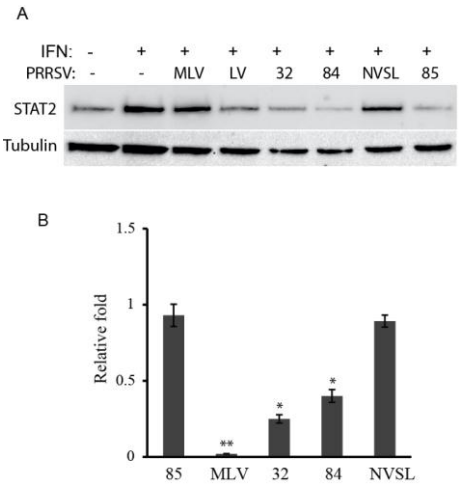

Fig. 3. Variable inhibition of IFN-activated JAK/STAT signaling in PAM cells by different PRRSV strains. A. Inhibition of IFN-induced STAT2 elevation by different PRRSV strains. The cells were infected with PRRSV at 0.05 MOI, treated with porcine IFN-α at 17 hpi, and harvested at 25 hpi. Uninfected cells were included as controls. Western blotting with antibodies against STAT2 and tubulin was conducted. MLV: Ingelvac PRRS MLV; LV: LeLystad; 32: VR-2332; 84: MN-184; NV: NVSL; and 85: VR-2385. B. PRRSV RNA level in infected PAM cells detected by RT-qPCR. Relative folds in comparison with VR-2385-infected cells are shown. Significant differences from VR-2385-infected cells are denoted by "*" and "**", which indicates $P < 0.05$ and $P < 0.01$, respectively.

Objective 2. To identify the structural components of PRRSV that cause inhibition of IFN signaling

Screening of PRRSV proteins in inhibiting the activity of IFN

We speculated that more than one PRRSV protein might be able to interfere with the activity of IFN. To determine the effect of individual PRRSV proteins on JAK/STAT signaling, we cloned VR-2385 nsps and ORFs into pCAGEN-HA vector. Nsp6 was small and was not included in the cloning and expression. Nsp8 was cloned into VenusC1 vector. Expression of the proteins in HEK293 was confirmed by Western blotting or immunofluorescence assay. IFN-stimulated response element (ISRE) luciferase reporter assay was conducted. Compared with empty vector control, nsp1β significantly reduced ISRE reporter expression (Fig. 4A). Nsp7 and nsp12 led to limited reduction of the reporter. Nsp8 had minimal effect on the reporter expression (data not shown).

Among the PRRSV ORFs, expression of ORFs 3, 4 and 7 led to significant reduction of ISRE reporter expression in comparison with empty vector control (Fig. 4B). ORF3 and ORF4 encode membrane-associated minor glycoproteins. ORF7 encodes N, the capsid protein. The expression of ORF7 resulted in lowest luciferase activity, indicating its inhibition of IFN-activated signaling.

As nsp1β had the most significant inhibition of the IFN-activated signaling, it was selected for further study to define the mechanisms of the interference.

Comparison of nsp1β from different strains in blocking the activity of IFN

As different strains showed variable effect on IFN signaling, we wonder whether the nsp1β from these strains would have similar or different effect on JAK/STAT signaling. First we conducted alignment of amino acid sequences of nsp1β of VR-2385, VR-2332, MLV, MN-184, NVSL, and Jiangxi-2 (an isolate of China high pathogenic strain) (Fig. 5A). Due to big variation of Lelystad strain, it was not included in the alignment. We cloned nsp1β of VR-2385, VR-2332, MLV, and Jiangxi-2 strains into pCAGEN vector for overexpression. Their expression in HEK293 cells was confirmed by Western blotting. ISRE luciferase reporter assay with these nsp1β plasmids showed that all except MLV nsp1β inhibited the IFN-activated reporter expression (Fig. 5B). The nsp1βs of MN-184 and NVSL were cloned into other expression vectors and were not included in this reporter assay.

