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ORIGINAL ARTICLE

The activation of the IFNβ induction/signaling pathway in porcine alveolar macrophages by porcine reproductive and respiratory syndrome virus is variable

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Abstract
Background It has been recognized that the expression of type I interferon (IFNα/β) may be suppressed during infection with porcine reproductive, respiratory syndrome virus (PRRSV). This causes profound negative effects on both the innate and adaptive immunity of the host resulting in persistence of infection.
Objective Test the effects of PRRSV infection of porcine alveolar macrophages (PAMs), the main target cell, on the expression of interferon beta (IFNβ) and downstream signaling events.
Methods In order to examine those effects, PAMs harvested from lungs of healthy PRRSV-free animals were infected with virulent, attenuated, infectious clone-derived chimeric viruses, or field PRRS virus strains. Culture supernatants from the infected PAMs were tested for IFNβ protein expression by means of indirect ELISA and for bioactivity by a vesicular stomatitis virus plaque reduction assay. The expression of the Mx protein was assayed to ascertain signaling events.
Results These experiments demonstrated that PRRSV does induce variably, the expression of bioactive IFNβ protein in the natural host cell. To further elucidate the effects of PRRSV infection on IFNβ signaling, Mx-1 an interferon stimulated gene (ISG), was also tested for expression. Interestingly, Mx-1 expression by infected PAMs generally correlated with IFNβ production.
Conclusion The results of this study demonstrate that the induction of IFNβ and signaling in PAMs after PRRSV infection is variable.

Keywords Porcine reproductive respiratory syndrome virus · Interferon beta · Mx-1 innate immunity

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Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive sense, single-stranded RNA virus in the *Arteriviridae* family within the order Nidovirales (Cavanaugh 1997). PRRSV causes respiratory disease in neonatal pigs and reproductive failure in pregnant sows. The disease has devastating effects on the swine industry worldwide because of reduced birth rates with high mortality of offspring from infected sows, as well as lost reproductive cycles. [Christianson et al. 1992]. Direct losses due to PRRS amount to over $664 million yearly to U.S. farms alone (Holtkamp et al . 2013).

Evasion of type I IFN (IFNα/β) innate responses and delayed and relatively inefficient adaptive responses are observed in PRRS. These deficiencies include reduced or lack of interferon alpha (IFNα) (Miller et al. 2004; Loving et al. 2007); blocking of IFNβ at both the inductive and signaling phases (Darwich et al. 2010; Yoo et al. 2010); mutations on key viral ORFs promoted by swIFNβ that may play a role in evasion from its effects (He et al. 2013). Delayed and suboptimal neutralizing antibody and IFN gamma (IFNγ) responses; induction of regulatory T cells (Tregs) (Wongyanin et al. 2010; Silva-Campa et al. 2012; Huang and Meng 2010)

escape variants from the host immune response, and immunosuppression leading to persistent infections (Murtaugh et al. 2002) are also recognized. In addition to the generation of an innate antiviral state (Le Bon et al. 2001; Van Uden et al. 2001) type I IFN responses are crucial for linking the innate and adaptive immune responses.

Interferon beta (IFNβ), is produced and secreted in response to viral infections of host cells. Once secreted, IFNβ binds to type I IFN receptor (IFNAR) on cells to initiate a signaling that activates the JAK/STAT pathway. This results in transcription and translation of approximately 300 interferon stimulated genes (ISGs) including Mx-1, PKR, 2′,5′-OAS, ISG15, ISG20, as well as IFNα and a second wave of IFNβ (Friedemann and Haller 2007). PRRSV induction of IFNα in MARC-145 cells and porcine alveolar macrophages (PAMs) is variable and strain-dependent (Lee et al. 2004). The induction of IFNβ mRNA expression during PRRSV infection remains somewhat controversial as it was demonstrated to occur in some studies (Miller et al. 2004; Loving et al. 2007; Genini et al. 2008; Gudmundsdottir and Risatti 2009) but not in others (Song et al. 2010; Li et al. 2010; Kim et al. 2010). These data underline the notion that different effects may reflect true differences between strains, rather than being absolute observations applicable to all strains. Therefore, it is of interest to examine further the expression of IFNβ in response to PRRSV infection with several strains and determine how this expression correlates with downstream signaling. It has been observed that poly I:C induced IFNβ pathway is strongly inhibited by PRRSV non-structural protein 1 (nsp1) although there was a weak activation of the IFNβ response to the live virus via IRF3 phosphorylation (Shi et al. 2010). This highlights the importance of studying the effects of both infectious virus versus isolated proteins. In spite of the attention given to the IFNβ response pathways, direct demonstration of IFNβ protein expression in PAMs is hampered by a lack of commercially available reagents for swine IFNβ protein detection. Research on IFNβ induction by viral infection of the natural swine host cells, will provide biologically relevant insights on the mechanisms and pitfalls behind it.

