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Short communication

Porcine reproductive and respiratory syndrome virus activates the transcription of interferon alpha/beta (IFN- α/β) in monocyte-derived dendritic cells (Mo-DC)

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is known to be a poor inducer of interferon alpha/beta (IFN- α/β), which may contribute to the delayed development of adaptive immunity and the resultant viral persistence. However, the exact mechanism by which PRRSV inhibits the induction of IFN- α/β during infection of its natural host cells remains less well defined. Here, we show that PRRSV efficiently activates the transcription of IFN- α/β in porcine monocyte-derived dendritic cells (Mo-DC) in a time-dependent and transient manner; and this effect is dependent on the activation of phosphatidylinositol 3-kinase (PI3K). Despite the abundant IFN- α transcripts detected in PRRSV-infected Mo-DC, little or no detectable IFN- α is found in the supernatants and cell lysates of PRRSV-infected Mo-DC, suggesting that PRRSV either blocks the translation of IFN- α or inhibits the RNA processing and transport. Furthermore, we observed that PRRSV infection significantly reduced the induction of IFN- α by Poly I:C treatment; and virus replication is essential to the effect since heat-inactivated PRRSV has no effect on IFN- α induction by Poly I:C. Overall, our data provide evidence for the possible role of PI3K in the activation of the transcription of IFN- α/β by PRRSV. We conclude that PRRSV inhibits the induction of IFN- α in Mo-DC by as yet undefined post-transcriptional mechanisms.

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1. Introduction

Interferon alpha/beta (IFN- α/β) production by virus-infected cells represents a critical part of host's innate immunity. IFN- α/β not only induces an antiviral state in cells by activating the expression of IFN stimulated genes (ISGs) but also modulates the subsequent development of adaptive immunity (Iwasaki and Medzhitov, 2004; Welsh et al., 2012). However, a number of viruses have evolved

strategies to block the induction of IFN- α/β by interfering with the transcriptional and/or translational regulatory mechanisms (Lyles, 2000; Mibayashi et al., 2007; Ruggli et al., 2005). In most of the cases, the interference with the IFN- α/β induction pathway contributes to the establishment of persistent virus infections in hosts (Haller et al., 2006).

Porcine reproductive and respiratory syndrome virus (PRRSV) is a plus-sense, single-stranded RNA virus with a genome length of approximately 15 kb. PRRSV infection is characterized by reproductive failure including abortion and stillborn piglets in infected pregnant sows and by acute respiratory disease in neonatal and young pigs. Approximately 10 million animals are infected with PRRSV each year in the US with the economic impact reaching 560 million dollars (Neumann et al., 2005). PRRSV primarily

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infects immune cells including alveolar macrophages and dendritic cells, which is believed to contribute to the immune suppression and viral persistence (Halbur et al., 1996; Suarez, 2000; Wang et al., 2007). PRRSV infections induce little or no IFN- α/β *in vivo* and *in vitro* (Albina et al., 1998; Buddaert et al., 1998). Studies have further suggested that PRRSV not only failed to induce IFN- α , but also was capable of blocking the IFN- α induction ability of other viruses such as porcine transmissible gastroenteritis coronavirus (TGEV) or dsRNA (Poly I:C) *in vitro* (Albina et al., 1998; Calzada-Nova et al., 2011; Miller et al., 2004). Overall, the existing evidence clearly suggests that PRRSV may have intrinsic properties to inhibit or reduce the induction of IFN- α/β . Several recent studies have shown the role of nonstructural proteins of PRRSV, especially Nsp1 α and Nsp1 β , in antagonizing the transcriptional activation of IFN- β *in vitro* (Beura et al., 2010; Chen et al., 2010; Kim et al., 2010; Song et al., 2010), but their relevance to the natural host cells such as macrophages and Mo-DC remains unknown. Here, we intended to explore the mechanisms by which PRRSV inhibits the induction of IFN- α/β in its natural host cells, porcine Mo-DC.

