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Short communication

Porcine reproductive and respiratory syndrome virus inhibition of interferon-β transcription by IRF3-independent mechanisms in MARC-145 cells in early infection

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ABSTRACT

Interferon β is an important antiviral molecule whose expression is triggered through recognition of viral components by pattern recognition receptors via a cascade of signaling molecules, while viruses could target these molecules to evade from innate immunity. IFN regulatory factor 3 (IRF3) plays a crucial role in innate immune responses. Here, we demonstrate that PRRSV infection did not induce IFN-β gene transcription in MARC-145 cells, but inhibited poly (I:C) stimulated IFN-β gene transcription instead. Such inhibition is time-dependent with the progression of PRRSV infection. We also show that the inhibition of IFN-β transcription in the early stage of infection could not be due to inhibition of phosphorylation and nuclear translocation of IRF3, though significant decrease of p-IRF3 and its nuclear translocation in PRRSV-infected and poly (I:C) cells was observed later at 48 h post-infection. The different patterns of inhibition for IFN-β transcription and IRF3 phosphorylation have important implications as to the mechanism(s) by which PRRSV suppresses the type I IFN signaling at early stage of infection. There could be mechanism(s) other than effecting on IRF3 or molecules upstream that require further investigation.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease characterized by reproductive failure in sows and respiratory disease in pigs of all ages (Botner et al., 1997). The causal virus PRRSV could escape from innate immunity and causes persistent infections (Miller et al., 2004). Type I IFNs are important against PRRSV infection (Albina et al., 1998; Overend et al., 2007). However, PRRSV was found to be a poor IFN-inducer as compared with porcine respiratory coronavirus (Buddaert et al., 1998).

Expression of type I IFNs are affected by transcription factors, such as interferon regulatory factor 3 (IRF3), IRF7 and nuclear factor-κB (NF-κB) upon recognition of pathogen-associated molecular patterns (PAMP) by pattern-recognition receptors (PRR) (Akira et al., 2006; Bowie and Haga, 2005). IRF3 and NF-κB are also activated by post-translational modification (Honda and Taniguchi, 2006). A number of viruses develop different mechanisms to inhibit expression of IFNs or antagonize their actions (Goodbourn et al., 2000; Miller et al., 2004). The immediate early protein ORF62 of Varicella-zoster virus blocks IRF3 phosphorylation to disarm the IFN-dependent antiviral defense (Sen et al., 2010), while its ORF61 protein antagonizes the IFN-β pathway by degrading IRF3 (Zhu

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et al., 2011). PRRSV NSP1 was found to strongly inhibit phosphorylation and nuclear translocation of IRF3 induced by dsRNA or Sendai virus (Beura et al., 2010). Others suggested that PRRSV NSP1 did not appear to modify IRF3 phosphorylation or to prevent its nuclear translocation, but rather to block its full activation with subsequent inhibition of assembly of the IFN enhancers through degrading histone acetyltransferase nuclear protein CBP in the nucleus (Eksioglu et al., 2011; Kim et al., 2010). Therefore, definite mechanisms by which PRRSV inhibits expression of IFN-β are poorly understood. Given the apparent contradictory results regarding the role of PRRSV in evading IRF3-mediated responses, we assessed the effects and patterns of PRRSV on IFN-β expression and IRF3 activation over an extended period of time in MARC-145 cells treated with poly(I:C).

2. Materials and methods

2.1. Virus and cells

The PRRSV JX07 isolated from a clinically diseased pig is the North American genotype strain (Hu et al., 2009). MARC-145 cells were cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle medium (DMEM, Gibco-Invitrogen, CA, USA) supplemented with 8% fetal bovine serum (FBS, Gibco-Invitrogen).

2.2. Viral infection and transfection

MARC-145 cells seeded in 24-well plates were infected with PRRSV at a multiplicity of infection (MOI) of 0.1 (except for those explained in figure captions). At indicated time points, the cells were transfected with poly(I:C) (0.5 μg/ml) (InvivoGen, San Diego, CA, USA) using Lipofectamine 2000 (Invitrogen) for 10 h before being harvested for IFN-β transcription analysis, Western blotting or immunofluorescence assay.