Recent studies in our laboratory demonstrate that some PRRSV strains are sensitive to the antiviral effects of exogenous IFNβ, whereas others are moderately to highly resistant in MARC-145 cells, but appear to be equally and highly sensitive in PAMs (Overend et al. 2007; He et al. 2011). These marked host cell differences in type I IFN effects (He et al. 2011) underscore the necessity of using a system that most closely replicates the natural host. The differences between strains in induction of or sensitivity to type I IFN provide a potential model to examine mechanisms of subversion of the host innate immune response. In the present study a set of infectious clone-derived parental, chimeric viruses (Kwon et al. 2006), and field strains of PRRSV were examined for activation of type I IFN induction/signaling pathway in PAMs. This investigation demonstrated that PRRSV is capable, to varying degrees and depending on the virus strain being tested, of inducing expression of bioactive IFNβ in PAMs. the natural host cell system. Expression of IFNβ and downstream signaling effects in PRRSV infected PAMs are briefly discussed.

Materials and methods

Viruses and cells

NVSL PDV30–9301 (9301), MO-8981, and strain VR-2332, and a series of chimeric PRRS viruses, kindly provided by Drs. F. Osorio and A. Pattnaik (University of Nebraska, Lincoln, NE), were used. The construction and characterization of the chimeric viruses was described before (Kwon et al. 2008). Briefly, the infectious clone derived chimeric viruses are based on a highly pathogenic (FL-12) virus backbone replaced with genomic segments of an attenuated vaccine strain of virus (PP-18). Eight FL-12/PP-18 chimeric viruses designated here CH1-CH5 or "non-structural chimeras" are those carrying PP-18 nsp genes 1–12 and CH-6,CH-7 and CH-67 or "structural chimeras" those carrying ORFs 2–7.

Porcine alveolar macrophages (PAMs) were collected by bronchial lavage from the lungs of healthy 4–6-week old castrated male Landrace/Yorkshire cross piglets. Donor animals were determined to be PRRSV-free both by RT-PCR and serology prior to testing. A total of 6 PAM donor animals were used for this study. The use of experimental animals was under an approved IACUC protocol.

Infection and sampling of PAMs

PAMs donated by healthy PRRSV-free animals as described above were cultured in 6-well tissue culture plates in RPMI media supplemented with glucose, 2× antibiotics, and 10 % FBS. For induction assays, PAMs were infected with PRRSV at different MOIs, 24 h after seeding the cells into the plates. Culture supernatants were sampled at 12, 24, and 48 h post-infection (hpi) for analysis of secreted cytokines.

Plaque assay

Vesicular stomatitis virus (VSV) plaque assays were performed on confluent monolayers of Vero cells seeded in 6-well plates. Ten-fold dilutions of stock virus were inoculated to the cells and incubated for 1.5 h at 37 °C. The inoculum was removed, and wells were overlaid with DMEM containing 0.8 % agarose, and incubated until plaques were visible, about 48 h later. Agarose overlays were removed, and the cells were fixed with 90 % ice-cold acetone, and stained with 1 % crystal violet in methanol.

IFNβ bioassay

Bioassays were performed essentially as described before with some modifications (Overend et al. 2007). To induce IFNβ, PAMs were infected with PRRSV, as described above, culture supernatants were collected, acid treated overnight to pH 2 at 4 °C, and subsequently the pH was readjusted back to 7. These acid-treated supernatants were then placed on fresh Vero cell monolayers and incubated at 37 °C for 18 h. The cells thus primed, were then infected with 100 PFU/well of VSV for 1.5 h, the monolayers were overlaid with agar, and after incubation for several days at 37 °C were fixed and stained. The numbers of plaques were compared to those counted in wells treated with culture supernatants from uninfected PAMs (100 % plaques). To test the specificity of the antiviral activity, culture supernatants of PRRSV-infected PAMs were incubated with either an in-house made anti-swine IFNβ monoclonal antibody (mAb) or an anti-swine IFNα mAb (AbCam Cambridge, MA) overnight at 4 °C, at a concentration of 5 μg/ml prior to priming Vero cells and infecting them with VSV as described above.

