




# Effect of an 88-amino-acid deletion in nsp2 of porcine reproductive and respiratory syndrome virus on virus replication and cytokine responses *in vitro*

Wei He<sup>1</sup> · Ying Wei<sup>1</sup> · Jing Yao<sup>1</sup> · Xin Xie<sup>1</sup> · Jiabin Huang<sup>1</sup> · Siyuan Lin<sup>1</sup> · Kang Ouyang<sup>1</sup> · Ying Chen<sup>1</sup> · Weijian Huang<sup>1</sup> · Zuzhang Wei<sup>1</sup> 

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## Abstract

Previously, a spontaneous 88-amino-acid (aa) deletion in nsp2 was associated with cell-adaptation of porcine reproductive and respiratory syndrome virus (PRRSV) strain JXM100, which arose during passaging of the highly pathogenic PRRSV (HP-PRRSV) strain JX143 in MARC-145 cells. Here, to elucidate the biological role of this deletion, we specifically deleted the region of a cDNA clone of HP-PRRSV strain JX143 (pJX143) corresponding to these 88 amino acids. The effect of the deletion on virus replication in cultured cells and transcriptional activation of inflammatory cytokines and chemokines in pulmonary alveolar macrophages (PAMs) was examined. Mutant virus with the 88-aa deletion in nsp2 (rJX143-D88) had faster growth kinetics and produced larger plaques in MARC-145 cells than the parental virus (rJX143), suggesting that the deletion enhanced virus replication in MARC-145 cells. In contrast, the overall yield of rJX143 was almost 1 log higher than that of rJX143-D88, suggesting that the 88-aa deletion in nsp2 decreased the production of infectious viruses in PAMs. Infection with the mutant virus with the 88-aa deletion resulted in increased mRNA expression of type I interferon (IFN- $\alpha$  and IFN- $\beta$ ) and chemokines genes. In addition, the mRNA expression of antiviral genes (ISG15, ISG54 and PKR) regulated by the IFN response was upregulated in PAMs infected with the mutant virus rJX143-D88. Our results demonstrate that virus-specific host immunity can be enhanced by modifying certain nsp2 epitope regions. These findings provide important insights for understanding virus pathogenesis and development of future vaccines.

## Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) was first recognized in 1987 in the USA and shortly afterwards in the Netherlands [50], and it has spread worldwide to become the most economically significant swine disease [29]. In 2006, a large outbreak of porcine high

fever syndrome (PHFD), caused by a highly pathogenic form of PRRSV (HP-PRRSV), emerged in China and Southeast Asian countries and has increased the threat to the swine industry worldwide [4, 43, 45].

PRRSV is a member of the family *Arteriviridae*, order *Nidovirales*. PRRSV contains a positive-stranded RNA genome of approximately 15.4 kb consisting of at least 10 open reading frames (ORFs). Approximately two-thirds of the 5' end of the viral genome contains ORF1a and ORF1b, which encode polyproteins that are processed by viral proteases into 14 nonstructural proteins (nsps) [11]. The 3' third of the genome contains the ORFs 2a, 2b, and 3–7, which encode the PRRSV structural proteins, namely GP2a, 2b (or E), GP3, GP4, ORF5a, GP5, matrix protein (M) and nucleocapsid (N), respectively [12, 19, 37, 51].

The nsp2 of PRRSV is a highly heterogeneous protein. Natural deletions and insertions have occurred in the hypervariable region (HVR) of nsp2, and these lead to genome size differences among PRRSV strains [21, 36, 54]. PRRSV strains with different types of aa deletion patterns in nsp2

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Wei He and Ying Wei contributed equally to this study.

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✉ Weijian Huang  
weijianhuang@163.com

✉ Zuzhang Wei  
zuzhangwei@gxu.edu.cn

<sup>1</sup> Laboratory of Animal infectious Diseases and Molecular Immunology, College of Animal Science and Technology, Guangxi University, Nanning 530005, People's Republic of China

have been prevalent in the field. Intriguingly, most of the so-called hypervirulent PRRSV strains, (e.g., MN184 and some Asian PRRSV isolates) have been found to have a variety of deletions in the nsp2 coding region [3, 15, 27, 43, 45]. Some Chinese HP-PRRSV isolates contain a discontinuous deletion of 30 aa in the variable region of nsp2, but this hallmark deletion was shown not to be a factor in the increased virulence of HP-PRRSV [58]. Some studies have also shown that some PRRSV strains and engineered mutants with large deletions in nsp2 do not appear to have increased pathogenicity [1, 5, 58]. Prolonged *in vitro* viral passaging is the usual method for attenuating HP-PRRSV and the development of live-attenuated vaccines (JXA1-R, HuN4-F112 and TJM) [17, 24, 25, 44]. Interestingly, spontaneous aa deletions in nsp2 have also occurred during the process of serial passages of a virulent PRRSV strain. A spontaneous 120-aa deletion located downstream of the 30-aa deletion in the nsp2 region was identified in the vaccine strain TJM after 19 passages (P19) of a cell-adapted virus of strain HP-PRRSV TJ [25].

A mutant carrying a 131-aa deletion that partially overlapped with the 120-aa deletion of TJM was found to be less virulent than the parental virus [22]. It was suggested that this aa deletion in nsp2 could affect the pathogenicity of PRRSV. A large 145-aa deletion in the C-terminus of nsp2 was identified after continuous passaging of the PRRSV strain VR2385 in cell culture. It was shown that this spontaneous nsp2 deletion played a role in enhancing PRRSV replication in MARC-145 cells, whereas it had no effect on the pathogenicity of the virus *in vivo* [30]. Overall, the mechanisms underlying spontaneous deletions during viral passage in cells *in vitro* as well as *in vivo* and their effects on viral replication and pathogenicity remain unclear.

Nsp2 is the most variable and highly immunogenic non-structural protein in PRRSV. It contains several putative B-cell and T-cell epitopes. Some of these epitopes have been mapped to the HVR of nsp2, which is the region where substitutions, natural deletions and insertions usually occur [31, 53]. Several studies have demonstrated that nsp2 contains a stretch of amino acids that is dispensable for virus viability [16, 22, 25, 30, 43]. A marker PRRSV strain that can differentiate infected from vaccinated animals could potentially be developed through engineered deletions of specific individual immunodominant epitopes or large fragments in nsp2 that are capable of eliciting specific antibody production during viral infection [8, 10, 22, 47, 52]. Numerous studies have also shown that natural or engineered amino acid deletions in nsp2 of PRRSV play an important role in modulating the induction of inflammatory cytokines *in vitro* [6, 7, 9, 26].