2. Materials and methods

2.1. Cells and viruses

Porcine monocyte-derived dendritic cells (Mo-DC) were prepared as described previously (Wang et al., 2007). Briefly, pig peripheral blood was obtained from the state-inspected South Dakota State University Meat Laboratory. Serum samples were examined for the presence of PRRSV specific antibodies using the IDEXX HerdChek[®] PRRS X3 Ab ELISA (Westbrook, MA) at the South Dakota State University's Animal Disease Research and Diagnostic Laboratory. Peripheral blood mononuclear cells (PBMC) were isolated from pig peripheral blood by density centrifugation using Histopaque-1077 (Sigma Inc., St. Louis, MO). CD14 positive monocytes were purified from PBMC by staining with mouse-anti-swine CD14 (AbD Serotec, UK) and goat-anti-mouse IgG microbeads (Miltenyi Biotec, Auburn, CA) followed by an immunomagnetic separation method. Purified CD14 positive monocytes were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin solution, 25 ng/ml of IL-4 (R&D systems Inc., Minneapolis, MN) and 10 ng/ml of GM-CSF (R&D systems Inc.) for 7 days at 37 °C in a 5% CO₂ atmosphere.

PRRSV-23983 was used in this study. It was propagated in MARC-145 cells and the supernatant of infected cells was collected and stored at –80 °C. Virus titers were determined by transferring a 10-fold serially diluted supernatant to MARC-145 cells and incubating for 5–7 days. 50% tissue culture infective doses were then calculated on the basis of the cytopathic effects caused by the virus-containing supernatants at different dilutions. Heat inactivated virus was obtained by incubating virus at 56 °C for 1 h. The loss of infectivity was confirmed by its inability to cause a cytopathic effect on MARC-145 cells.

2.2. Real-time RT-PCR

1×10^6 Mo-DC were either mock infected or infected with 0.05 MOI of PRRSV-23983. In some of the groups, cells were infected with heat-inactivated PRRSV-23983 to investigate the effect of virus replication on cellular gene transcription. In other groups, cells were treated with 25 μ g/ml poly I:C alone or together with either PRRSV-23983 or heat-inactivated PRRSV-23983. Cells treated with 1 μ g/ml Pam3Csk4, a TLR1/2 agonist, (Invivogen, San Diego, CA) were also included as a negative control. At 4, 12, 24, and 48 h after infection, cells were harvested and total RNAs were extracted using RNeasy protect mini kit (Qiagen, Valencia, CA) by following the manufacturer's instructions. The concentrations of RNA were determined using a NanoDrop ND-1000 spectrometer (Thermo Scientific). The same amount of RNA in each treatment group was reverse transcribed into complementary DNA (cDNA) using high-capacity cDNA reverse transcription kits (Applied Biosystem, Foster City, CA) by following the manufacturer's instructions. Real-time PCR was then performed on a Mx3000P Real-time thermocycler (Agilent Technologies). Primer sequences used in this study are shown in Table 1. For PCR amplifications, 4 μ l of cDNA was added to a mixture containing 10 μ l of SYBR green master mix (Brilliant II SYBR green QPCR master mix, Stratagene, LaJolla, CA), 0.3 μ l of ROX reference dye (Stratagene), 0.2 μ l of forward primer (50 pmol/ μ l) and 0.2 μ l of reverse primer (50 pmol/ μ l). All samples were tested in duplicate and the cycling conditions were 90 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. At the end of amplifications, cycle threshold (Ct) values were obtained. Transcripts of porcine GAPDH were also examined to normalize the amount of input RNA. Relative transcript levels were quantified by the $\Delta\Delta$ CT method as described previously (Guo et al., 2008).