Enzyme linked immunosorbent assay

Secreted IFNβ and Mx-1 were detected in culture supernatants by indirect ELISA using a mouse anti-swine IFNβ monoclonal antibody, produced in our laboratory, or an anti-swine Mx-1 antibody from (AbCam Cambridge, MA) respectively as primary antibodies. Briefly, 96-well plates were coated overnight at 4 °C with culture supernatants from infected PAMs. The plates were washed 3 times with phosphate buffered saline/Tween-20 (PBS-T), and blocked for 3 h using 5 % BSA fraction V. Primary antibodies were added at appropriate concentrations, and incubated for 1 h at room temperature. After washing, goat-anti-mouse IgG-HRP was added to the wells and incubated for 1 h at room temperature. Plates were washed 6 times prior to adding the substrate. TMB well substrate (KPL, Gaithersburg, MD) was added to the wells, and incubated in the dark for 20 min, and then a 1 N H_2SO_4 stop solution was added. Absorbance was measured using a Biotek microplate reader (Biotek Instruments Inc., Winooski, VT) at a wavelength of 450 nm.

Statistical analysis

Statistical significance in all experiments was determined using paired t-test. The correlation coefficient was calculated using GraphPad Prism (version 5.0).

P-values under 0.05 were considered significant.

Results

Expression of IFNβ protein by PAMs is variable

PAMs were infected at an MOI of .02 as infection with MOIs >.02 did not induce any greater expression of IFNβ. Poly I:C served as a positive control. Twelve hours post-infection, significant increases in IFNβ were detected in poly I:C stimulated cells, and cells infected with chimeras CH-4, and CH-67. Cells infected with strains 9301, VR-2332 or chimeric viruses CH-1 or CH-6 had significantly lower expression compared to unstimulated cells (Fig. 1a). The cells infected with the parental viruses (virulent FL-12 and attenuated PP-18), had increased levels at 12 hpi although these were not statistically significant. However, at 24 hpi, FL-12 and PP-18 induced significant increases in the amount of IFNβ secreted, which persisted up to 48 hpi (Fig. 1c and e). Chimeras CH-7 and CH-67 also induced significant IFNβ responses at 24 hpi, which returned to baseline levels by 48 hpi. At none of the time-points tested did the field strains VR-2332, 9301, MO-8981 induced statistically significant IFNβ responses. Nevertheless, in comparison to the other field strains tested, strain MO-8981 induced higher levels of IFNβ and the level at 48 hpi was comparable to that induced by PP-18. Cells infected with CH-1 or strain 9301 at 12 hpi or CH-6 at 48 hpi, yielded significant lower IFNβ levels compared to those of unstimulated cells.

Mx-1 expressed by infected PAMs roughly follows IFNβ production

Mx-1 was detected in culture fluids of PRRSV-infected or poly I:C treated cells by ELISA (Fig. 1). Infection with chimera CH-4 or poly I:C treatment of PAMs induced a significant increase in Mx-1 protein expression, whereas infections with chimera CH-6 or strains 9301 and VR-2332 showed significantly decreased Mx-1 expression at 12 hpi (Fig. 1b) . At 24 hpi, an increase in Mx-1 expression was detected with parental viruses FL-12 or PP-18 (Fig. 1d), which continued to 48 hpi (Fig. 1f) and roughly coincided with IFNβ increases. Infection with chimeras CH-3, CH-4, CH-5, Ch-7 and CH-67 resulted in an increased expression of Mx-1 at 24 hpi (Fig. 1d) although the increases were only statistically significant in CH-4 infected cells. By 48 hpi, the levels of Mx-1 protein were consistently lower than background levels observed in unstimulated cells with most strains tested in repeated experiments (Fig. 1f). Individual levels of Mx-1 generally followed the corresponding levels of IFNβ produced (Fig.1) and showed a level of direct correlation (Fig. 2a, b, and c). Levels of Mx-1 induced by exogenous recombinant swine IFNβ were similar to those observed in poly I:C treated cells (data not shown).

