**Downregulation of Protein KinasePKR Activationby Porcine Reproductive and
Respiratory Syndrome Virusat Its Early Stage Infection**

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Abstract

The interferon-induced double-strand RNA activated protein kinase (PKR) plays an important role in antiviral response. The objective of this study was to assess the effect of porcine reproductive and respiratory syndrome virus (PRRSV) on PKR activation. Here we report that PRRSV inhibited PKR activation during its early stage infection of primary pulmonary alveolar macrophages (PAMs). PRRSV infection led to lower level of phosphorylated PKR in comparison with mock-infected cells. The PKR inhibition was sustained until 10 hours post infection in the presence of polyI:C, a synthetic analog of double-stranded RNA activating PKR. PKR-mediated phosphorylation of the eukaryotic translation initiation factor eIF2 α was also lower in the PRRSV-infected PAMs during the early stage infection. Interestingly, inactivated PRRSV was capable to inhibit the PKR activation until 6 hours post infection. This suggests that structural components of PRRSV virions were responsible for the inhibition, although PRRSV replication was needed for longer inhibition. These results indicate that the downregulation of PKR activation during early infection stages should be essential for PRRSV to avoid the antiviral response to initiate replication. This finding contributes to our understanding on PRRSV interaction with host innate immune response and reveal a target for control of PRRSV infection.

Key words: Protein kinase PKR, PKR inhibition, porcine reproductive and respiratory syndrome virus (PRRSV), pulmonary alveolar macrophages (PAMs), eIF2 α , innate immunity.

1. Introduction

PKR is a serine-threonine kinase that is activated by double-stranded RNA (dsRNA) of cellular, viral or synthetic (such as polyI:C) origins (Garcia et al., 2006; Garcia et al., 2007). PKR is induced by interferon and expressed as a latent state. PKR contains two N-terminal dsRNA binding domains and one C-terminal kinase domain. In non-stressed cells, PKR exists as a monomeric latent state due to the autoinhibitory effect of the dsRNA binding domains on the kinase domain. Binding of dsRNA to PKR results in dimerization and subsequent autophosphorylation of PKR (Clemens, 1997; Dey et al., 2014; Garcia et al., 2006). The PKR activation leads to phosphorylation of serine 51 of eukaryotic translation initiation factor 2 α (eIF2 α), one of the three subunits of eIF2 (Garcia et al., 2007). Under permissive conditions, eIF2 binds to guanosine-5'-triphosphate (GTP) and initiator methionyl-tRNA to form a ternary complex, which further associates with the 40S ribosomal subunit and results in initiation of polypeptide synthesis in mammals (Majumdar and Maitra, 2005). GTP hydrolysis leads to release of eIF2 and generation of inactive eIF2-GDP. Regeneration of eIF2-GDP is catalyzed by GTP exchange factor eIF2B for GDP-to-GTP exchange. The eIF2B functions only if eIF2 is in an unphosphorylated state. Phosphorylation of eIF2 α causes increased affinity of eIF2 to eIF2B up to 100-fold, leading to competitive inhibition of eIF2B (Sudhakar et al., 2000). The cellular concentration of eIF2B is much lower than eIF2. Thus sequestration of eIF2B by even a small amount of phosphorylated eIF2 leads to abolishment of eIF2B function and consequent translation inhibition. As PKR is an important antiviral protein, viruses have evolved a number of strategies to counteract its deleterious effects (Garcia et al., 2007). However, it is unknown whether porcine reproductive and respiratory syndrome virus (PRRSV) thwarts the effect of PKR during the virus replication cycle.

PRRSV is the causative agent of porcine reproductive and respiratory syndrome (PRRS), an economically devastating swine contagious disease across the world that has resulted in an estimated \$664 million loss per year to the swine industry in the United States alone (Holtkamp et al., 2013). PRRSV is a positive-sense single-stranded RNA virus of the family *Arteriviridae*, order *Nidovirales* (Faaberg et al., 2011; Meulenberg, 2000). PRRSV genome is approximately 15 kb in length and encodes over ten open reading frames. PRRSV virions are spherical and enveloped, with a diameter of approximately 50 nm. Pulmonary alveolar macrophages (PAMs) are the main target cells for PRRSV infection in pigs (Rossow et al., 1995). Propagation of PRRSV *in vitro* is generally conducted on MARC-145 cell line, which is derived from MA-104, a kidney cell line established from an African green monkey (Kim et al., 1993).