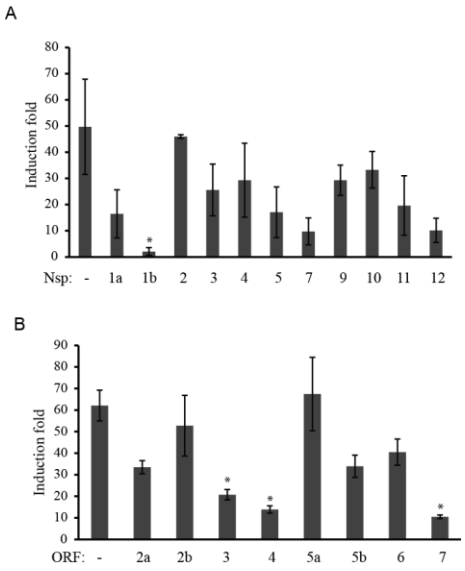

Fig. 4. Screening of VR-2385 proteins in ISRE luciferase reporter assay in HEK293 cells. A. Effect of PRRSV nsps on IFN signaling. The cells were transfected with ISRE reporter and PRRSV nsp plasmids. IFN-α was added 24 h after the transfection. Luciferase activity was detected 20 h after the IFN treatment. Relative induction folds are shown in comparison with untreated control cells. Significant differences from the control are denoted by "*", which indicates $P < 0.05$. B. Effect of PRRSV structural proteins on IFN signaling. The experiment was done similarly as in "A" except using PRRSV plasmids of ORFs 2-7.

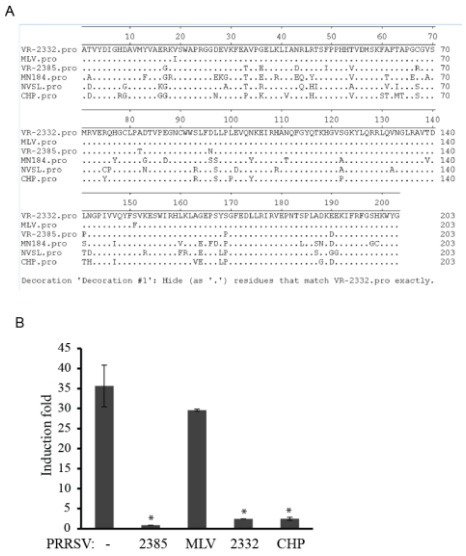

Fig. 5. Comparison of nsp1β from different strains in ISRE reporter assay. A. Alignment of amino acid sequence of nsp1β from VR-2332, MLV, VR-2385, MN184, NVSL, and Jiangxi-2 (a China high pathogenic strain (CHP)). The numbers above the sequence and on the right side indicate amino acid position of nsp1β. The residues that match nsp1β of VR-2332 are shown as ".". B. ISRE reporter assay in HEK293 cells transfected with nsp1β and treated with IFN-α. Relative induction folds in comparison with untreated control cells are shown. Significant differences from the control with empty vector are denoted by "*", which indicates $P < 0.05$.

In addition, we cloned the nsp1βs of these strains into VenusC1 vector. Their expression in HEK293 cells was confirmed. They had similar pattern in subcellular localization but variable intensity. ISRE luciferase reporter assay with these VenusC1-nsp1β plasmids was also conducted. Results showed except MLV, all others had significant inhibition of the IFN-activated reporter expression (data not shown), which was consistent with the reporter assay with pCAGEN-nsp1β plasmids.

Objective 3. To determine the mechanism of PRRSV-mediated inhibition of IFN signaling

Nsp1 β from virulent strain VR-2385 blocks the nuclear translocation of STAT1

Since nsp1β from VR2385 inhibits IFN signaling, we further tested at which step of IFNs signaling it interferes with. After transient expression of both nsp1α and nsp1β, HEK293 cells were treated with IFN-α, and harvested at 1h post treatment. Phosphorylation status of STAT1 and STAT2 was detected with rabbit anti-STAT1(Y701) and STAT2(Y690) phosphorylation-specific antibodies. Our result showed that both nsp1α and nsp1β had no effect on phosphorylation of STAT1 and STAT2 after IFN-α treatment (Fig. 6A). The phosphorylation level of the two proteins after IFN induction was similar to cells with empty vector.