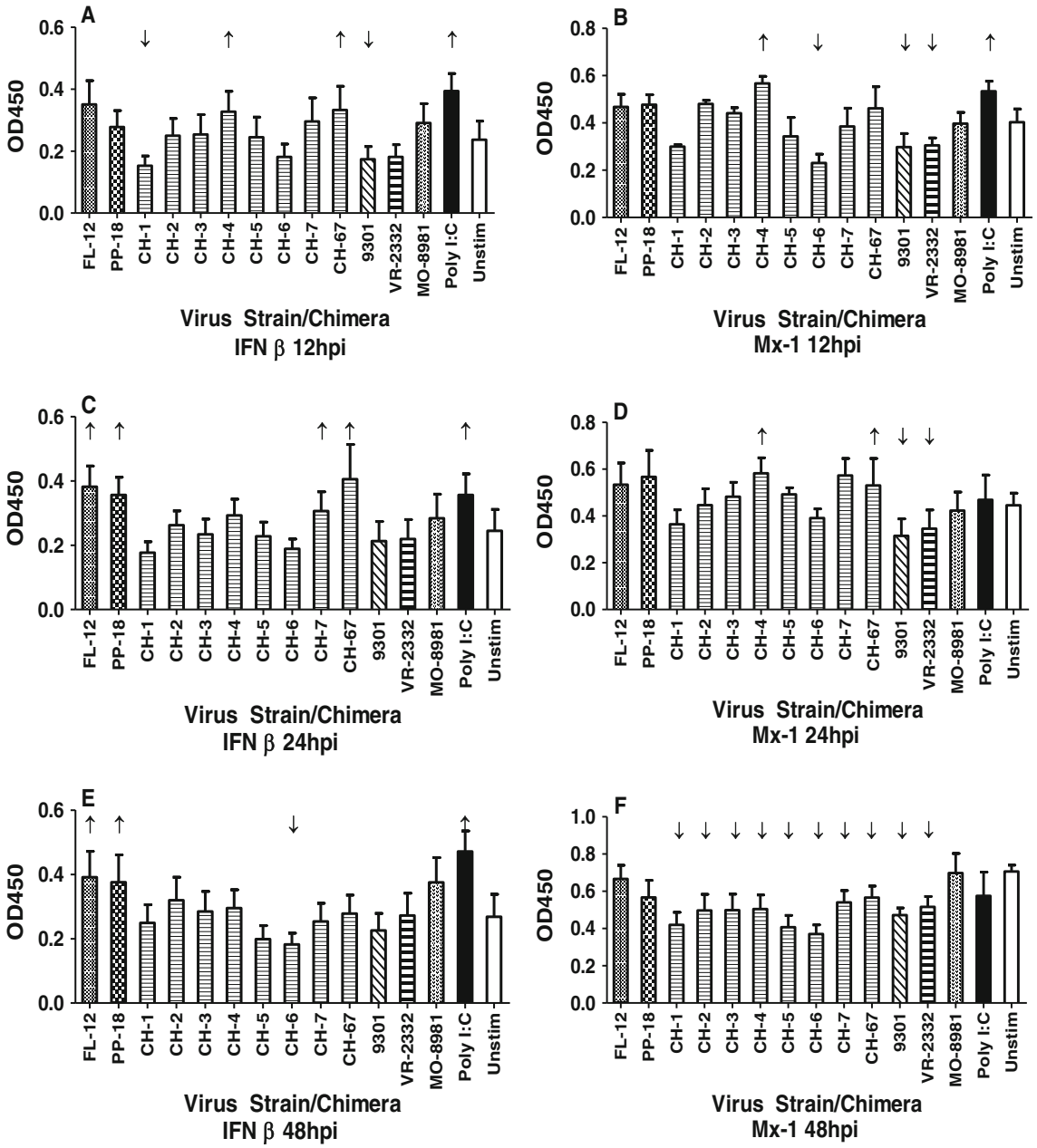

Fig. 1 Detection of IFNβ and MX-1 secreted by PRRSV-Infected-PAMs by indirect ELISA at 12 hpi (**a,b**); 24 hpi (**c,d**); and 48 hpi (**e,f**). Culture supernatants from PRRSV infected PAMs (MOI.02) were acid treated (pH 2) overnight at 4 °C and neutralized to pH 7.2 for IFNβ assays. Bars represent OD_{450} values that were significantly greater (*arrow up*) or lesser (*arrow down*) than that of unstimulated control at $p < 0.05$