2.3. ELISA for IFN- α

Mo-DC were treated as described above in 2.2. At 4, 12, 24, and 48 h after infection, supernatants and cells were both collected. Cytoplasmic extracts were prepared with NucleoBuster protein extraction kit (EMD Bioscience, San Diego, CA) by following the manufacturer's instruction. The concentration of IFN- α in both of the supernatants and cytoplasmic extracts were measured with a porcine IFN- α specific ELISA as previously described (Diaz de Arce et al., 1992). Briefly, HRP was conjugated to the K9 MAb (PBL InterferonSource Inc., Piscataway, NJ), a porcine IFN- α specific monoclonal antibody, with a labeling kit (Lightning

Table 1
Primers used for real time RT-PCR analysis.

Primers	5'-3' sequence	Gene accession #
GAPDH FP	AGG TCA TCC ATG ACA ACT TCG GCA	AF017079
GAPDH RP	AGC ACC AGT AGA AGC AGG GAT GAT	AF017079
IFN- α FP	ACT CCA TCC TGG CTG TGA GGA AAT	NM214393
IFN- α RP	ATC TCA TGA CTT CTG CCC TGA CGA	NM214393
IFN- β FP	TGC AAC CAC CAC AAT TCC AGA AGG	NM001003923
IFN- β RP	TCT GCC CAT CAA GTT CCA CAA GGA	NM001003923

link conjugation kit HRP, Novus Biological, LLC.). Each well of a flat-bottomed 96-well-plate was coated overnight at 4 °C with F17 MAb (PBL InterferonSource), a porcine IFN- α specific monoclonal antibody, at a concentration of 5 μ g/ml in 50 μ l ELISA coating buffer (eBioscience, San Diego, CA). Plates were then washed 3 times with PBST and non-specific binding sites were blocked by incubating plates with 1% BSA in phosphate buffered saline for 1 h at RT. After washing for 5 times, 100 μ l of IFN- α standards (Quantified recombinant porcine IFN- α , PBL InterferonSource) or samples were added to the wells in duplicates and incubated overnight at 4 °C. Following 5 washes, 100 μ l of HRP conjugated K9 MAb was added to each well at a concentration of 1 μ g/ml and incubated for 2 h at RT. Plates were washed for 7 times and 100 μ l of substrate solution (1 \times TMB substrate solution, eBioscience) was added to the wells. 15 min later the reaction was terminated by adding 50 μ l of 2 N H₂SO₄ to each well. The absorbance at 450 nm was then measured in Synergy 2 Multi-mode microplate reader (BioTek) and the concentration of IFN- α was calculated based on the standard curve generated using IFN- α standard.

2.4. Statistical analysis

The Student's *t*-test was used in this study. *P* values less than 0.05 were considered significantly different.

3. Results

3.1. PRRSV activates the transcription of IFN- α in Mo-DC

To further elucidate the mechanisms by which PRRSV interferes with the induction of IFN- α/β in its natural susceptible cells, we first examined the kinetics of IFN- α transcription in Mo-DC by real-time RT-PCR. The results showed that PRRSV induced a comparable amount of IFN- α transcripts as Poly I:C treatment in Mo-DC (Fig. 1A). Furthermore, PRRSV activated the transcription of IFN- α in a time dependent manner. The transcription of IFN- α started to increase at 12 h after infection and reached the highest level at 24 h after infection, which is about 12 h delayed compared to poly I:C treatment. The transcription of IFN- α in Poly I:C treated cells increased at 4 h after

treatment and reached the highest level at 12 h after treatment, then started to decline (Fig. 1A). As expected, Pam3CSK4 treatment down-regulated the transcription of IFN- α , suggesting that TLR1/2 activation possibly suppresses the IFN- α/β induction pathway (Fig. 1A).

3.2. PRRSV blocks the induction of IFN- α in Mo-DC via post-transcriptional mechanisms

We next examined whether IFN- α is actually produced by PRRSV infection of Mo-DC. The supernatants we collected from the above experiments were subjected to a quantitative ELISA to quantify the amount of IFN- α . No detectable IFN- α was observed at all time points in PRRSV infected Mo-DC (Fig. 1B). As expected, IFN- α was detected in Poly I:C treated Mo-DC at 12, 24 and 48 h after treatment. Pam3CSK4 treatment also did not induce any IFN- α production, which is consistent with the down-regulated IFN- α transcription (Fig. 1A). We also examined the intracellular level of IFN- α in PRRSV and Poly I:C treated groups at 24 and 48 h after treatment. Very low or no IFN- α was detected in PRRSV-infected cells (Fig. 1C). It is evident that PRRSV interferes with the induction of IFN- α via post-transcriptional mechanisms.