2.3. Quantitative PCR

Total RNA was extracted with RNA kit (Tiangen Biotech Co. Ltd., China) from the cell lysates. cDNA was synthesized with oligo(dT_{15}) (Takara Biotech Co., Ltd., China) and M-MLV reverse transcriptase (Promega). Quantitatve PCR was performed in triplicate using SYBR Green I dye (Takara) on a Bio-Rad iQ5 system. Primers used for qPCR are listed in Table 1. Results for the target gene were presented after normalization to β-actin. Relative transcription levels were quantified by the $2^{-\Delta\Delta CT}$ method and shown as relative fold changes in comparison with mock-treated control (Livak and Schmittgen, 2001). To quantitate PRRSV RNA replication levels, dilutions of plasmids (10^0–10^8 copies) containing the PRRSV subgenomic ORF7 were used to construction standard curves and run in parallel with viral cDNA or cellular β-actin gene from the cells (primers in Table 1).

2.4. Immunofluorescence assay

Immunofluorescence assay (IFA) was performed to examine subcellular localization of IRF3 in MARC-145 cells. The cells treated as above were harvested, fixed with 80% acetone for 20 min at −20 °C, and then probed with an IRF3-specific rabbit polyclonal antibody (sc-9082) (Santa Cruz Biotechnology, CA, USA) and a murine monoclonal antibody to PRRSV-NSP2 (mAb1D8, prepared in our laboratory and suitable for immunofluorescence and Western blotting with titer from 1:5000–1:10,000) for 60 min at 37 °C. The bound antibodies were revealed by incubation with an Alexa Fluor 488-labeled goat anti-mouse IgG and an Alexa Fluor 555-labeled goat anti-rabbit IgG (Invitrogen) for 60 min at 37 °C. Cells were counterstained with 4′,6-diamidino-2-phenylindol (DAPI, Invitrogen) for 5 min at room temperature and examined with a fluorescent microscope at 320× magnification.

2.5. Western blot analyses

The MARC-145 cells were lysed in cell lysis buffer (Beyotime Biotechnol, Shanghai, China) supplemented with 1× protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma–Aldrich, MO, USA). Whole cell lysate proteins, fractionated nuclear or cytoplasmic proteins were quantified using the BCA assay and prepared for SDS-PAGE/Western blot analyses as described elsewhere (Zhu et al., 2012). Specific antibodies used included those to IRF3 (4302s, rabbit mAb D83B9 to detect total IRF3), phospho-IRF3 (4947s, rabbit mAb 4D4G to detect phosphorylation at Ser396) (Cell Signaling Technology Inc., MA, USA), tubulin, histone H3 and β-actin (Santa Cruz Biotechnology, CA, USA) as well as a monoclonal antibody to PRRSV-N protein.

2.6. Statistical analysis

Two-tailed Student's *t*-test was performed using Microsoft Excel software (2003).

3. Results and discussion

Previous studies suggest that PRRSV does not induce, or actively suppresses type I IFN induction (Beura et al., 2010; Miller et al., 2004). We did not find significant IFN-β expression in virus infected cells throughout the infection period, which was in sharp contrast with marked increase of IFN-β transcription in poly (I:C) stimulated cells (Fig. 1), suggesting that PRRSV did not seem to initiate PRR signaling in MARC-145 cells. The virus also did not activate IFN-β promoter activity as revealed by the dual luciferase assay (Promega) (data not shown). The 5′ end of the PRRSV genome is reported to be methyl-capped (Sagripanti et al., 1986; Yoo et al., 2004). Similar to coronavirus (Zust et al., 2011) or dengue virus (Rodriguez-Madoz et al., 2010), PRRSV might hide or modify their 5′-RNA structures to escape from innate immune recognition such as by RNA capping. Another possibility could be that PRRSV might be able to induce IFN-I signaling in infected cells via its dsRNA replication intermediate, but could be inhibited by its nonstructural or structural proteins produced during

Table 1
The primer pairs used for this study.