PRRS remains one of the top challenges for swine producers, despite substantial efforts to control PRRS in pig populations. So far there is no production or vaccination regimen that has demonstrated sustaining success. Biosecurity challenges and both antigenic and genomic variations among PRRSV strains contribute to frequent transmission between pig populations and persistence of the virus in infected pigs. Further studies on PRRSV pathogenesis and induced immune responses are needed to facilitate development of superior strategies for PRRS control.

The objective of this study was to examine the PRRSV modulation of PKR signaling in primary PAM cells. Here we report that PRRSV infection of primary PAMs led to lower level of phosphorylated PKR in comparison with mock-infected cells. The PKR inhibition sustained until 10 hours post infection (hpi) in the presence of polyI:C. PKR-mediated phosphorylation of eIF2 α was also lower in the PRRSV-infected PAMs during the early stage infection. Inactivated PRRSV was capable to inhibit the PKR activation until 6 hpi. This suggests that some structural components of PRRSV virions are responsible for the inhibition. These results indicate that

84 inhibition of PKR activation during early infection stages should be essential for PRRSV to avoid
85 the antiviral response and initiate replication.

2. Materials and methods

2.1. Cells and virus

MARC-145 (Kim et al., 1993) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Primary PAM cells were prepared and cultured in RPMI 1640 medium as previously described (Patel et al., 2008). The cryopreserved PAM cells were revived and pre-cultured for 24 hours before used for virus inoculation. PolyI:C (Invivogen, San Diego, CA) was used to induce PKR activation at a concentration of 1 µg/ml for transfection of the PAM cells by using transfection agent polyethylenimine (Polysciences, Inc., Warrington, PA).

PRRSVVR-2385 strain was propagated in MARC-145 cells and multiplicity of infection (MOI) was based on the median tissue culture infectious dose (TCID₅₀), as previously described (Zhang et al., 2006). UV-inactivation of PRRSV was conducted and verified as described previously (Nan et al., 2012).

2.2. Western blotting

Cell lysate preparation, total protein separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting were conducted as described previously (Zhang et al., 2007). Antibodies used in the Western blotting were rabbit anti-phospho-PKR (Thr451) antibody (EMD Millipore, Billerica, MA, USA), rabbit anti-PKR antibody (Sigma-Aldrich Corp, St. Louis, MO, USA), mouse anti-tubulin monoclonal antibody (Sigma), mouse anti-eIF2α monoclonal antibody (Cell Signaling Technology, Danvers, MA), phospho-eIF2α (Ser51) (D9G8) XP rabbit mAb (Cell Signaling), and mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mAb (Santa Cruz Biotechnology, Dallas, TX, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies used in the study were

goat anti-rabbit IgG-HRP (Rockland Immunochemicals Inc., Limerick, PA) and goat anti-mouse IgG-HRP (Rockland). The chemiluminescence signal was recorded digitally using a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA). Densitometry analyses of the digital images were conducted with the Quantity One Program, Version 4.6 (Bio-Rad).

2.3.RNA isolation, reverse transcription, and real-time PCR

Total RNA was isolated with TRIzol® Reagent (Life Technologies, Grand Island, NY) following the manufacturer's instructions. Reverse transcription and real-time PCR(RT-qPCR) were conducted as previously described (Patel et al., 2008; Patel et al., 2009). Detection of house-keeping gene ribosomal protein L32 (RPL32) transcript was also conducted for normalization of total input RNA. Primers used in this study and analysis of relative PRRSV RNA levels were performed as previously described (Patel et al., 2010).

2.4.Statistical analysis

Differences in PRRSV RNA level between treatment samples and a control were assessed by the Student *t*-test. A two-tailed *P*-value of less than 0.05 was considered significant.