3.3. PRRSV activates the transcription of IFN- α in a PI3K dependent manner

Recently, we have observed that PRRSV activates the PI3K-dependent Akt during early infection and inhibits the PI3K/Akt during late infection in Mo-DC (Zhang and Wang, 2010). We have also shown that inhibition of PI3K/Akt by a PI3K specific inhibitor reduced PRRSV replication in Mo-DC (Zhang and Wang, 2010). Since some previous studies have suggested the possible role of PI3K/Akt activation in mediating the IFN- α induction in plasmacytoid dendritic cells (Guiducci et al., 2008), it is reasonable to speculate that PRRSV may activate the IFN- α transcription in a PI3K-dependent manner. We sought to determine whether PI3K/Akt activation actually contributes to the activated transcription of IFN- α by PRRSV. We infected Mo-DC with PRRSV in the presence of DMSO or LY294002, a PI3K specific inhibitor. At 4, 12, 24, 48 h after virus infection,

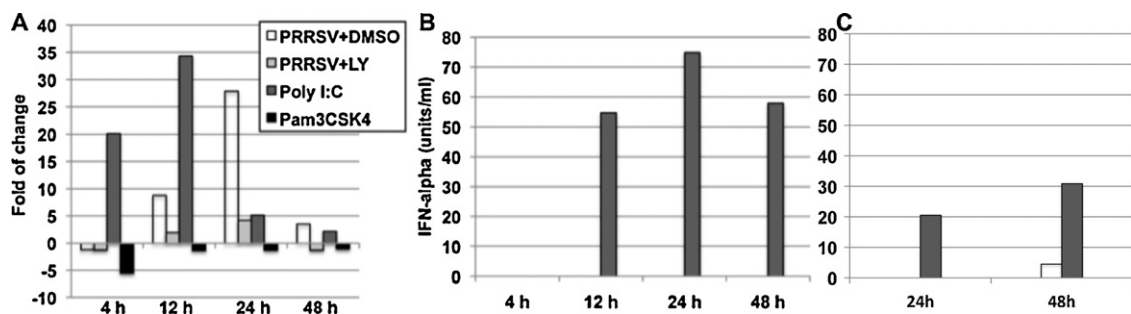


Fig. 1. PRRSV blocks the induction of IFN- α in Mo-DC via a post-transcriptional mechanism. A: PRRSV activates the transcription of IFN- α in a PI3K-dependent manner in Mo-DC. Mo-DC were infected with 0.05 MOI of PRRSV in the presence of DMSO (PRRSV + DMSO) or 20 μ M of LY294002 (PRRSV + LY). Mo-DC treated with Poly I:C and Pam3CSK4 were included as controls. At 4, 12, 24 and 48 h after infection, cells were harvested and used for RNA extraction and real-time RT-PCR as described in the materials and methods. B: Supernatants were collected from the same experiments and used in a quantitative ELISA to determine the production of IFN- α . C: Cell lysates from the same experiments were prepared and used in a quantitative ELISA to determine the production of IFN- α . Representative results of two experiments are shown.

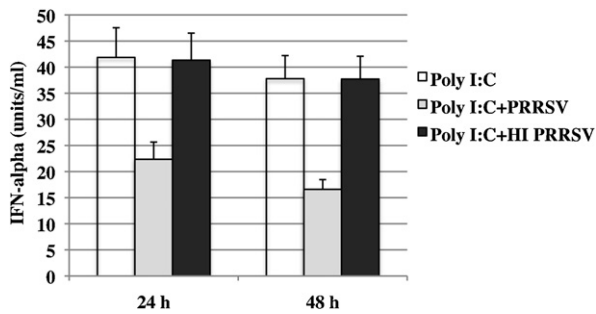


Fig. 2. Virus replication is essential to the reduced production of IFN- α induced by Poly I:C. Mo-DC were treated with Poly I:C alone, or treated simultaneously with Poly I:C and 0.05 MOI of live or heat-inactivated (HI) PRRSV-23983. At 24 and 48 h after treatment, supernatants were harvested and used in ELISA to quantify the amount of IFN- α . Averages and standard deviations of 6 replicates and 3 independent experiments are shown.