Gene	Primer	Sequence (5′–3′)
4 × PRDIII	Template	CAATACGGGGTACCGGAAAACTGAAAGG GAAAACTGAAAGGGAAAACTGAAAGGGAAAACTGAAAGGTCTGAATAGAGAGA
	Forward	GTACCAATACGGGGTACCG
	Reverse	CCCAAGCTTAGCAAGTTGTAGCTCATG
IFN-β promoter	Forward	GTACCAATACGGGGTACCTGCCTTCTGAGTTCTCCATC
	Reverse	CCCAAGCTTAGCAAGTTGTAGCTCATG
IFN-β (real time PCR)	Forward	TAAGCAGCTGCAGCAGTTCCAGAAG
	Reverse	GTCTCATTCCAGCCAGTGCT
β-Actin (real time PCR)	Forward	CGTGCGTGACATCAAAGAGAAG
	Reverse	CGTTGCCAATAGTGATGACCTG
PRRSV (real time PCR)	Forward	ATGGCCAGCCAGTCAATC
	Reverse	TCAGTCGCTAGAGGAAAATGG

infection. This has been seen with hepatitis C virus (Cheng et al., 2006). However, the mechanisms by which PRRSV deploys for evasion, though extensively studied in recently years, remain to be determined.

We used poly (I:C) as an inducer of type I IFN transcription in MARC-145 cells and analyzed the IFN-β expression patterns during PRRSV infection. We found that even high MOI up to 4.0 did not stimulate IFN-β expression, while MOI as low as 0.1 suppressed poly (I:C)-induced IFN-β gene transcription (Fig. 2A). Such inhibition was time-dependent, being markedly significant with PRRSV infection from 12 to 60 h post-infection (hpi) (Fig. 2B).

To further investigate this inhibitory mechanism, we analyzed IFN-β gene transcription and IRF3 phosphorylation in response to poly(I:C) stimulation over an extended period of time from 12 to 60 hpi, instead of examining activation of the type I IFN signaling pathway between 30 and 40 h (Kim et al., 2010; Luo et al., 2008). IRF3 was examined because it is a key cellular transcription factor in the type I IFN pathway (Iwamura et al., 2001). IRF3 usually retains in the cytoplasm of unstimulated cells. Upon stimulation of the cells, such as infection by viruses, it is phosphorylated (Panne et al., 2007b; Yoneyama et al., 2004), dimerized and translocates to the nuclei where it forms complex with CBP/p300 (Panne et al., 2007a; Wathelet et al., 1998). Such nuclear recruitment promotes histone acetylation and changes chromatin architecture that are permissive to transcriptional activation (Sharma and Nyborg, 2008). Although our results were in general

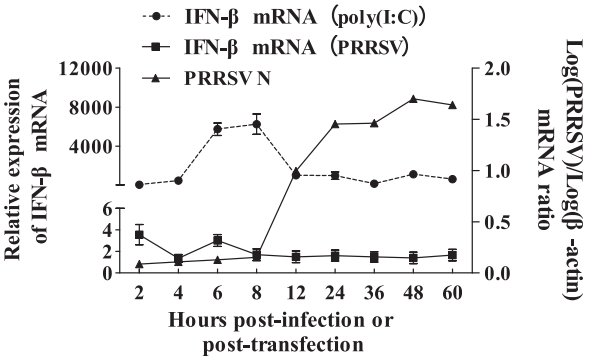

Fig. 1. PRRSV infection did not activate IFN-β gene transcription. Quantification of cellular IFN-β transcription by RT-qPCR presented as fold induction relative to the basal level in mock-infected cells (MOI = 0.1). Data represent mean ± SD of three replicates of a typical experiment from four repeated experiments.