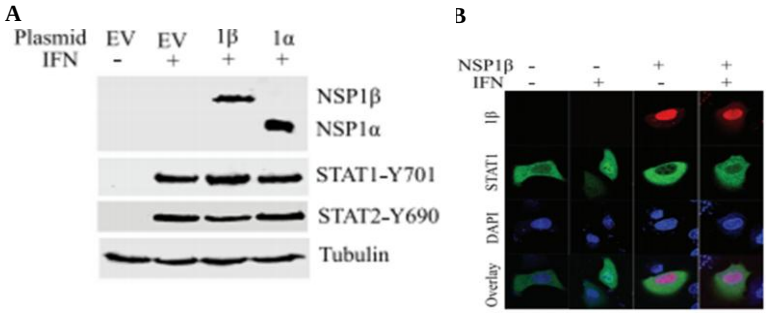

Fig. 6. NSP1β from VR2385 block nuclear translocation of STAT1. (A) NSP1α and NSP1β play no role in phosphorylation of STAT1 and STAT2 in HEK293 cells. The cells were harvested for STAT1-Y701 detection 1 h after IFN treatment. (B) NSP1β inhibits nuclear translocation of STAT1-eGFP in HeLa cells observed 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.

Since nsp1β does not affect the IFN-stimulated phosphorylation of STAT1, we speculated that it might interfere with the STAT1 nuclear translocation. 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. 6B), indicating that NSP1β inhibits the STAT1 nuclear translocation.

PRRSV nsp1β reduces the complexes of STAT1 interacting KPNA1 after interferon stimulation.

As shown in Fig 6, nsp1β blocks the IFN-activated nuclear translocation of STAT1/STAT2 heterodimers (24). Here the mechanism of inhibition was further examined. STAT1 nuclear translocation mainly involves in KPNA1, which functions

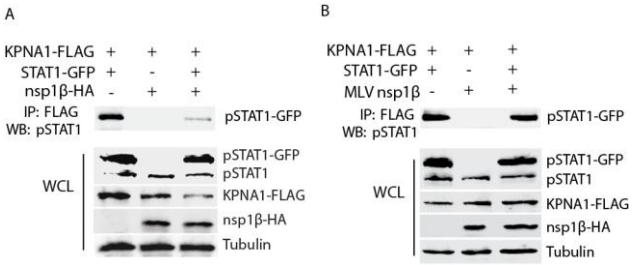

Fig. 7. VR-2385 nsp1β reduces the complexes of phosphorylated STAT1 interacting with karyopherin alpha 1 (KPNA1). HEK293 cells were transfected with plasmids of KPNA1-FLAG, STAT1-GFP, and nsp1β-HA. At 48 h post-transfection, the cells were treated with IFN-α for 1.25 h before lyzed for further analysis. **A**. VR-2385 nsp1β reduces complexes of phosphorylated STAT1 interacting with KPNA1. Immunoprecipitation (IP) was done with FLAG antibody and Western blotting was performed with antibody against phosphorylated STAT1 at tyr701 (pSTAT1). The lower panel images show Western blotting results of whole cell lysate (WCL) with antibodies against pSTAT1, FLAG, HA, and tubulin. **B**. Ingelvac PRRS MLV nsp1β has little effect on the interaction of pSTAT1 and KPNA1. IP was done with FLAG antibody and Western blotting was conducted with antibody against pSTAT1. The lower panel images show Western blotting results of WCL of HEK293 cells with MLV nsp1β expression with antibodies against pSTAT1, FLAG, HA, and tubulin.

in transferring proteins from cytoplasm into the nucleus. Initially, we tested whether the interaction of STAT1 and KPNA1 was affected in cells with nsp1β expression. HEK293 cells were transfected with plasmids of VR-2385 nsp1β, KPNA1-FLAG, and STAT1-GFP. IFN-α was added to the cells to activate the JAK/STAT signaling pathway 48 h after the transfection. The cells were then harvested for immunoprecipitation with FLAG antibody. Next, Western blotting was conducted with antibodies against tyrosine phosphorylated STAT1 (pSTAT1). The blotting results revealed that there was significantly less pSTAT1 in complexes with KPNA1 in cells with nsp1β expression than in cells without nsp1β expression (Fig. 7A). Western blotting of whole cell lysate showed similar level of pSTAT1 in the cells with or without nsp1β expression, whereas KPNA1 level in cells with nsp1β was lower than control cells (Fig. 7A).