3. Results

3.1. PRRSV inhibits PKR activation in PAMs

In our study of PRRSV effect on interferon-activated signaling, we noticed that level of phosphorylated PKR (p-PKR) was reduced in PRRSV-infected PAM cells. To confirm the reduction of p-PKR by PRRSV infection, we inoculated PAM cells with PRRSV strain VR-2385 at an MOI of 3 and harvested 5 hpi. Western blotting result showed that the level of p-PKR in PRRSV-infected cells is lower than a quarter of that in mock-infected cells, whereas total PKR level had minimum change (Fig. 1A).

To further confirm the observation, we transfected the cells with polyI:C to induce the PKR phosphorylation and assess PRRSV effect on the PKR activation. Addition of polyI:C led to 2-fold elevation of PKR phosphorylation, while PRRSV infection blocked the polyI:C-induced PKR activation (Fig. 1B). The results indicate that PRRSV blocks PKR activation.

3.2. The PRRSV-mediated blocking of PKR activation occurs at early stage after inoculation

To delineate the temporal kinetics of the PRRSV-mediated inhibition of PKR activation, we inoculated PAMs with PRRSV strain VR-2385 at an MOI of 3 and harvested the cells at different time points until 12 hpi. Western blotting results showed that the p-PKR levels remained low from 2 to 8 hpi, and increased at 10 and 12 hpi, whereas the total PKR levels had minimum change in the time course (Fig. 2A). Compared with PAMs treated with polyI:C, the PRRSV-infected PAMs harvested at 2, 4, 6, 8, 10 and 12 hpi had relative levels of p-PKR at 0.26, 0.10, 0.14, 0.16, 0.73, and 0.71-fold, respectively. The direct target of PKR activation is translation initiator eIF2 α , leading to its phosphorylation and consequent translation inhibition (Garcia et al., 2006). The phosphor-eIF2 α (p-eIF2 α) levels remained low from 2 to 6 hpi, had a little elevation 8 hpi and increased 10 and 12 hpi, while the total eIF2 α level had minimum

change (Fig. 2A). The relative levels of p-eIF2 α in comparison with PAMs treated with polyI:C are 0.23, 0.16, 0.23, 0.33, 0.49, and 0.71-fold for PRRSV-infected PAMs harvested at 2, 4, 6, 8, 10 and 12 hpi, respectively.

As the cells were harvested early after inoculation, we wondered about PRRSV replication status in the cells. Real-time PCR was conducted to determine the PRRSV RNA levels in the cells. Results showed that PRRSV RNA levels significantly increased along with time extension from 2 to 12 hpi (Fig. 2B). The virus replicated fast in the cells after inoculation (0 hpi indicates the moment of virus inoculum addition to the cells).

To further confirm the temporal kinetics, we transfected the cells with polyI:C to induce the PKR phosphorylation and assess PRRSV effect on the PKR activation. To ensure proper comparison, all the PAM cells with indicated polyI:C treatment were conducted for 3 hours before harvested for further analysis with the exception of 2 hpi sample (treated for 2 h). The p-PKR levels remained low from 2 to 8 hpi (Fig. 3A), which indicate that PRRSV infection blocked the polyI:C-induced elevation of PKR phosphorylation. Upon polyI:C treatment, the relative levels of p-PKR in comparison with uninfected PAMs are 0.27, 0.07, 0.11, 0.23, 0.47, and 0.49-fold for PRRSV-infected PAMs harvested at 2, 4, 6, 8, 10 and 12 hpi, respectively. Likewise, the p-eIF2 α levels remained low and the relative levels of the p-eIF2 α in comparison with uninfected PAMs are 0.38, 0.15, 0.17, 0.24, 0.58, and 0.62-fold for PRRSV-infected PAMs harvested at 2, 4, 6, 8, 10 and 12 hpi, respectively. The levels of both p-PKR and p-eIF2 α remained low from 2 to 8 hpi, indicating the polyI:C activation was blocked soon after PRRSV inoculation.