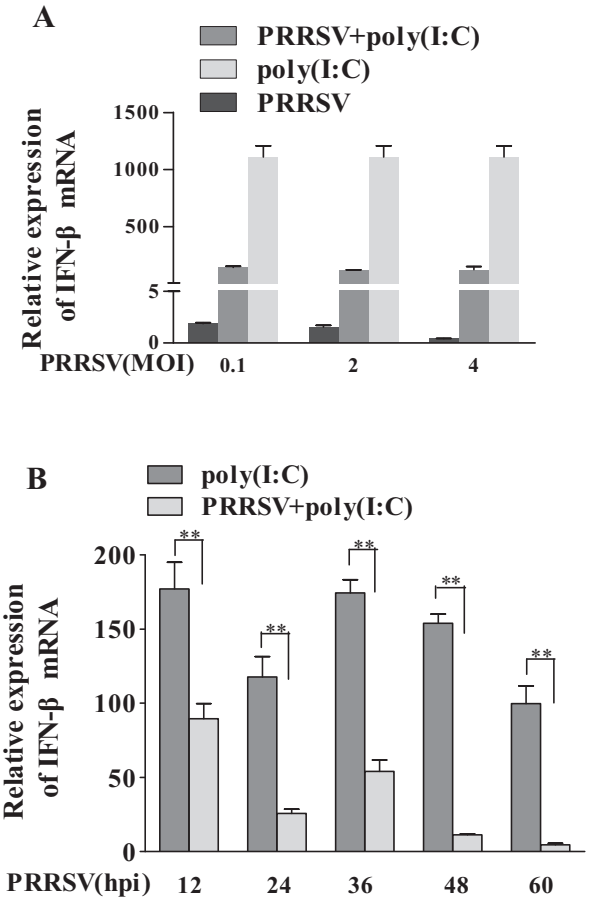

Fig. 2. PRRSV blocked poly(I:C)-induced IFN-β gene transcription in MARC-145 cells. MARC-145 cells were infected with PRRSV for 36 h at different MOI (A) or with 0.1 MOI for different times (B). Poly(I:C) was then transfected for 10 h, and total cellular RNA was extracted for quantitative PCR analysis of IFN-β gene transcription. Data represent mean ± SD of three experiments, each in triplicate (*$P < 0.05$; **$P < 0.01$, same for Fig. 3).