MLV nsp1β does not to block IFN-activated STAT1/STAT2 nuclear translocation(24). Whether MLV nsp1β would have any effect on the interaction between STAT1 and KPNA1 was tested to exclude the possibility that the KPNA1 reduction was due to nsp1β's non-specific effect. IP and Western blotting showed that pSTAT1 level was similar in cells with and without MLV nsp1β (Fig. 7B). Western blotting of whole cell lysate showed that the cells with or without MLV nsp1β had similar levels of pSTAT1 and KPNA1. These results indicate that VR-2385 nsp1β reduced complexes of pSTAT1 interacting with KPNA1 in cells after IFN stimulation, whereas MLV nsp1β had little effect.

Nsp1β reduces KPNA1 and the ubiquitin-proteasome pathway is involved in KPNA1 reduction.

We noticed that cells with nsp1β expression had lower KPNA1 level (Fig. 7A). To confirm this observation, we tested whether nsp1β caused the reduction of KPNA1. HEK293 cells were transfected with nsp1β plasmids of VR-2385, VR-2332 and MLV. Western blotting showed that KPNA1 levels were reduced in cells with nsp1β of VR-2385 or VR-2332, however, minimal change in cells with MLV nsp1β, in comparison to cells with empty vector (Fig. 8A). The relative KPNA1 levels were 0.44-, 1.18-, and 0.34-fold for cells with nsp1β of VR-2385, MLV and VR-2332, respectively. Moreover, along with incremental amount of VR-2385 nsp1β plasmid DNA from 0.125 to 1 μg in the transfection, the KPNA1 protein level was reduced from 0.88- to 0.4-fold, in comparison with empty vector control (Fig.

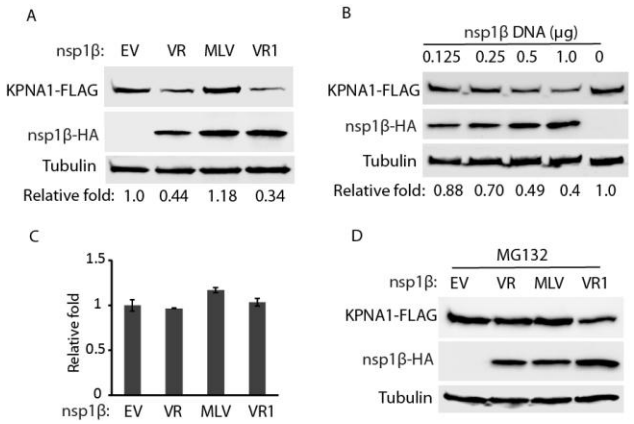

Fig. 8. PRRSV nsp1β reduces KPNA1 expression via proteasome pathway. **A**. KPNA1 level is reduced in HEK293 cells with expression of nsp1β of VR-2385 and VR-2332. The cells were transfected with plasmids of KPNA1-FLAG and nsp1β-HA. At 48 h after transfection, the cells were harvested for Western blotting. Relative folds of KPNA1 level were shown below the images. EV: empty vector; VR: VR-2385; VR1: VR-2332. **B**. Dose-dependent reduction of KPNA1 by VR-2385 nsp1β. HEK293 cells were transfected with KPNA1-FLAG and incremental amount of nsp1β-HA DNA. At 48 h after transfection, the cells were harvested for Western blotting. Relative folds of KPNA1 level were shown below the images. **C**. KPNA1 mRNA level is not affected by nsp1β expression. HEK293 cells were transfected with nsp1β plasmid. At 24 h after transfection, the cells were harvested for RNA isolation and RT-qPCR. Error bars represent variation of three repeated experiments. **D**. The nsp1β-induced KPNA1 reduction in HEK293 cells involves in proteosome pathway. At 48 h after transfection, the cells were treated with MG132 and harvested for detection of KPNA1-FLAG, nsp1β-HA and tubulin by Western blotting.

8B), which shows the nsp1β-mediated dose-dependent inhibitory effect.