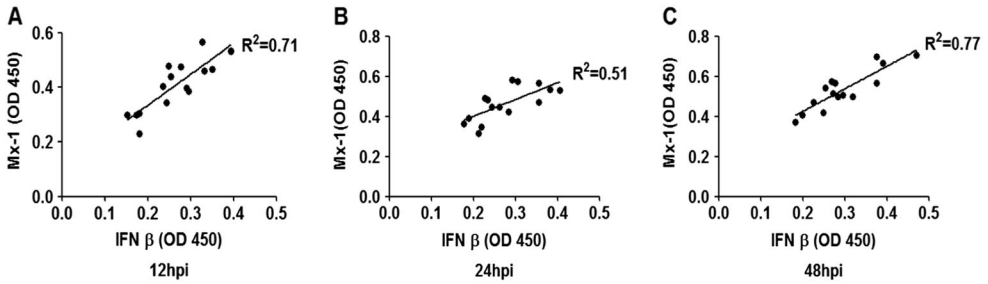

Fig. 2 IFN and Mx-1 expression in PRRSV-infected PAMs at 12 hpi (**a**), 24 hpi (**b**) and 48 hpi (**c**). Regression analysis with data points for each individual IFN measurement plotted against the corresponding Mx-1 values and R values calculated for each time point

The IFNβ induced by infection of PAMs is bioactive

Vero cells primed with acid-treated culture supernatants from PRRSV infected PAMs developed an antiviral state to infection with VSV. The data represent percentages of plaques compared to the control plaques recorded (100 %) with unstimulated non-infected PAM supernatants. Thus, a reduction in the number of plaques is indicative of the presence of functional type I IFN. For plaque reduction analysis, samples were selected based upon the 48 hpi IFNβ ELISA data, using FL-12, PP-18 and a field strain MO-8981 which showed an intermediate expression of IFNβ. At lower sample dilutions (1:2 and 1:4), PP-18 supernatants reduced the number of VSV plaques significantly, as did strain MO-8981 and the poly I:C positive control (Fig. 3a). At the highest dilution (1:8), FL-12 and poly I:C supernatants had significant reductions in the number of plaques.

The number of VSV plaques increased to numbers comparable to the negative control when anti-IFNβ antibodies were present whereas, in the presence of anti-IFNα the number of VSV plaques was similar to treatment of the culture supernatants with no antibody or were slightly increased (Fig. 3b). This confirmed that the antiviral activity was mediated predominantly by IFNβ.

Discussion

This investigation demonstrates that IFNβ protein is produced by PAMs in response to PRRSV infection. Furthermore, downstream signaling also occurs as Mx-1 protein, an ISG, was also detected which generally followed IFNβ levels. However, it appeared that different PRRSV strains do differ in their ability to induce IFNβ in PAMs as shown previously with IFNα (Lee et al. 2004). It has been demonstrated previously that following infection with PRRSV, IFNα expression is induced or suppressed in PAMs in a strain-dependent manner (Lee et al. 2004). In analogy, transcription of the IFNβ gene by PRRSV-infected PAMs also occurs in a strain and time dependent fashion (Gudmundsdottir and Risatti 2009). The differences between strains could reflect differences in replication efficiency. However, it is difficult to picture a virus that replicates to high titers as being a better inducer of type I IFN as this would nullify it. The parental viruses FL-12 and PP-18 behaved similarly despite their differences in virulence.