In our previous study, a spontaneous 88-aa deletion located immediately upstream of the 29+1-aa deletion in the nsp2 region was identified in a cell-adapted strain, JXM100,

after 100 passages of a cell-adapted virus of HP-PRRSV strain JX143 [47]. To investigate further the biological role of the 88-aa deletion in nsp2, in this study, we deleted 88 aa of nsp2 using an infectious clone of HP-PRRSV strain JX143 (pJX143) [27]. The effect of the 88-aa deletion on virus replication in MARC-145 cells and the host immune response were investigated in pulmonary alveolar macrophages (PAMs). The results suggest that this specific 88-aa deletion can enhance virus replication in MARC-145 cells and also plays an important role in the modulation of mRNA expression of IFN-related genes in infected PAMs.

## Materials and methods

### Cells, viruses and plasmids

MARC-145 and BHK-21 cells (ATCC, CCL10) were maintained as described in our previous study [42]. PAMs were harvested from lungs of 6-week-old PRRSV-negative piglets as described previously and maintained at 37 °C in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) [49]. The infectious PRRSV cDNA clone pJX143 was constructed from the highly pathogenic PRRSV JX143 strain (EU708726), the parental virus of the cell-attenuated virus with an 88-aa deletion in nsp2, and was used as a backbone for genetic manipulation in this study [27]. For convenience of genetic manipulations, a shuttle plasmid (pTOPO-SA) was generated. Briefly, the pJX143 infectious clone was used as template, and a fragment was amplified by PCR using primers JX451F and JX6463R. The PCR product was cloned into the Zero Blunt® PCR vector. Site-directed mutagenesis was done by splicing overlapping extension (SOE) PCR as reported previously [48]. To generate the mutant plasmid carrying the 88-aa deletion in nsp2, mutagenic primers (nsp2D88F and nsp2D88R) and flanking primers (JX718F and JX2939R) were used to make the intermediate PCR products JF-D88R and D88F-JR, which were then denatured and used as templates for a second round of fusion PCR. The resulting PCR fragments were digested with the appropriate restriction enzymes (*ScaI* and *XhoI*) and ligated into the plasmid shuttle vector pTOPO-SA digested with the same enzymes. The fragments carrying the verified 88-aa deletion in the shuttle vector were digested with the appropriate restriction enzymes (*SpeI* and *AflIII*) and ligated into the corresponding region of pJX143 after treatment with the same enzymes.

### RNA transfection and recovery of mutant viruses

Plasmids were purified using a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). RNA transcripts were generated *in vitro* from 1 µg of non-linearized DNA template

using the T7 RNA polymerase provided in the mMessage mMachine Kit (Ambion Inc., USA). After transcription, RNase-free DNase I was added to eliminate the DNA template. BHK-21 cell monolayers in 6-well-plates were transfected with 1 µg of the *in vitro* RNA transcripts, using DMRIE-C transfection reagent (Invitrogen, MA) according to the manufacturer's instructions. The transfection mixture was replaced with EMEM containing 2% FBS at 4 hours post-transfection (hpt) and further incubated at 37 °C in the presence of 5% CO<sub>2</sub> for 24 hours. One hundred µL of the culture supernatant was used to inoculate fresh MARC-145 cells, and after a 1-hour adsorption period at room temperature, 3 mL of EMEM containing 2% FBS was added, and the cells were incubated at 37 °C, in the presence of 5% CO<sub>2</sub> for 6 days. Rescued viruses were designated as the primary passage (P0) and used to infect MARC-145 cells for five subsequent passages (P1 to P5) using a 10<sup>2</sup> dilution at each passage.

### Indirect immunofluorescence assay (IFA)

MARC-145 cell monolayers were used for assessing viral protein expression as described previously [57]. MARC-145 cells were infected with the mutant or wild-type (wt) virus. At 48 hours post-inoculation (hpi), the cell monolayer was washed twice with PBS and then fixed with cold methanol for 15 min at room temperature, followed by treatment with 1% bovine serum albumin (BSA). The cells were incubated at 37 °C for 2 h with a monoclonal antibody against PRRSV N protein, SR30-A (Rural Technologies Inc., IA). The cells were washed five times with PBS, and the stained cell monolayers were observed under an Olympus inverted fluorescence microscope fitted with a CCD camera.

### Multi-step growth curve

To assess the growth kinetics of the virus, MARC-145 cells in six-well plates were infected with the rescued viruses (P3) at a multiplicity of infection (MOI) of 0.1 as described previously [55]. After a 1-h incubation at 37 °C, cells were washed three times with PBS and incubated at 37 °C in 3 mL of EMEM containing 2% FBS in a CO<sub>2</sub> incubator. Two hundred µL of supernatant was harvested at 6, 12, 24, 48, 72, 96, and 120 hpi. and frozen at -70 °C until used. The viral titers (TCID<sub>50</sub>/mL) were measured in MARC-145 cells and calculated using the method Reed and Muench [33].

To assess the growth kinetics of the virus in PAMs, PAMs in six-well plates were infected with the rescued viruses (P3) at a multiplicity of infection (MOI) of 0.1. Two hundred µL of supernatant was harvested at 6, 12, 24 and 48 hpi. Viral RNA was extracted from 140 µL of supernatant followed by one-step qRT-PCR as described previously [49]. Primers and probes were designed using Primer Express 2.0 software and

are listed in Table 1. One-step qRT-PCR was carried out in a 25-µL reaction volume containing 2 µL of extracted RNA, 12.5 µL of 2× RT-PCR reaction mix for probes, 10 pmoles of forward and reverse primers, 10 pmoles of probe, and 0.5 µL of iScript reverse transcriptase (Bio-Rad).

### Viral plaque assay

To examine the plaques produced by the mutant virus with the 88-aa deletion in nsp2, tenfold serially diluted virus suspensions (P3) were inoculated onto MARC-145 cells in six-well plates. After 1 h of adsorption, cell monolayers were washed three times with PBS and then overlaid with a mixture of EMEM containing 2% FBS and 1% low-melting agarose (Cam-brex, Rockland, ME, USA). The plate with solidified agarose was placed upside-down in an incubator at 37 °C with 5% CO<sub>2</sub>. At 5 days postinfection (dpi), the plaques were visualized by crystal violet staining.