KPNA1 reduction could be due to a decrease of transcription and/or translation, and accelerated protein degradation. To determine the mRNA level of KPNA1 in the cells with nsp1β expression, we conducted RT-qPCR. The KPNA1 mRNA levels were similar in cells with and without nsp1β expression (Fig. 8C). The results indicate that the reduction of KPNA1 protein was not due to its mRNA level change. Next we tested whether the KPNA1 reduction was due to degradation by ubiquitin-proteasome pathway. MG132, a proteasome inhibitor, was added to the cells 48 h after nsp1β transfection. The cells were harvested 6 h later for Western blotting. The MG132 treatment resulted in the restoration of KPNA1 in the cells with expression of VR-2385 nsp1β to a level similar to that in the cells with empty vector (Fig. 8D). These results indicated that KPNA1 reduction in the cells with VR-2385 nsp1β was due to the degradation by the ubiquitin-mediated proteasomal pathway.

VR-2385 nsp1β increases KPNA1 ubiquitination and shortens its half-life.

As MG132 treatment restored KPNA1 level, we expected that KPNA1 ubiquitination levels in the cells with nsp1β expression would increase. HEK293 cells transfected with KPNA1-FLAG, Ubiquitin-Myc, and VR-2385 nsp1β-HA plasmids. The cells were lysed for IP with antibody against FLAG. Western blotting with ubiquitin antibody was then performed to detect ubiquitinated KPNA1. The blotting results showed that ubiquitinated KPNA1 in the cells with nsp1β appeared to be smear, as expected, and that its density was 4.18-fold higher than the cells transfected with empty vector (Fig. 9A).

As nsp1β increased KPNA1 ubiquitination, we speculated that the half-life of KPNA1 would be shortened. HEK293 cells were transfected with KPNA1-FLAG and VR-2385 nsp1β. At 24 h after the transfection, the cells were treated with a translation inhibitor, cycloheximide, at a final concentration of 100 μg per ml. The cells were then harvested at specified time points for Western blotting. The KPNA1 level 3 h after the cycloheximide treatment was reduced to 0.49-fold in the cells with nsp1β expression, while it remained at 0.79-fold in cells with empty vector (Fig. 9B). The nsp1β expression led to a shortening of the

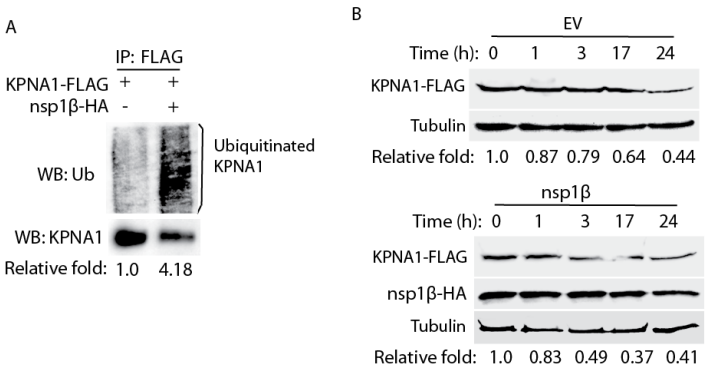

Fig. 9. VR-2385 nsp1β increases KPNA1 ubiquitination. **A**. KPNA1 ubiquitination in HEK293 cells with nsp1β expression increases. The cells transfected with KPNA1-FLAG and VR-2385 nsp1β plasmids were lysed for IP. Western blotting with ubiquitin antibody was done after IP. Relative folds of ubiquitin level in comparison with the cells without nsp1β are shown below the images after normalization with KPNA1 level. **B**. KPNA1 half life is shortened in the presence of nsp1β expression. HEK293 cells transfected with KPNA1-FLAG and VR-2385 nsp1β plasmids were treated with cycloheximide and harvested at indicated time (h). KPNA1, nsp1β and tubulin were detected by Western blotting. Relative folds of KPNA1 level in comparison to 0 h post-cycloheximide addition are shown below the images.

KPNA1 half-life from approximately 22 h to about 3 h. This result is consistent with the increased ubiquitination of KPNA1 in the cells with nsp1β expression.

Reduction of endogenous KPNA1 by PRRSV infection.