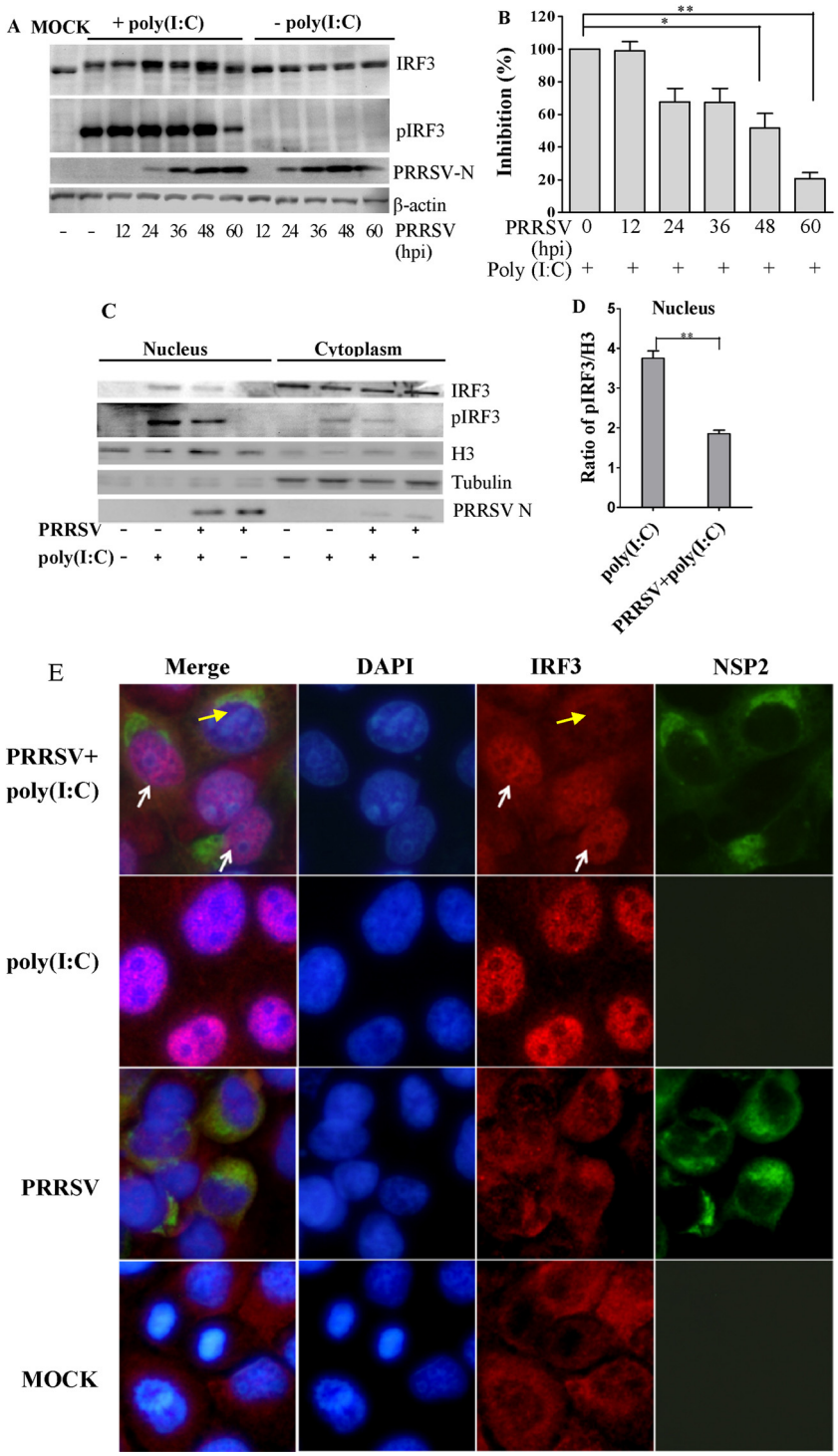

Fig. 3. Effects of PRRSV infection on IRF3 phosphorylation and nuclear translocation in MARC-145 cells treated with poly(I:C). MARC-145 cells were infected with PRRSV at 0.1 MOI for indicated times (A and B) or for 50 h (C–E) and poly(I:C) was transfected for 10 h (60 h of infection altogether). The cells were harvested for SDS-PAGE/Western blotting to visualize the total and phospho-IRF3 (ser-396) levels (A) or density-based quantification of the changes of relative ratios of pIRF3 to IRF3 over time with the poly(I:C)-induced mock control set as 100% (B). Nuclear and cytoplasmic proteins were resolved by SDS-PAGE/Western blotting (C) for total and phospho-IRF3 (ser-396) levels as well as for quantification of the ratios of pIRF3 inside the nuclei to the nuclear protein histone H3 (D). Alternatively, treated cells were fixed and incubated with anti-IRF3 and anti-PRRSV NSP2 antibodies for immunofluorescence analysis (E): white arrows indicate the PRRSV infected cells with IRF3 nuclear translocation, and the upper yellow arrows indicate the PRRSV infected cell without IRF3 nuclear translocation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

agreement with several previous reports that PRRSV was inhibitory to poly(I:C)-induced IFN-I signaling (Kim et al., 2010; Luo et al., 2008), we found that PRRSV exhibited different patterns of inhibition: significant inhibition of IFN-β gene transcription seen as early as 12 hpi, marked down-regulation of IRF3 phosphorylation from 48 hpi. Such inhibition became more pronounced with the progression of viral infection up to 60 h (Fig. 3A and B). Moreover, suppression of IRF3 phosphorylation or its nuclear translocation was still partial at 60 hpi (Fig. 3C–E). The different patterns of inhibition for IFN-β transcription and IRF3 phosphorylation have important implications as to the mechanism(s) by which PRRSV suppresses the type I IFN signaling at early stage of infection (e.g. within 24 h). There should be mechanism(s) other than effecting on IRF3 or molecules upstream. It may do so either through degradation of CBP (Han et al., 2012; Kim et al., 2010), or by decreased IRF3 nuclear translocation (Beura et al., 2010), or possibly by direct blocking of IRF3 binding to the IFN-β promoter region within the nucleus which is yet to be studied in the future.

In summary, PRRSV could abrogate INF-β transcription in MARC-145 cells stimulated by poly(I:C). The different profiles for IFN-β, its promoter/enhancer activity, and IRF3 phosphorylation indicate complexity of the host–virus interactions. Further research should focus on the mechanisms of lack of type I IFN responses to PRRSV infection as well as the signaling pathways other than IRF3 that suppress IFN-β expression to poly(I:C) induction at early stages of infection in different host cell types where PRRSV is persistent.

Conflict of interests

The authors have declared no conflict of interests.

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