### RT-PCR

Sequencing of RT-PCR products was carried out to analyze the introduced mutations in nsp2. Viral genomic RNA was isolated directly from the supernatant of the P3-virus-infected cell cultures using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. First-strand cDNA synthesis was carried out at 42 °C for 1 h using avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa, Dalian, China) with the anchored poly(T) primer Qst (Table 1). The fragments containing the nsp2 region were amplified using LA-Taq DNA polymerase (TaKaRa Dalian, China) with the forward primer nsp2F and the reverse primer nsp2R flanking nsp2 (Table 1). The PCR products were purified using a TIANGel Mini Purification Kit (TIANGEN) and subjected to nucleotide sequencing.

### Infection of PAMs

To investigate the effects of PRRSV on transcriptional activation of inflammatory cytokines/chemokines, an equal number of virus particles (MOI = 1) was used to infect PAMs. After 1 h of incubation, fresh culture medium was added, and the infected cells were maintained in fresh medium for 24 h. Total RNA was extracted from lysates of the infected cells at 6, 12 and 24 h hpi using TRIzol Reagent (Invitrogen). The concentration of the extracted RNA was measured using a NanoVue spectrophotometer (GE Healthcare, Piscataway, NJ). Real-time PCR was conducted with 1 µg of cDNA in a total volume of 20 µL with iQ SYBR Green Supermix (Bio-Rad, CA, USA), following the manufacturer's instructions. The target genes of the primers used in this study have been reported previously, and the sequences of

**Table 1** Primers used in this study

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Accession no./reference
88-amino-acid deletion in nsp2			
JX718F/JX2939R	GGACTAGTATACAGTACTTCTCTG GTATACAGTACTTCT CTGTAAAG	GGGCGGCCGCGTGTCTCGAGAATC ATCTTTG	EU708726
nsp2D88F/R	AGGGCTCGGTCCCTCTAAAAGCTT GGGTCA	TGACCCAAGCTTTTAGAGGGACCG AGCCCT	EU708726, EF536003
nsp2-F/R	AATGTTGTTCTTCTCGGGTTGAG	AAGCTGCAAAACCCCAATCACCCG	EU708726, EF536003
Viral RNA detection			
PRRSV-F/R	CCAAGTCTACTGCACACGATG	TTTCCACTAGTCATTCGTGC	[49]
PRRSV-P	FAM-CTCCGGTGGACGTTGCCAC-TAMRA		
Cytokines			
IL-1 $\alpha$	GTGCTCAAAACGAAGACGAACC	CATATTGCCATGCTTTTCCAGAA	[23]
IL-1 $\beta$	AACGTGCAGTCTATGGAGT	GAACACCACTTCTCTCTTCA	[23]
IL-6	ACT GGC AGA AAA CAA CCT GA	CCT CGA CAT TTC CCT TAT TGC T	NM_001252429
IL-8	CCGTGTCAACATGACTTCCAA	GCCTCACAGAGAGCTGCAGAA	[23]
IL-10	CGGCGCTGTCATCAATTTCTG	CCCCTCTTTGGAGCTTGCTA	[23]
IL-12p40	AACTCTTCACGGACCAAATCTCA	GGTCCC GGCTTGCA	[23]
TNF- $\alpha$	AACCTCAGATAAGCCCGTGC	ACCACCAGCTGGTTGTCTTT	[23]
Chemokines			
AMCF-1	GCTCGTGTCAACATGACTTCCA	GCCTCACAGAGAGCTGCAGAA	[23]
MCP-1	GCAGCAAGTGTCTAAAGAAGCA	GCTTGGTTCTGCACAGATCT	[23]
MCP-2	AAGACCAAAGCCGACAAGGA	TCATGGAATTCTGGACCCACTT	[23]
Interferons (IFNs) and interferon regulatory factors (IRFs)			
IFN- $\alpha$	ACAGACTCACCTCTATCTG	TCCTTCTTCTGAGTCTGTC	JQ839262
IFN- $\beta$	GCTCTCTGATGTGTTTCTC	AGGGACCTCAAAGTTCATCC	KJ147517
IFN- $\gamma$	AATGGTAGCTCTGGGAAACTG	ACTTCTCTCCGCTTCTTAGG	[23]
IRF-3	CGCTTCTTGCCCCAACCT	TCCACTCGTCGTCATTCG	[23]
IRF-7	CGCTCTGGAAAACCAA	CCCTGAGTTGTCCTGCAACA	[23]
Antiviral activity			
PKR	AAAGCGGACAAGTCGAAAGG	TCCACTTCATTTCCATAGTCTTCTGA	[23]
ISG-15	AGCATGGTCCTGTTGATGGT	TGACACACCTGCTGCTTGAG	EU584557
ISG-54	AAGAACTCCTTGAGAGCTG	CCTGTATGTTGCACATCGTG	JX070559
Toll-like receptors (TLRs)			
TLR3	GACTCAAATACCCGATGACC	TTGAGATGGCGTTAAATCCTC	GU013759

the primers are given in Table 1. Three independent experiments were conducted in duplicate. Relative expression values were normalized using an internal  $\beta$ -actin control. The fold change of relative gene expression levels was calculated as  $2^{-\Delta\Delta C_t}$  ( $\Delta C_t$  of gene -  $\Delta C_t$  of  $\beta$ -actin).

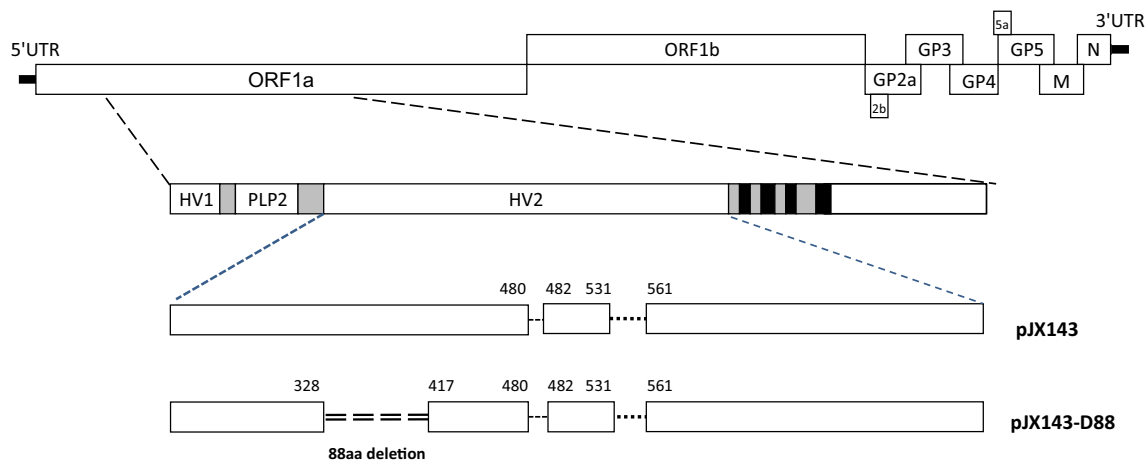
### Statistical analysis

Statistical analysis of gene expression levels and virus titers was performed using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to evaluate the differences among cytokines/chemokines levels and virus titers. Subsequently, the Duncan honestly significant difference test was used to examine multiple comparisons.