3.3. Inactivated PRRSV is capable to inhibit PKR activation at early time points after inoculation

Since the results above showed PKR activation was blocked during early stage PRRSV infection, we presumed that the blocking occurred soon after viral entry. We wondered if inactivated PRRSV had any effect on the PKR activation. UV-inactivated PRRSV was used to inoculate PAM cells and polyI:C treatment was conducted. Results showed that the inactivated PRRSV also led to lower p-PKR in comparison to the polyI:C-treated PRRSV-negative control (Fig. 3B). Compared with the uninfected PAMs without polyI:C, the PAMs infected with UV-inactivated PRRSV and treated with polyI:C had relative levels of p-PKR at 1.11, 0.73, 1.24, 1.63, 1.55 and 1.85-fold for samples of 2, 4, 6, 8, 10 and 12 hpi, respectively, whereas the relative level of p-PKR in uninfected PAMs with polyI:C treatment increased to 2.35-fold. This indicates that some structural components in PRRSV virions prevents the polyI:C from activating PKR.

3.4. PRRSV replication is needed to sustain the blocking of polyI:C-induced phosphorylation of eIF2 α in PAM cells

As UV-inactivated PRRSV was capable to inhibit the polyI:C-induced activation of PKR, we wondered if it could block the activation of eIF2 α and whether virus replication is needed to prolong the inhibition. PAM cells were inoculated with live and UV-inactivated PRRSV. PolyI:C treatment was conducted. Immunoblotting results showed that p-eIF2 α levels in PAMs inoculated with live PRRSV and treated with polyI:C remained low 4 and 8 hpi (Fig. 4A). Compared with the uninfected PAMs without polyI:C, the PRRSV-infected PAMs treated with polyI:C had relative levels of p-eIF2 α at 0.69 and 0.79-fold for samples of 4 and 8 hpi, respectively, whereas the relative level of p-PKR in uninfected PAMs with polyI:C treatment increased to 2.25-fold. In PAMs inoculated with inactivated PRRSV and treated with polyI:C, the relative p-eIF2 α levels were 0.56 and 1.61-fold for samples of 4 and 8 hpi, respectively. These results suggest that

192 the virus replication was needed to maintain the inhibition of polyI:C-induced eIF2 α
193 phosphorylation at 8 hpi.

194 Detection of PRRSV RNA in the PAM cells was conducted. Compared with the cells
195 inoculated with inactivated virus, the PRRSV RNA level in the PAMs inoculated with live virus
196 significantly increased to 1,903 and 16,731-fold higher at 4 and 8 hpi, respectively (Fig. 4B). The
197 virus appeared to rapidly initiate replication in the cells soon after entry.

4. Discussion

As a dsRNA sensor, PKR plays an important role in antiviral response. To initiate infection, PRRSV needs to thwart the PKR activation. Our results demonstrate that PRRSV inhibits PKR activation during its early stage infection of physiologically relevant primary PAMs. Interestingly, the UV-inactivated PRRSV is also capable to block the PKR activation, indicating components of the PRRSV virions are responsible for the inhibitory effect. Besides antiviral response, PKR induces apoptosis and functions to control cell growth and cell differentiation (Garcia et al., 2007). Therefore, effects on PKR activation could lead to different consequences depending on context during infection.

PolyI:C induces phosphorylation of PKR in the primary PAM cells. PRRSV is able to inhibit the PKR activation induced by polyI:C, while having no effect on total PKR protein level. This suggests that the PRRSV inhibition occurs before PKR autophosphorylation, possible via blocking PKR binding of dsRNA. PRRSV was found to block the PKR activation from 2 to 8 hpi in the PAM cells. The PRRSV replication in the cells appeared fast, shown by the rapid increase of viral RNA level. The temporal kinetics of PRRSV-mediated inhibition of PKR suggest that the inhibition occurred soon after the virus entry until PRRSV finished its full cycle of replication or generated enough viral proteins for packaging. PRRSV genomic RNA is positive-sense and can be used as mRNA template for translation after virion unpacking post entry. The first PRRSV viral product to be synthesized in the cells would be the non-structural proteins (nsps) from ORF1a and ORF1b, which encode replicase products. Some of the nsps are known to interfere with interferon induction and signaling (Sun et al., 2012; Wang and Zhang, 2014). It is unknown if any of the nsps inhibits PKR activation.

The PKR phosphorylation levels in the PRRSV-infected PAM cells increased 10 and 12

hpi, compared to the samples of 2 to 8 hpi. The reason for the increase is unknown. It is possibly because that more dsRNA of PRRSV are generated, or that the viral protein responsible for the inhibition is sequestered by other viral proteins. The later speculation seems more plausible as PRRSV viral protein production should increase along with PRRSV replication and the viral proteins are used for packaging at certain stage. The viral protein responsible for the PKR inhibition might be sequestered and its level as free molecules is reduced to a level that has no effect on PKR activation, possibly 10 hpi.