The chimeric viruses used here are composed of non-structural and structural protein coding regions from a vaccine virus inserted into a virulent parental virus backbone (Kwon et al. 2008). Previously, it was demonstrated that the parental viruses FL-12 and PP-18 were highly virulent, and attenuated respectively while the chimeric viruses exhibited intermediate virulence phenotypes (Kwon et al. 2006). Thus, it was hypothesized that induction of IFNβ could inversely correlate to virulence and that valuable insights as to the genome segments responsible for induction/evasion could be identified with parental and chimeric viruses. The chimeric viruses utilized here, while less efficient in replication than parental viruses in MARC-145 cells, yielded comparable titers among themselves (Kwon et al. 2008). The chimeras were used primarily as a tool to discern genomic areas that may be involved in modulating the host expression of IFNβ. However, the observed effects are complex as to permit locating such genomic areas with precision.

IFNα and IFNβ are often regarded as functionally identical proteins, likely due to the fact that they signal through the same type I IFN receptor, but it is important to distinguish between the two proteins. While several of the effector functions overlap, IFNα and IFNβ do indeed behave differently.

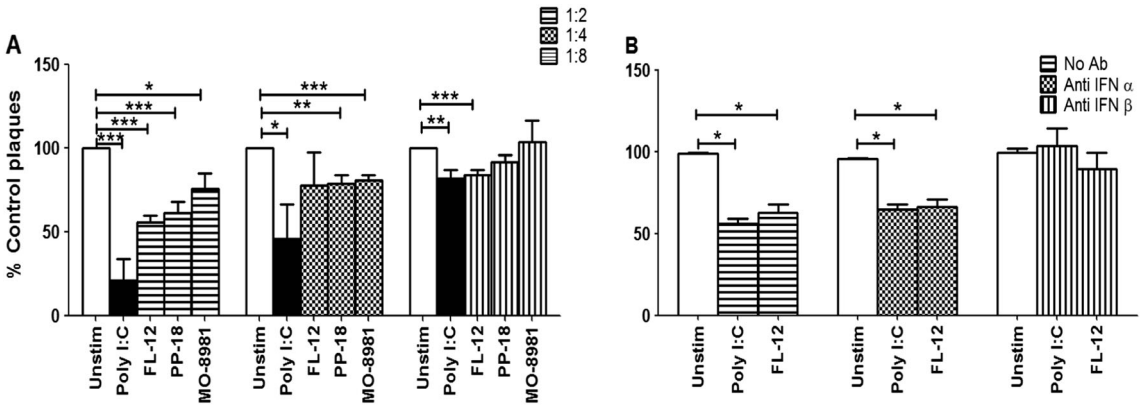

Fig. 3 VSV neutralization by IFNβ in Vero cells (**a**) primed with acid-treated culture supernatants from PAMs infected with FL-12 or treated with poly I:C (high IFNβ inducing) or PP-18 and MO-8981 (intermediate IFNβ inducing) strains of PRRSV. Bars represent percentages of plaques compared to unstimulated control (100 % plaques). Significant changes in the number of VSV plaques: at $p < 0.05$*; $p < 0.01$**; $p < 0.001$*** .Specificity of the antiviral activity (**b**). Plaque assays were done in the presence of anti-IFNα, anti-IFNβ antibody or no antibody using culture supernatants from poly I:C treated or FL-12-infected PAMs. The values shown are changes in percentages of plaques compared to the negative control (100 %) as influenced by antibody

First, it has been demonstrated that IFNβ has a greater binding affinity to IFNAR than IFNα resulting in more potent effects on gene expression (Jaitin et al. 2006). IFNα engineered to match the IFNAR binding affinity of IFNβ, induced gene expression profiles similar to each other. Furthermore, it has been demonstrated that IFNα and IFNβ exhibit different capabilities in reducing viral loads in patients chronically infected with hepatitis C virus (HCV) (Furusyo et al. 1999), and thus influence in the incidence of HCV associated hepatocellular carcinoma (Kashiwagi et al. 2003). Other studies have demonstrated differences among type I IFNs in their abilities to induce activation of hepatic stellate cells (Shen et al. 2002), their abilities to induce subsets of chemokines and ISGs (Coelho et al. 2005), and that IFNβ is produced first in response to Newcastle disease virus infection, and that IFNα is produced following IFNβ induced activation of TLR7 (Marie et al. 1998).