The results above showed that nsp1β of both VR-2385 and VR-2332 reduced KPNA1. One might speculate whether endogenous KPNA1 protein in MARC-145 cells would decrease after PRRSV infection. MARC-145 cells were infected with PRRSV strain VR-2385, MLV, or VR-2332 and were harvested 24 h post-infection (hpi) for Western blotting. Compared to uninfected cells, KPNA1 levels in the cells infected with VR-2385 and VR-2332 were reduced to 0.48- and 0.65-fold, respectively, while the protein remained at 0.84-fold in the cells infected with MLV (Fig. 10A). To test if KPNA1 mRNA level in MARC-145 cells after PRRSV infection had any change, RT-qPCR was conducted. Results showed that KPNA1 transcripts in the cells both with and without PRRSV infection had no significant difference (Fig. 10B). PRRSV genomic RNA levels in the cells with PRRSV infection were also detected. The cells with MLV infection produced significantly more copies of viral RNA than the other two strains (Fig. 10C). This suggested that, as expected, MLV replicated faster than the other two strains in MARC-145 cells.

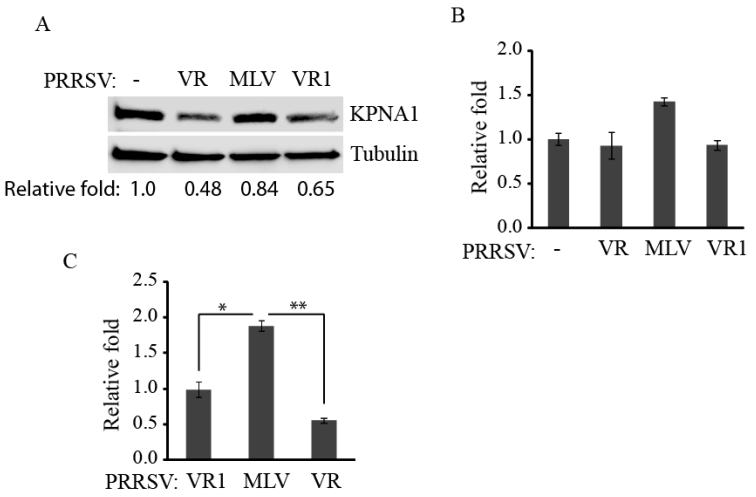

Fig. 10. Reduction of endogenous KPNA1 level by infection with PRRSV strain VR-2385 and VR-2332. **A**. Endogenous KPNA1 protein level in MARC-145 cells after PRRSV infection. MARC-145 cells were infected with PRRSV strain VR-2385, MLV, or VR-2332 and harvested 24 hpi for Western blotting. Lysate of uninfected cells was included as a control. Relative folds of KPNA1 protein level were shown below the images. VR: VR-2385; VR1: VR-2332. **B**. KPNA1 mRNA level in MARC-145 cells after PRRSV infection. RT-qPCR was done to quantify KPNA1 mRNA at 24 hpi. Relative folds in comparison with uninfected cells are shown. Error bars represent repeated experiments **C**. PRRSV RNA in MARC-145 cells detected by RT-qPCR. Relative folds in comparison with VR-2332-infected cells are shown. Significant differences between viral RNA levels are denoted by "*" and "**", which indicates $P < 0.05$ and $P < 0.01$, respectively.

IX. Discussion: Explain your research results and include a summary of the results that is of immediate or future benefit to pork producers.

PRRSV inhibits the ability of type I IFNs in inducing antiviral response in MARC-145 and PAM cells (24). In this study, variable effects of different PRRSV strains on the inhibition were assessed. Three of the six strains tested had consistent inhibitory effect in both MARC-145 and PAM cells, while the three others had different effect in both cell types. In the case of MLV, it may be because the virus was adapted to grow in MARC-145 cells and grew poorly in PAM cells. For MN-184 and NVSL, the reason for the different effects in both cell types is not known

yet. We speculate that these two strains might have different mechanism of interacting with cellular proteins in the two cell types.

As the different strains had variable effect on the IFN signaling in different cell types, we wondered which viral protein(s) were responsible for the virus-mediated inhibition. We cloned and expressed all viral proteins from VR-2385, a virulent strain that consistently inhibits IFN signaling in both cell types. In screening viral proteins in ISRE reporter assay, we noticed several viral proteins, including nsp1β, nsp7, nsp12, GP3, GP4 and N, inhibited IFN-activated ISRE reporter expression. These proteins are speculated to cooperatively thwart host IFN-mediated antiviral response to gain time for virus replication and spread. Among the proteins, nsp1β showed the most significant and consistent inhibition of IFN signaling. We selected it for further study to define the mechanism of the inhibition.