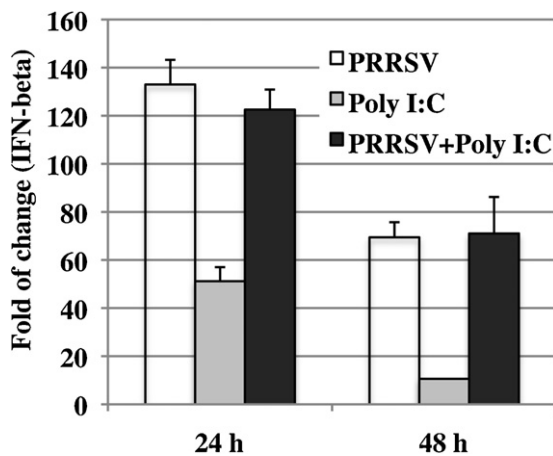


Fig. 3. PRRSV activates the transcription of IFN- β in Mo-DC. Mo-DC were infected with 0.05 MOI of PRRSV, treated with Poly I:C, or treated with Poly I:C and 0.05 MOI of PRRSV simultaneously. At 24 and 48 h later, cells were harvested and used for total RNA extraction. Real-time RT-PCR was performed to quantify the transcript level of IFN- β . GAPDH was used to normalize the gene transcript level. Data represents mean \pm standard deviation of three experiments.

cells were harvested for real-time RT-PCR. Supernatants were collected for IFN- α ELISA. We found that treatment of Mo-DC with a PI3K inhibitor prior to PRRSV infection dramatically reduced the transcription of IFN- α (Fig. 1A). This suggests that PRRSV activates the transcription of IFN- α in a PI3K dependent manner in Mo-DC.

3.4. PRRSV reduces the production of IFN- α by Poly I:C treated Mo-DC

Our data suggest that PRRSV inhibits the induction of IFN- α by as yet undefined post-transcriptional mechanisms. To further determine whether PRRSV is able to inhibit the induction of IFN- α by Poly I:C treated Mo-DC, we infected Poly I:C treated Mo-DC with 0.05 MOI of live or heat-inactivated PRRSV. At 24 and 48 h later, the production of IFN- α in the supernatants of treated cells was determined by a quantitative ELISA assay. As shown in Fig. 2, the amount of

IFN- α was significantly lower in PRRSV infected group compared to the Poly I:C treated group ($P = 0.0003$) and Poly I:C plus heat-inactivated PRRSV treated group ($P = 0.0001$) at 24 h after infection. A similar significant reduction of IFN- α in the PRRSV infected group was observed at 48 h after infection ($P = 0.000004$). This result suggests that virus replication is essential to the reduction of IFN- α induced by Poly I:C.

3.5. PRRSV activates the transcription of IFN- β in Mo-DC

Several recent studies have shown the role of non-structural proteins in inhibiting the transcriptional activation of IFN- β in MARC-145 and human cell culture systems (Beura et al., 2010; Chen et al., 2010; Kim et al., 2010; Song et al., 2010). We intended to examine whether PRRSV is also capable of activating the transcription of IFN- β in Mo-DC. As shown in Fig. 3, PRRSV efficiently activated the transcription of IFN- β in a time-dependent and transient manner. No synergistic effect in IFN- β transcription was observed between PRRSV infection and Poly I:C treatment. A similar phenomenon was observed for IFN- α (data not shown). Due to the lack of porcine IFN- β specific antibodies for use in ELISA or Western blot to detect the protein level of IFN- β , we are not sure whether IFN- β is also blocked at the post-transcriptional step.