## Results

### Construction and recovery of a PRRSV mutant with a 88-aa deletion in nsp2

To investigate the role of the 88-aa deletion in nsp2 on the replication of PRRSV in cultured cells, a mutant (pJX143-D88) containing this deletion was constructed based on the infectious cDNA clone, pJX143, which was derived from the parental virus of JXM100, the attenuated mutant in which the original deletion was found (Fig. 1). BHK-21 cells were transfected with the RNA transcript of mutant plasmid pJX143-D88 or wt plasmid pJX143. The supernatants of the transfected BHK-21 cells were collected 24 hours after transfection and were used to infect fresh MARC-145 cells. A



**Fig. 1** The schematic diagram of the construction of a full-length cDNA mutant carrying an 88-aa deletion in nsp2. Site-directed mutagenesis was accomplished by splicing the overlapping exten-

sions to generate the mutant plasmid (pJX143-D88) carrying the 88-aa deletion in nsp2. The double dotted line represents the position of the deletion

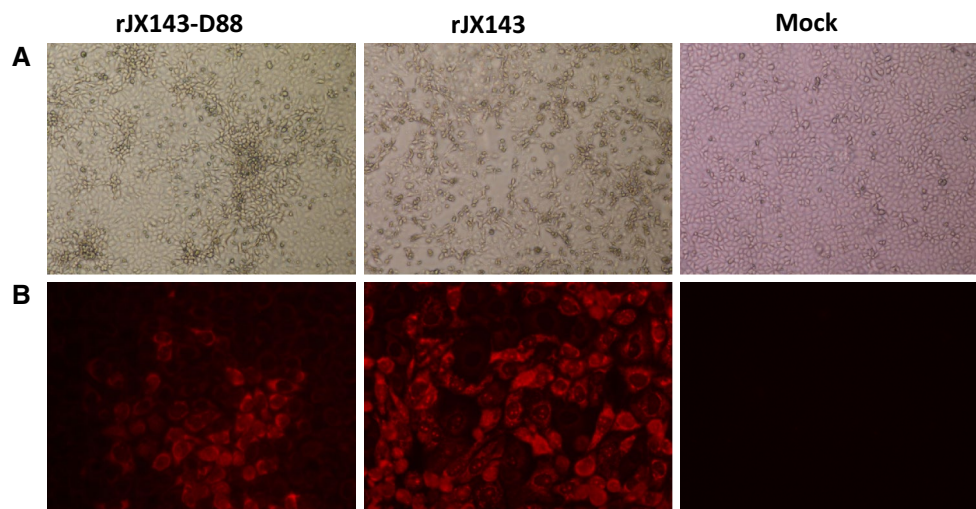
typical cell cytopathic effect (CPE) was observed in MARC-145 cells at 72 hpi. As shown in Fig. 2, the specificity of the CPE was confirmed by IFA with antibodies against PRRSV N protein. The IFA results showed that staining specific for N was clearly evident in MARC-145 cells inoculated with supernatants harvested from BHK-21 cells transfected with mutant or wt plasmids. This suggested that infection was spread by the mutant virus to neighboring cells.

To determine whether the mutations introduced into the viral RNA were maintained in the replicating viruses, the nsp2 regions of the mutant virus (rJX143-D88) and wt virus (rJX143) were amplified by RT-PCR and sequenced.

The results showed that the engineered 88-aa deletion was present in all of the passaged viruses (P0-P8), and no other mutations were detected in the nsp2 coding region (data not shown).

### The 88-aa deletion in nsp2 enhances virus replication in MARC-145 cells

To examine the virological properties of the mutant virus, MARC-145 cells were infected with the mutant virus (rJX143-D88) and parental virus (rJX143) at an MOI of 0.1. The viral titers in supernatants harvested at



**Fig. 2** Recovery of a PRRSV mutant with the 88-aa deletion in nsp2. The mutant plasmids, along with the parental plasmids, were used to transfect BHK-21 cells, and the supernatants were harvested at 24 hpt, and used to inoculate fresh MARC-145 cells. (A) CPE in MARC-145 cells produced by mutant and wild type plasmids. (B)

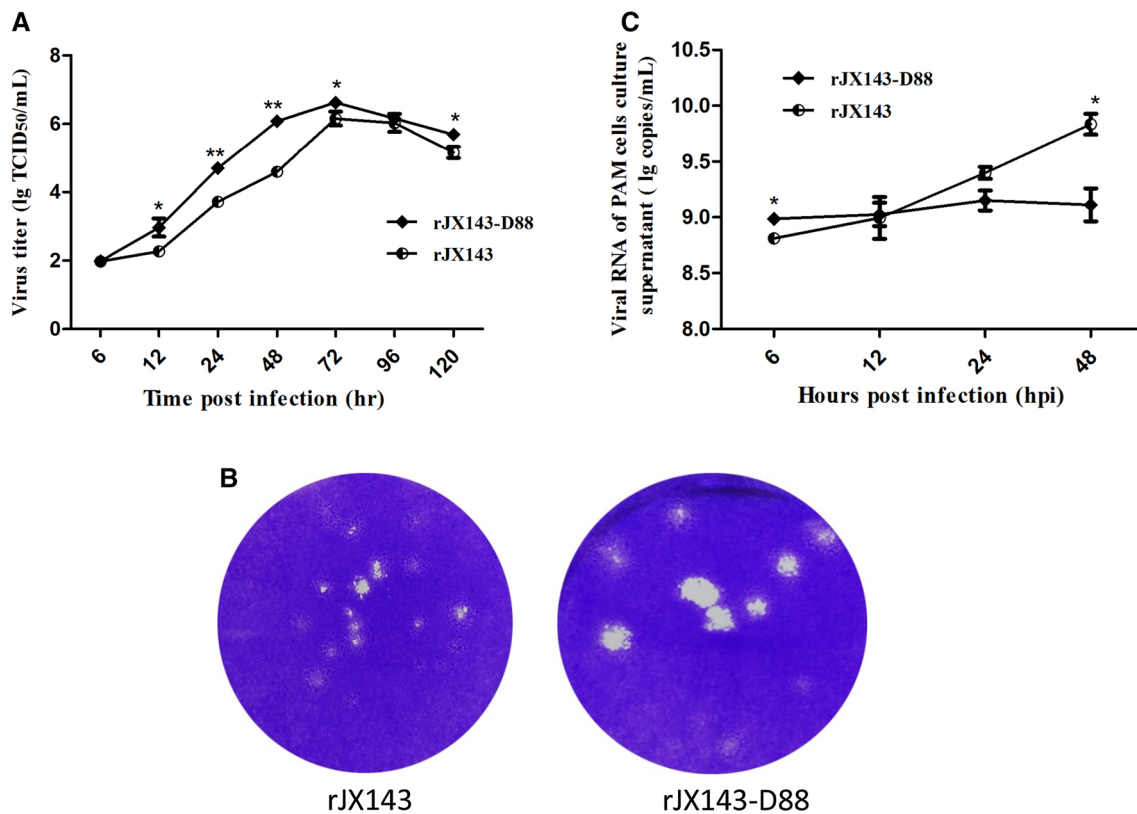
Detection of N protein expression using an indirect fluorescence assay. The infected MARC-145 cells were fixed and stained using a monoclonal antibody against the PRRSV N protein and an anti-mouse secondary antibody labeled with Alexa Fluor (Sigma). Images were taken at  $\times 200$  magnification