VR-2385 nsp1β was found to inhibit the IFN signaling by blocking STAT1 nuclear translocation. Overexpression of the protein did not have any effect on IFN-induced phosphorylation of both STAT1 and STAT2. The nuclear translocation of STAT1-GFP in cells with nsp1β expression was blocked. This interference appears to be the reason for the inhibition of IFN-activated signaling.

The nsp1βs of several strains were cloned and expressed. Their effects on IFN signaling were compared. It was found that except MLV nsp1β, all others had significant inhibition of IFN-activated ISRE reporter expression. This result indicates that nsp1β plays a role in the inhibition of IFN signaling in virus-infected cells by all these strains except MLV. In MLV-infected MARC-145 cells, other viral proteins might be responsible for the inhibition. In PAM cells, MLV had no effect on the IFN signaling partly because of its nsp1β's inability and low level expression of the other viral proteins due to low viral replication.

We further discovered that the mechanism for nsp1β-mediated inhibition of JAK/STAT signaling pathway was due to the increased degradation of KPNA1. Several lines of evidence were provided in this study to support the conclusion. First, nsp1β leads to lower levels of exogenous overexpressed KPNA1. The interaction of pSTAT1 with KPNA1 after IFN stimulation was examined. The cells with nsp1β expression had lower amount of pSTAT1 in complexes with KPNA1. The total level of pSTAT1 remained unchanged, while the total KPNA1 level was reduced in the cells with nsp1β expression. As MLV nsp1β does not inhibit pSTAT1 nuclear translocation, we included it in this experiment to exclude the possibility that nsp1β has any non-specific effects on KPNA1. The expression of MLV nsp1β in HEK293 cells did not affect KPNA1 level or its interaction with pSTAT1.

KPNA1 degradation is due to the ubiquitin-mediated proteasome pathway. Nsp1β induced KPNA1 degradation in a dose-dependent manner. The mechanism for the KPNA1 reduction was explored at mRNA and protein levels. The KPNA1 transcript level had minimal change in the cells with nsp1β expression. Blocking the ubiquitin-mediated proteasomal degradation pathway with MG132 led to the restoration of KNPA1 level in the cells with VR-2385 nsp1β. These results indicated that the proteasome pathway was involved in the nsp1β-induced degradation of KPNA1. IP result showed that ubiquitination of KPNA1 was significantly increased. Moreover, the KPNA1 half-life was reduced from 22 h to about 3 h, which further substantiates the observation of KPNA1 degradation. Since the nsp1β reduced KPNA1, we wondered whether it would interact with KPNA1 or not. IP with FLAG antibody to pull down KPNA1-FLAG and then Western blotting with HA antibody failed to detect nsp1β-

HA (result not shown). On the other hand, IP with HA antibody and then Western blotting with FLAG antibody did not detect KPNA1 either. The results indicated that KPNA1 and nsp1β either had transient or weak interaction that is below detectable level by IP, or had no interaction with each other.

PRRSV infection leads to a reduction of endogenous KPNA1. Overexpression of nsp1β might lead to unexpected change in the cells. To exclude this possibility, we conducted analysis of endogenous KPNA1 in PRRSV-infected MARC-145 cells. KPNA1 level was reduced in the cells infected with VR-2385 and VR-2332, while MLV replication had little effect. The KPNA1 reduction was not due to KPNA1 mRNA changes, as cells infected with different strains had similar levels of the transcript.

Nsp1β is the second protein in non-structural proteins encoded by PRRSV ORF1a and has drawn attention due to its ability to inhibit interferon induction and IFN-activated JAK/STAT signaling (4, 7, 12, 24), interact with cellular poly(C) binding protein (3), and suppresses tumor necrosis factor-alpha promoter activation (32).

In conclusion, we found that different PRRSV strains have variable effects on IFN-activated antiviral response and identified the mechanism of PRRSV nsp1β in inhibition of JAK/STAT signaling in this study. The nsp1β inhibits the IFN signaling by inducing degradation of KPNA1, which leads to blocking of STAT1 nuclear translocation, a key step in the IFN signaling. Our study provides further insight to the PRRSV interference with IFN-mediated antiviral responses. These results provide insight of the viral pathogenesis and will be useful for development of a better vaccine against PRRSV infection.

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