UV-inactivated PRRSV is capable to inhibit the PKR activation, which indicates that structural proteins of PRRSV play the role. But the PRRSV nsps cannot be completely excluded as nsp2 was found to be packaged in PRRSV virions (Kappes et al., 2013). Further work is needed to elucidate the PRRSV component and mechanism for the PKR inhibition.

PKR activation leads to phosphorylation of eIF2 α and consequent translation inhibition. Our results showed that p-eIF2 α level in PRRSV-infected PAMs was lower than mock-infected control. The results of the temporal kinetics of p-eIF2 α level are consistent with PKR phosphorylation status at corresponding time points post PRRSV infection. It suggests that the eIF2 α phosphorylation in the PRRSV-infected PAMs in this study was due to PKR activation, not by the other three eIF2 α kinases, which act as a result of stress such as amino acid deprivation (GCN2 (general control nonderepressible 2)), endoplasmic reticulum stress (PERK (PKR-like ER-localized eIF2 α kinase)), or heme deficiency (HRI (heme-regulated eIF2 α kinase)) (Garcia et al., 2006; Williams, 1999).

PRRSV replication was found to extend the inhibition of eIF2 α phosphorylation from 4 to 8 hpi compared to the UV-inactivated PRRSV. This suggests that some viral proteins newly synthesized were able to prolong the inhibition. It also suggests that the virion component that

isable to inhibit the PKR activation was degraded to a threshold that was unable to exert the blockage. But it appeared that the elevation of p-PKR and p-eIF2 α at 10 or 12 hpi had minimum effect on PRRSV replication as viral RNA levels increased. The possible reason is that the virus made enough proteins for virus replication and packaging before 10 hpi or that PRRSV might have other strategies to thwart the inhibitory effect of eIF2 α on translation.

A previous report showed that addition of PKR inhibitor 2-aminopurine (2-AP) to IFN- γ primed MRAC-145 cells partially restored PRRSV replication (Rowland et al., 2001). Another study using recombinant swine IFN- β (rswIFN- β) showed that addition of 2-AP to rswIFN- β primed MARC-145 cells restored PRRSV replication, but had minimum effect on rswIFN- β primed PAM cells, which suggests that the two kinds of cells use different rswIFN- β -activated antiviral pathways (He et al., 2011). Interferon priming of the cells leads to PKR expression in a latent state. Addition of 2-AP inhibits PKR activation. Our results demonstrate that PRRSV inhibits PKR activation during its early stage infection of PAMs. We did not test addition of 2-AP as it might have limited or minimum effect on replication of PRRSV VR-2385 unless a variant strain that is unable to inhibit PKR activation is used. A recent paper showed that PKR plays a pro-viral role in PRRSV-infected MARC-145 cells (Wang et al., 2016). Our results showed that PKR activation was inhibited during early stage of PRRSV infection and resumed after 10 hpi. It is unknown if the PKR would play a pro-viral role in PAMs after 10 hpi.

Because of PKR's role in antiviral response, viruses use a number of strategies to counteract the PKR pathway (Garcia et al., 2007; Li et al., 2006; Tu et al., 2012; Zhang et al., 2013). There are viral proteins that interfere with PKR activation at different levels by sequestering dsRNA, inhibiting PKR dimerization, being PKR pseudosubstrates, activating antagonist phosphatases or degrading PKR (Garcia et al., 2007). Influenza virus NS1 protein

267 sequesters dsRNA and directly interact with PKR (Dauber et al., 2006; Li et al., 2006; Lu et al.,
268 1995). Our study showed that PRRSV inhibits PKR activation but has no effect on total PKR
269 level. It appeared that PRRSV blocked the PKR binding with dsRNA. Further study is needed to
270 address the exact mechanism of the inhibition.