regular intervals (6 to 120 hpi.) were determined. The growth kinetics of rJX143-D88 showed that the peak titer was 1 log higher than that of rJX143 before 72 hpi (Fig. 3A). The plaque morphologies of mutant and parental virus were assessed using MARC-145 cells. As shown in Fig. 3B, the plaques generated by rJX143-D88 were slightly larger than the plaques generated by rJX143, suggesting that the 88aa-deletion in nsp2 increases the production of infectious viruses in MARC-145 cells. To further characterize the mutants carrying the 88-aa deletion in nsp2, the multi-step growth kinetics were also determined in infected PAM cells. As shown in Fig. 3C, rJX143 and rJX143-D88 displayed similar multi-step growth curves. However, the overall yield of rJX143 was nearly 1 log higher than that of rJX143-D88 at 48 hpi, suggesting that the 88-aa deletion in nsp2 decreases the production of infectious viruses in PAMs.

### Cytokine mRNA expression in PAM cells infected with mutant virus incorporating the 88-aa deletion in nsp2

To confirm that expression of the most relevant early innate response genes was induced at early times postinfection, real-time RT-PCR was performed with RNA samples from PAMs infected with parental virus rJX143 or mutant virus rJX143-D88. The expression levels of IL-6 and IL12-p40 were not significantly different in PAMs infected with rJX143 or rJX143-D88 at any time point (data not shown). As shown in Fig. 4, infection of PAMs with either rJX143 or rJX143-D88 resulted in higher levels of IL-10 expression than in mock-infected cells, with significant differences at 12 hpi, but IL-10 expression levels were similar to those in the controls at 6 and 24 hpi. PAMs infected with rJX143-D88 exhibited higher expression levels of IL-1 $\alpha$ , IL- $\beta$  and IL-8 than those infected with rJX143 cells, with significant differences seen at 6 hpi. No significant differences in the expression of these three genes were observed in rJX14-3,



**Fig. 3** Multi-step growth curves and viral plaque morphology of rJX143 and rJX143-D88 in MARC-145 cells and PAMs. The mutant rJX143-D88 and the parental strain rJX143 were used to infect monolayers of MARC cells and PAMs. Subsequently, 200  $\mu$ L of the supernatant was collected at the indicated time and stored at  $-70^{\circ}\text{C}$ . **A**) The viral titers were determined as TCID<sub>50</sub> and the values shown represent the mean from three separate experiments. An asterisk (\*)

indicates a significant difference ( $P < 0.05$ ). **B**) The viral titers were determined as copy numbers, and data were obtained from three separate experiments. An asterisk (\*) indicates a significant difference ( $P < 0.05$ ). **C**) Monolayers of MARC-145 cells in six-well plates were infected with mutant rJX143-D88 or the parental strain rJX143. The cell monolayers were overlaid with 1% agarose and stained with crystal violet at 96 hpi

**Fig. 4** Expression of cytokines, chemokines, and TLR and antiviral gene mRNAs. PAMs were mock infected or infected with rJX143-D88 or the parental strain rJX143 at 6, 12 and 24 hpi. The transcriptional level of each gene was assessed by quantitative real-time RT-PCR and normalized to that of porcine  $\beta$ -actin. The results are presented as fold changes in cytokine mRNA levels compared to those in uninfected controls (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). The fold change was calculated by the  $2^{-\Delta\Delta Ct}$  method