In pigs, a lack of commercially available reagents to detect IFNβ protein has limited investigation. Monoclonal antibodies specific for swine-IFNβ developed in our laboratory (Overend et al. 2007) enabled us to demonstrate here that infection of PAMs with PRRSV does result in a detectable expression of functional IFNβ protein in vitro. Nevertheless, it is clear that expression is variable and the strain and time play a role in such variability. Significant increases in IFNβ expression were observed relatively late following infection (24 hpi for viruses FL-12, PP-18 and CH-7, compared to 12 hpi for poly I:C treated cells and viruses CH-4 and CH-67). Although the reason for this rapid, and in the case of CH-4, transient induction of IFNβ is not clear, it appears as though the PRRSV non-structural protein nsp9 (CH-4) may not have an inhibitory effect on IFNβ induction, whereas other non-structural proteins apparently do (Song et al. 2010; Li et al. 2010; Kim et al. 2010; Beura et al. 2010). However, Beura et al. (2010), demonstrated that while the nsp1 protein of PRRSV inhibited activation of the IFNβ promoter in HEK293 cells, several other non-structural proteins exhibited a robust activation of the same promoter. In light of the findings by Shi et al. (2010) in MARC-145 cells, it is possible that individual non-structural proteins produced by PRRSV may be capable of producing a single effect, whereas the infectious virus as a whole exerts different effects. Additionally, differences between cells in handling infection with PRRSV, notably between MARC-145 cells and alveolar macrophages the natural host cell, need consideration (Lee et al. 2004; He et al. 2011).

Given the patterns of IFNβ expression, the growth characteristics, the effect of gene exchanges on the chimeric viruses (Gudmundsdottir and Risatti 2009) and the present data, it could be speculated that multiple genes are involved in conferring the phenotypes observed. Thus, only some combinations of genes from parental viruses may be capable of reproducing the parental phenotype as shown here with some of the chimeric viruses.

The IFNβ induced in PAMs by infection with PRRSV in the present study was functional as demonstrated its antiviral activity against VSV which confirmed and strengthen the ELISA data. Although interferon activities are generally species-specific, our experiments demonstrate that swine IFNβ is capable of inducing an antiviral state in Vero cells. This is an advantage since Vero cells are not capable of producing interferon, so any antiviral effects are directly related to the experimental treatments. Similarly, previous investigations including ours have shown that swine type I IFN is capable of inducing antiviral states in MARC-145 cells, also of simian origin (Lee et al. 2004; Overend et al. 2007).

Supernatants from poly I:C stimulated cells induced strong antiviral state. Also, supernatants from PAMs infected with the parental strain FL-12 did confer a significant protective effect from VSV challenge, whereas those from PP-18 and strain MO-8981 did induce protection, but to an intermediate level. The antiviral activity of PP-18 and MO-8981 supernatants diluted out more quickly than those of poly I:C treated or FL-12 infected PAMs, suggesting that PRRSV, while capable of inducing IFNβ, it does so to variable extents. Data showing that anti-IFNβ antibody neutralized the anti-viral activity significantly, whereas the anti-IFNα antibody had a minimal effect, confirmed that IFNβ was induced to greater levels than IFNα by some of the PRRSV strains.

Consistent with findings previously reported (Lopez-Fuertes et al. 2000; Subramaniam et al. 2010), low levels of TNFα without any distinct pattern were detected in our study with all viruses tested (data not shown). TNFα levels induced by PRRSV infection in PAMs may be related to the stimulation of TLR3 which appears to be variable among strains (Kuzemtseva et al. 2014).

This investigation also showed that the expression of Mx-1 appeared to generally follow that of IFNβ produced by the infected cells. The detection of Mx-1 protein in this study is significant as it demonstrates that PRRSV infection can have effects on induction/signaling resulting in expression of functional IFNβ and downstream ISGs but this ability is variable.

Conclusions

This study demonstrates that PRRSV is capable of inducing expression of bioactive IFNβ in the natural host cell. Mx-1 expression by infected PAMs generally follow IFNβ production and confirmed IFNAR signaling. However, this induction/signaling process is clearly variable as some strains do induce IFNβ expression while others do not.

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Authors contributions C. Overend performed most of the experimental work and prepared the manuscript. J. Cui performed the statistical analysis and graphs. M. Grubman served as scientific collaborator and consultant, reviewed the data and the manuscript. A. Garmendia directed the study, contributed in the experimental work, reviewed the data and the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest

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