5. Conclusions

Our results demonstrate that PRRSV inhibits PKR activation during its early stage infection of physiologically relevant primary PAMs. PRRSV inhibited PKR phosphorylation from 2 to 8 hpi in the primary cells. PKR-mediated phosphorylation of eIF2 α was also blocked, which is consistent with the PKR inhibition. Inactivated PRRSV is capable to block the PKR activation soon after entry, which indicates the PRRSV structural components were involved in the PKR inhibition. PRRSV replicates fast in PAM cells after entry, shown by the quick increase of PRRSV RNA level.

The inhibition of PKR activation during early stage of PRRSV infection appears to occur in PKR interaction with dsRNA. The PKR inhibition should be essential for early stage of PRRSV replication in the primary PAM cells. This finding contributes to our understanding of PRRSV interaction with host innate immune responses and reveal a target for control of PRRSV infection.

284 **Conflicts of interest**

285 None

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Figure legends

Fig. 1. PRRSV inhibits PKR activation in PAM cells. A. PRRSV replication reduces PKR phosphorylation in the PAM cells. PAMs were inoculated with PRRSV strain VR-2385 at an MOI of 3 and harvested 5 hours post-infection (hpi) for Western blotting. Western blotting with antibodies against phosphorylated PKR (p-PKR), PKR, and tubulin was conducted. Relative level of p-PKR is indicated in folds below the images after normalization with tubulin. B. PRRSV inhibits polyI:C-induced activation of PKR in the PAM cells. PAMs were infected with PRRSV strain VR-2385 and, at 2 hpi, induced with polyI:C for 3 h before harvested for Western blotting.

Fig. 2. Temporal kinetics of PRRSV inhibition of PKR activation in PAMs. A. Temporal kinetics of PRRSV inhibition of PKR phosphorylation in PAMs. PAM cells were inoculated with PRRSV strain VR-2385 at an MOI of 3 and harvested at different time points after infection. Western blotting with antibodies against p-PKR, PKR, phosphorylated eIF2 α (p-eIF2 α), eIF2 α , and GAPDH was conducted. Treatment of PAMs with 1 μ g polyI:C for 3 hours and normal PAMs without any treatment were included as controls. Relative levels of p-PKR and p-eIF2 α in comparison with PAMs treated with polyI:C are indicated in folds below the images after normalization with GAPDH. B. Real-time PCR detection of PRRSV viral RNA levels in the infected PAM cells. Error bars represent standard errors of mean of three experiments. There is significant difference ($P < 0.01$) in PRRSV RNA levels between 2 hpi and the other samples tested.

Fig. 3. PRRSV inhibition of PKR activation in PAMs in the presence of polyI:C. A. Temporal kinetics of PRRSV inhibition of polyI:C-induced PKR activation in PAMs. PAM cells were inoculated with PRRSV VR-2385 and transfected with 1 μ g polyI:C for 3 h (2 h for the

396 harvesting at 2 hpi) before harvested for Western blotting. Relative levels of p-PKR and p-eIF2 α
397 in comparison with PAMs treated with polyI:C are indicated in folds below the images after
398 normalization with GAPDH. B. UV-inactivated PRRSV is capable to inhibit PKR activation at
399 early time points after inoculation. PAM cells were inoculated with UV-inactivated VR-2385 at
400 an MOI of 3 and treated with 1 μ g polyI:C for 3 h (2 h for the harvesting at 2 hpi) before
401 harvested for Western blotting. Relative levels of p-PKR in comparison with untreated PAMs are
402 indicated in folds below the images.

403 Fig. 4. PRRSV replication is needed to prolong the blocking of polyI:C-induced phosphorylation
404 of eIF2 α in PAMs. A. PRRSV blocks polyI:C-induced phosphorylation of eIF2 α , while UV-
405 inactivated virus is unable to do so at 8 hpi. PAMs were inoculated with live or UV-inactivated
406 PRRSV VR-2385. Treatment of the cells with polyI:C was conducted for 3 h before they were
407 harvested for Western blotting. Relative levels of p-eIF2 α in comparison with untreated PAMs
408 are indicated in folds below the images after normalization with GAPDH. B. Real-time PCR
409 detection of PRRSV RNA in PAM cells. Relative levels of the viral RNA are shown as folds in
410 comparison with the 4 hpi sample from cells inoculated with UV-inactivated PRRSV.