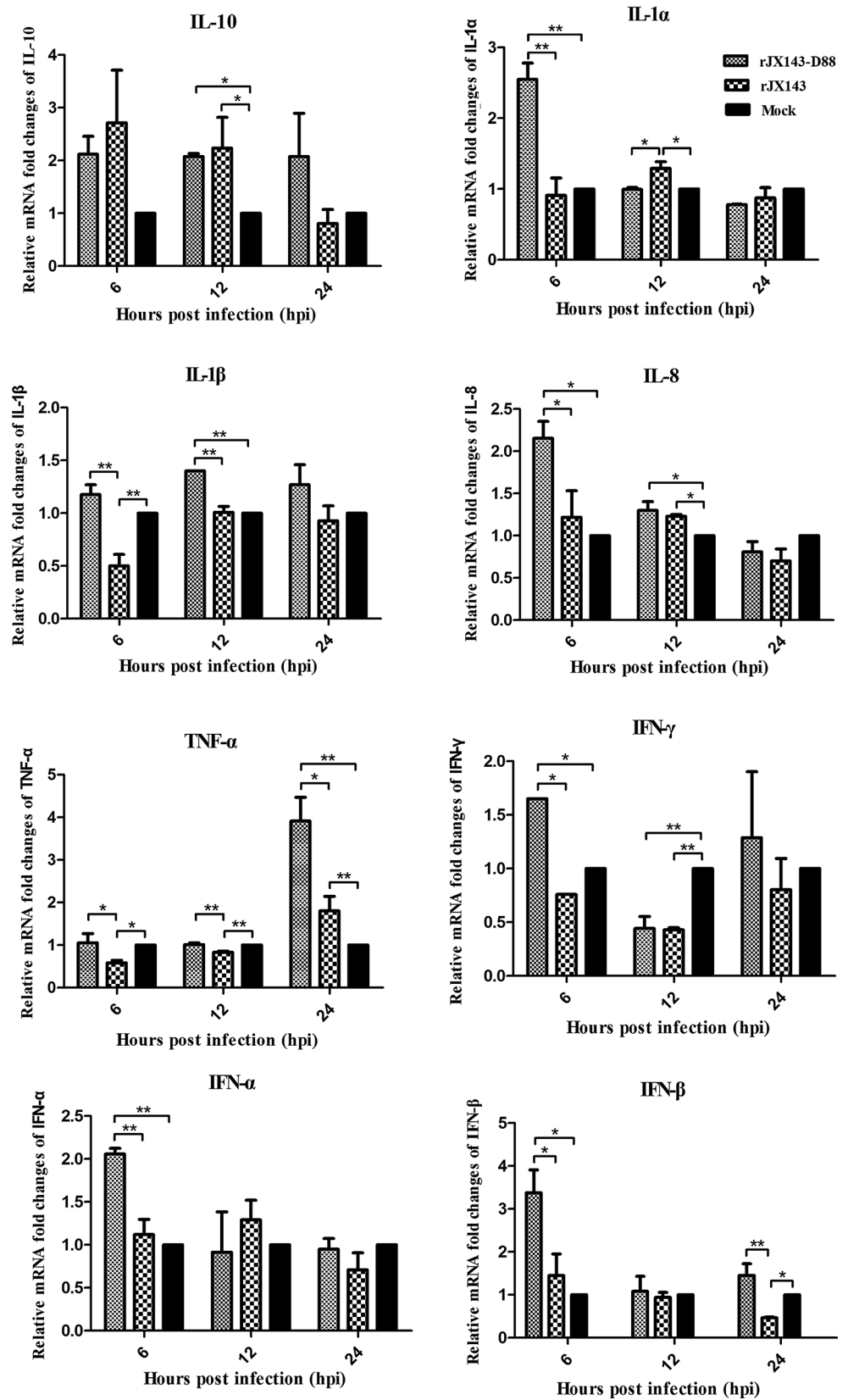


Fig. 4 (continued)

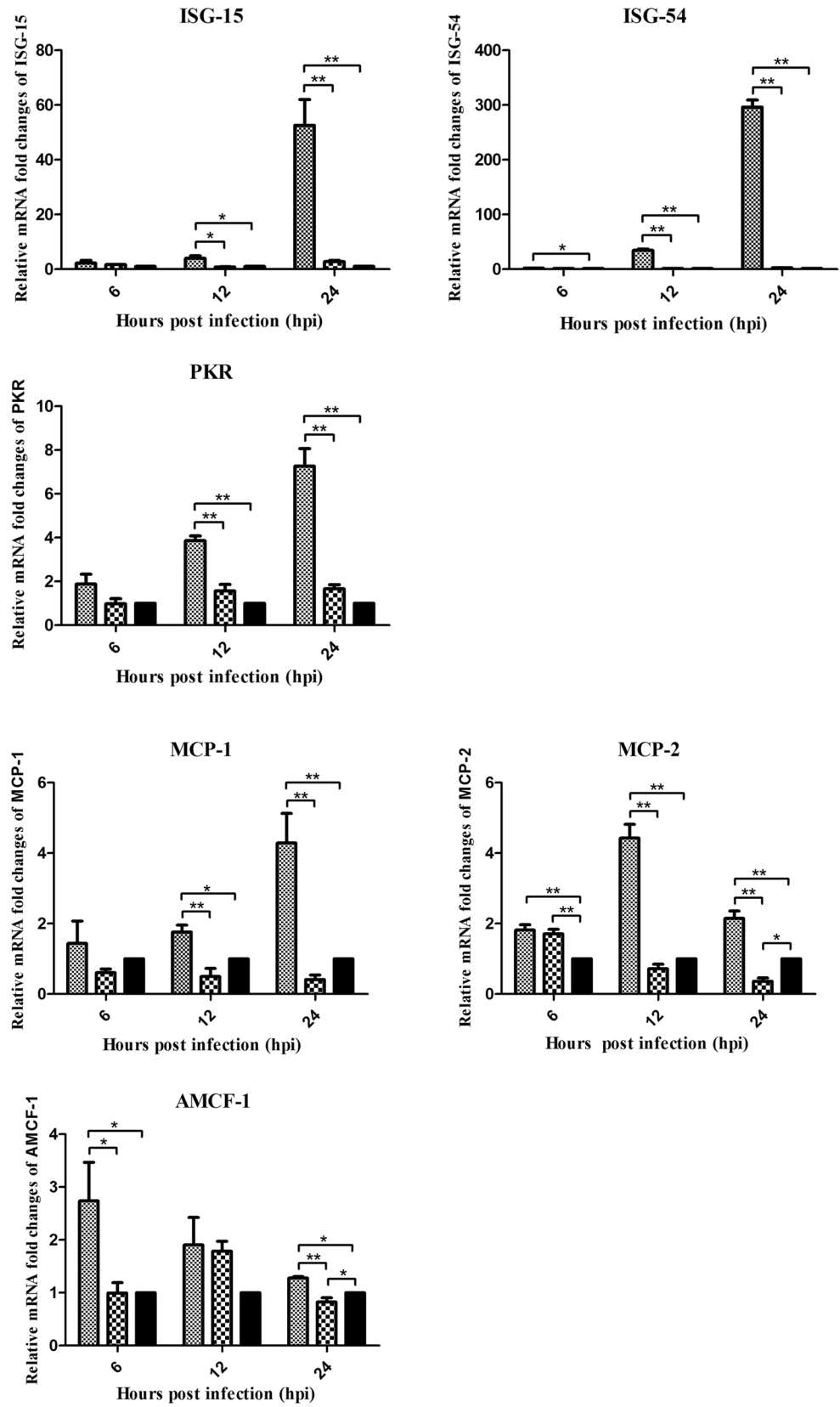
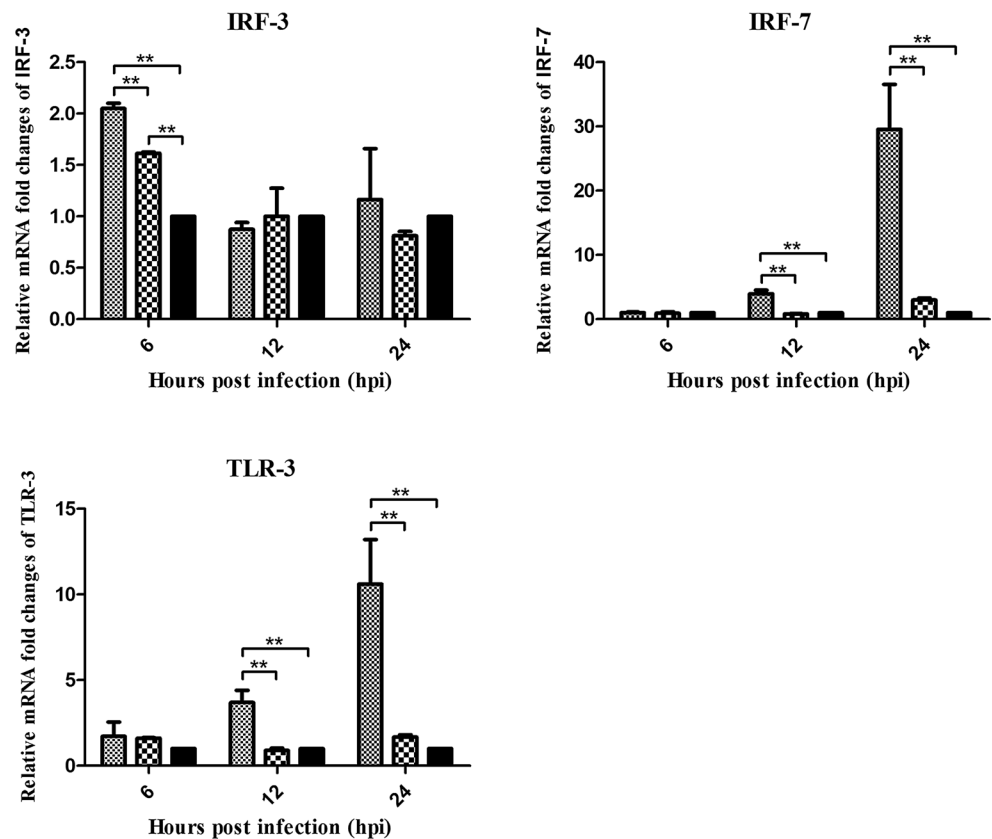