4. Discussion and conclusion

Numerous previous studies have consistently shown that PRRSV is a poor inducer of IFN- α/β both *in vitro* and *in vivo* (Albina et al., 1998; Buddaert et al., 1998; Calzada-Nova et al., 2011). However, the exact mechanism by which PRRSV inhibits the induction of IFN- α/β remains elusive, especially in porcine susceptible cells such as alveolar macrophages and Mo-DC. Although several recent studies have suggested the role of nonstructural proteins of PRRSV, especially Nsp1 α and Nsp1 β in antagonizing the promoter activity of IFN- β in MARC-145 and human cell culture systems (Beura et al., 2010; Chen et al., 2010; Kim et al., 2010; Song et al., 2010), their relevance to the PRRSV naturally susceptible porcine cells remains uncertain. More intriguingly, a couple previous studies have clearly demonstrated that PRRSV activated the transcription of IFN- α/β *in vitro* and *in vivo* as evidenced by the increased mRNA molecules detected by real-time RT-PCR (Chung et al., 2004; Lee et al., 2004). A similar observation was reported for mouse hepatitis virus (MHV) (Rose and Weiss, 2009). Although the nucleocapsid protein and two nonstructural proteins (nsp1 and nsp3) of MHV have been reported to antagonize the IFN promoter activity, IFN is induced in primary cell types such as plasmacytoid dendritic cells and macrophages. Here, we have observed that PRRSV efficiently activated the transcription of IFN- α/β in porcine Mo-DC. Abundant and a comparable level of IFN- α/β transcripts was induced by PRRSV and Poly I:C in Mo-DC. However, PRRSV blocked the induction of IFN- α in Mo-DC by as yet undefined post-transcriptional regulatory mechanisms. This agrees with the conclusions of the study on porcine alveolar macrophages by Lee et al. (Lee et al., 2004). Our results are contradictory to an earlier study, which showed that neither

Poly I:C nor PRRSV activated the transcription of IFN- α in Mo-DC at 12 and 24 h after treatment (Loving et al., 2007). Our results also support the conclusion that virus replication is essential to the inhibition of IFN- α/β induction by PRRSV as reported by Albina et al. (Albina et al., 1998), but contradictory to the results of a more recent study which showed that virus replication is not necessary to the suppressive activity of IFN- α/β induction by PRRSV (Calzada-Nova et al., 2011) in plasmacytoid dendritic cells. We have further demonstrated that the increased transcription of IFN- α is mainly mediated by the activation of PI3K by PRRSV since the use of a PI3K specific inhibitor prior to PRRSV infection significantly reduced the transcripts of IFN- α upon PRRSV infection. This is in agreement with the previous study showing the critical role of PI3K mediated IRF-7 in the induction of IFN- α in plasmacytoid dendritic cells by CpG ODN (a TLR9 ligand) and influenza A virus (a TLR7 ligand) (Guiducci et al., 2008). Our previous study has suggested that PRRSV activated the PI3K/Akt pathway during early infection to facilitate virus replication process. Here, we have further shown the positive correlation between PI3K activation and increased transcription of IFN- α . It seems that the PI3K/Akt pathway may be important in both virus's survival and replication and host's innate defense mechanism, suggesting a delicately controlled balance between virus's offense and host's defense mechanisms. A very similar role of PI3K/Akt in both virus replication and host defense has been suggested previously for influenza A virus (Ehrhardt et al., 2006).

In summary, we have demonstrated the possible role of PI3K in PRRSV mediated transcriptional activation of IFN- α/β in Mo-DC. Additionally, we have provided evidence for the novel post-transcriptional regulation of IFN- α induction exerted by PRRSV. Future studies will be focused on delineating the exact post-transcriptional mechanisms utilized by PRRSV to block the induction of IFN- α in infected Mo-DC and alveolar macrophages.

Conflict of Interest

The authors declare no conflict of interest.

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