Fig. 4 (continued)



rJX143-D88 and mock-infected cells at 24 hpi. The expression levels of IL-1 $\beta$  were slightly decreased, but IL-1 $\alpha$  levels were slightly increased in PAM cells infected with rJX143 at 12 hpi. The RNA levels of both genes remained unchanged at 6 and 24 hpi. The results also showed that infection of PAMs with rJX143 resulted in downregulation of TNF- $\alpha$  mRNA at early time points (6 and 12 hpi) and upregulation at late time points (24 hpi). rJX143-D88-infected cells induced more TNF- $\alpha$  mRNA than rJX143-infected cells. We also found that the RNA expression of IFN- $\gamma$  was not significantly higher at 6, 12, and 24 hpi in rJX143 virus-infected cells than in mock-infected cells. Upregulation of IFN- $\gamma$  gene expression was observed in cells infected with rJX143-D88 at 6 hpi, and then the expression declined significantly at 12 hpi and displayed similar expression levels without significant differences compared to the parental virus at 24 hpi.

Expression of the IFN- $\alpha$  and IFN- $\beta$  genes did not differ significantly at 6, 12, and 24 hpi in rJX143-virus-infected cells from that in mock-infected cells. Moreover, rJX143-virus-infected PAMs exhibited lower levels of IFN- $\beta$  expression than mock-infected cells. This indicates a strong ability of PRRSV to suppress IFN gene expression during the process of virus replication. However, a significant upregulation of IFN- $\alpha$  and IFN- $\beta$  gene expression was observed in cells infected with rJX143-D88 at 6 hpi, but thereafter, expression

declined significantly and no longer differed significantly from that observed with the parental virus at 12 hpi and 24 hpi. The interferon-stimulated genes (ISG) analyzed in this study consisted of ISG15, ISG54 and PKR, and these are downstream genes of IFN- $\alpha$  and IFN- $\beta$ . As shown in Fig. 4, there were no significant differences in RNA expression between the three interferon-stimulated genes (ISG) in rJX143 and mock infections at any of the time points examined. However, rJX143-D88 infection in PAM cells induced dramatically higher levels of ISG15, ISG54 and PKR gene expression than rJX143 infection at 12 and 24 hpi.

Little or no change in the expression of MCP-1 and AMCF-1 was observed in rJX143-infected cells when compared to mock-infected cells. In contrast, a dynamic upregulation of MCP-1 and AMCF-1 genes was observed in PAMs infected with the mutant rJX143-D88. Upregulation of MCP-2 genes was observed in PAMs infected with rJX143 or rJX143-D88 when compared to mock-infected cells at 6 hpi. rJX143-D88-infected cells induced high levels of MCP-2 gene expression when compared to rJX143 infection at 12 and 24 hpi.

The level of expression of IRF3 was slightly higher in cells infected with rJX143 and rJX143-D88 at 6 hpi than in mock-infected cells. RNA levels were not significantly different in rJX143-D88-infected and rJX143-infected PAMs at 12 hpi and 24 hpi. The expression of IRF7 remained

unchanged in rJX143-infected cells, while the expression of IRF7 was significantly upregulated in PAMs infected with rJX143-D88 at 12 and 24 hpi. No changes in the expression of TLR-3 mRNA were observed in rJX143-infected cells when compared to mock-infected cells. TLR-3 mRNA levels continuously increased to a much greater degree in PAMs infected with rJX143-D88 during the course of infection, resulting in much higher levels of TLR-3 mRNA at 24 hpi.

## Discussion

In previous a study, we conducted 100 serial cell passages using a HP-PRRSV JX143 isolate to determine the phenotypic and genetic changes associated with the cell adaptation process. We identified a total of 75 random nucleotide substitutions throughout the genome, including 33 non-silent mutations located in the ORF1, ORF2, ORF3, ORF4 and ORF6 regions. Interestingly, we also detected an extra 88-aa deletion in nsp2 of the highly cell-adapted virus (JXM100) [47]. It was suggested that some of these mutations may have a role in virus adaptation in the cultured cell model as well as in the attenuation of the HP-PRRSV strain (JX143) *in vivo*. In this study, we introduced this deletion into an infectious cDNA clone of HP-PRRSV strain vJX143 (pJX143) to investigate its effect on virus replication in cultured cells and the transcriptional activation of inflammatory cytokines and chemokines in PAMs.

We found that the 88-aa deletion mutant (rJX143-D88) grew to higher titers, exhibited faster growth kinetics, and produced larger plaques when compared to the parental virus (rJX143), indicating that the 88-aa deletion in nsp2 affects adaptation of the virus to cultured cells. Our results are consistent with previous studies that showed that a large 145-aa deletion in the C-terminus of nsp2 that was observed after continuous passage of the VR2385 virus in cell cultures appeared to enhance PRRSV replication in cultured MARC-145 cells [30]. However, the positions of the amino acid deletions in nsp2 of two attenuated strains (JXM100 and VR2385-CA) are different. The previous study also showed that a 145-aa deletion had no effect on the pathogenicity of the virus *in vivo* [30]. Our study also shows that the 88-aa deletion in nsp2 decreases the production of infectious viruses in PAMs at 48 hpi. The effect the 88-aa deletion in nsp2 on pathogenicity of HP-PRRSV *in vivo* needs to be studied in the future.

The innate immune response is the first line of defense of the host in response to infection. Viral infections result in the activation of pattern recognition receptors (PRRs) to initiate antiviral signaling cascades, triggering the production of cytokines, including interferons (IFNs) and chemokines [20, 32, 41]. The production of IFN- $\alpha$  and IFN- $\beta$  relies on activation of the transcription factors IRF-3/7, NF- $\kappa$ B and

AP-1 through either the TLR or RLR pathway. In addition to the role of amino acid deletions in nsp2 in virus replication, some studies have shown that natural or engineered deletions in nsp2 play an important role in modulating the induction of inflammatory cytokines *in vitro* [6, 7, 9, 26].

TLRs are key components of the host innate recognition system that lead to the transcription of inflammatory and anti-inflammatory cytokines. It has been shown that the expression of TLR-3, -4 and -7 mRNA is significantly suppressed or unchanged in PAMs infected with type 1 or type 2 PRRSV [56]. Our study also showed that the expression of TLR-3 mRNA was unchanged in rJX143 infection. However, rJX143-D88 infection resulted in a significant and dramatic upregulation of TLR-3 mRNA. This is consistent with another study, which showed that infection of pigs with PRRSV induced the upregulation of TLRs in at least one lymphoid tissue and cell type [56]. We also observed slight increases or unchanged levels in IRF-3 in both rJX143 and rJX143-D88-infected cells. However, the expression with IRF7 mRNA was significantly increased upon infection of rJX143-D88, suggesting that infection of cells with the nsp2 deleted mutation induced significant sustained transcriptional activation of IRF-7. Type I interferons (IFN- $\alpha$  and IFN- $\beta$ ) are the most potent antiviral cytokines against invading viruses, producing pleiotropic effects through the induction of expression of a very large number of IFN-stimulated genes (ISGs) [35].

Numerous studies have shown that the IFN- $\alpha$  and IFN- $\beta$  response is weak in PRRSV-infected pigs, MARC-145 cells, and PAMs, suggesting that the virus may actively suppress the IFN production both *in vivo* and *in vitro* [2, 23, 28]. In this study, we also found that PAMs infected with HP-PRRSV strain rJX143 do not reduce or downregulate the mRNA expression of IFN- $\alpha$  and IFN- $\beta$ . The expression of ISGs (PKP, ISG15 and ISG54), downstream genes of IFN- $\alpha$  and IFN- $\beta$ , also remained unchanged in rJX143-infected cells. This suggests that PRRSV is able to strongly suppress IFN gene expression during virus replication. In contrast, PAMs infected with mutant virus rJX143-D88 exhibited upregulation of IFN mRNA and high levels of ISG15, ISG54 and PKR gene expression. These results suggest that mutants with the 88-aa deletion in nsp2 can modulate the IFN signaling pathway for the promotion of an antiviral state in infected PAMs to limit viral replication.

One of the most remarkable features of PRRSV infection is that the virus fails to elicit any significant inflammatory cytokine expression in the lungs of swine infected with PRRSV. In this study, the expression levels of IL-1 $\alpha$ , IL- $\beta$  and IL-8 mRNA changed slightly or remain unchanged in both rJX143-D88- and rJX143-infected cells at most time points, whereas infection of PAM cells with rJX143-D88 resulted in significant transcriptional activation of these cytokines at 24 hpi. The expression levels of

IL-6, IL-10, and IL-12p40 were not significantly different between rJX143- and rJX143-D88-infected PAMs at any time point. However, we observed a slight increase at 12 hpi in cells infected with parental virus and nsp2 deletion mutant virus when compared to mock infection. Upregulation of IL-10, a crucial immunoregulatory phenomenon, can inhibit the production of numerous inflammatory cytokines and counter adaptive immunity [34]. Our results are consistent with previous data showing that PRRSV infection induces IL-10 expression *in vivo* and *in vitro* at both the mRNA and protein level [27, 38–40, 46].

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine secreted by a wide variety of cells, including macrophages. Previous studies have demonstrated that PRRSV infection downregulates TNF- $\alpha$  gene expression both *in vitro* and *in vivo* [1, 14], and TNF- $\alpha$  is poorly expressed in the acute phase of infection in PRRSV-infected pigs [13]. The HP-PRRSV strains HV and JXwn06 are weaker inducers of TNF- $\alpha$  production than the conventional strain CH-1a, which may partially contribute to the pathogenesis of HP-PRRSV [18]. Our data also showed that infection of PAMs with HP-PRRSV strain rJX143 resulted in slight downregulation of TNF- $\alpha$  mRNA at early time points. Interestingly, TNF- $\alpha$  mRNA levels were increased to a much greater degree in PAMs infected with mutant rJX143-D88 at 24 hpi.

Chemokines play important roles in regulation of the movement of leukocytes to the sites of inflammation. We observed little or no change in the expression of chemokines (MCP-1 MCP-2 and AMCF-1) in rJX143-infected cells. However, mutants produced much higher levels of MCP-1, AMCF-1 and MCP-2 mRNA in PAMs. Our data are consistent with those of a previous study which showed that chemokine mRNA was upregulated to a much greater degree in PAMs infected with nsp2 deletion isolates [7].

Overall, our data indicate that the HP-PRRSV strain rJX143 negatively affected the expression of IFN and IFN-related genes as well as other cytokine and chemokine genes, while a mutant with an 88-aa deletion in nsp2, rJX143-D88, more efficiently modulated expression of some cytokine and chemokine genes than the parental virus in PAMs, suggesting that the nsp2 deletion decreased viral replication. Our results demonstrate that virus-specific host immunity can be enhanced by modifying certain nsp2 epitope regions. These findings provide important insights into virus pathogenesis and will be useful for future vaccine development.

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## Compliance with ethical standards

**Conflict of interest** The authors declare they have no conflict of interest.

**Ethical approval** The animal experiment protocol complied with the Ethical Review Process and adhered to the guidelines of the Ethical and Animal Welfare Committee of Guangxi Province, China.

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