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Amino acid residues in the non-structural protein 1 of porcine reproductive and respiratory syndrome virus involved in down-regulation of TNF-α expression *in vitro* and attenuation *in vivo*

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) suppresses tumor necrosis factor-alpha (TNF-α) production at both transcriptional and post-transcriptional levels by its non-structural proteins 1α and 1β (Nsp1α and Nsp1β). To identify the amino acid residues responsible for this activity, we generated several alanine substitution mutants of Nsp1α and Nsp1β. Examination of the mutant proteins revealed that Nsp1α residues Gly90, Asn91, Arg97, Arg100 and Arg124 were necessary for TNF-α promoter suppression, whereas several amino acids spanning the entire Nsp1β were found to be required for this activity. Two mutant viruses, with mutations at Nsp1α Gly90 or Nsp1β residues 70–74, generated from infectious cDNA clones, exhibited attenuated viral replication *in vitro* and TNF-α was found to be up regulated in infected macrophages. In infected pigs, the Nsp1β mutant virus was attenuated in growth. These studies provide insights into how PRRSV evades the effector mechanisms of innate immunity during infection.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) causes late-term abortion in sows and respiratory disease in young pigs (Christianson et al., 1993; Christianson et al., 1992; Rossow et al., 1995). Following infection, PRRSV replicates in lungs and secondary lymphoid tissues in the host and establishes a viremic period of about 3–4 weeks (Duan, Nauwynck, and Pensaert, 1997; Rossow et al., 1994; Rossow et al., 1995). The viremic period is followed by a persistent period of 1–6 months, characterized by low levels of virus replication in secondary lymphoid tissues (Allende et al., 2000). The host immune response usually takes several months to clear the virus from persistently infected swine (Allende et al., 2000).

Previous studies demonstrated that the adaptive immune responses against PRRSV develop gradually, an important factor in less efficient clearance of the virus from the host (Allende et al., 2000; Meier et al., 2003). The ineffective adaptive immune responses against PRRSV are the result of various immune evasion strategies utilized by this virus (Ansari et al., 2006; Beura et al., 2010; Costers et al., 2009; Lopez and Osorio, 2004; Ostrowski et al., 2002; Subramaniam et al., 2010; Vu et al., 2011). One such strategy consists of inhibiting the key pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α) in infected cells (Lopez-Fuertes et al., 2000; Subramaniam et al., 2010). The TNF-α response against different PRRSV strains in pulmonary alveolar macrophages (PAMs) varies significantly depending on the strain (Darwich et al., 2011; Gimeno et al., 2011). Since TNF-α can inhibit PRRSV replication in macrophages (Lopez-Fuertes et al., 2000), we hypothesize that PRRSV-mediated TNF-α suppression would likely enhance virus production in the infected host.

Our laboratory has previously shown that PRRSV-vFL12 strain suppresses TNF-α during infection, at the promoter level and also post-transcriptionally (Subramaniam et al., 2010). Particularly, vFL12 strain suppresses the TNF-α promoter only at early times after infection. Even though TNF-α transcripts are abundant at later time points after vFL12 infection, secreted TNF-α is not detected in the infected culture supernatants (Subramaniam et al., 2010). The non-structural proteins 1α (Nsp1α) and 1β (Nsp1β) of the virus down-regulate NF-κB and Sp1 activities at TNF-α promoter, respectively (Subramaniam et al., 2010). In addition to Nsp1 proteins, PRRSV non-structural protein 2 (Nsp2) also regulates TNF-α expression in infected cells (Chen et al., 2010). The variations in Nsp2 sequences account for differences in TNF-α induction in response to various PRRSV field isolates (Darwich et al., 2011).

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Nsp1α and Nsp1β are non-structural proteins that participate in various aspects of the PRRSV life cycle such as transcription, virion biogenesis and innate immune evasion (Beura et al., 2010; Kroese et al., 2008; Tijms et al., 2007). Nsp1α, the proteolytically processed amino-terminal region of Nsp1, contains 180 amino acid residues and forms two domains, an N-terminal zinc finger domain (ZF domain) and a C-terminal papain-like cysteine protease (PCPα) domain (Sun et al., 2009). Nsp1β, the proteolytically processed carboxy-terminal region of Nsp1, contains 203 amino acid residues and forms three domains, an N-terminal nuclease domain, a linker domain and a C-terminal papain-like cysteine protease (PCPβ) domain (Xue et al., 2010). The PCPα and PCPβ domains auto-cleave Nsp1α and Nsp1β from the viral polyprotein, respectively (Kroese et al., 2008). Upon cleavage and activation, both of these proteins homo-dimerize (Sun et al., 2009; Xue et al., 2010). PCPα-mediated auto-cleavage of Nsp1α is essential for transcription of viral sub-genomic RNAs (Kroese et al., 2008). Likewise, PRRSV Nsp1α ZF domain may also directly participate in viral transcription (Fang and Snijder, 2010; Tijms et al., 2007). On the other hand, PCPβ-mediated auto-cleavage of Nsp1β is essential for PRRSV replication (Kroese et al., 2008). In addition, the Nsp1β nuclease domain cleaves double-stranded DNA, and single-stranded RNA *in vitro* (Xue et al., 2010). However, the Nsp1α and Nsp1β sequences necessary for down-regulating the TNF-α promoter activity are unknown.

In this study, we conducted mutagenesis studies to identify the amino acid residues in Nsp1α and Nsp1β that are necessary for affecting TNF-α promoter activity. Five Nsp1α amino acid residues, Gly90, Asn91, Arg97, Arg100, and Arg124 were identified as required for suppression of TNF-α promoter activity. Several Nsp1β amino acid residues spanning the entire protein were found to be necessary for suppression of TNF-α promoter activity. We subsequently recovered two mutant viruses from infectious cDNA clones with alanine substitution at Nsp1α Gly90 residue or Nsp1β 70–74 amino acid positions. These mutant viruses induced TNF-α mRNAs efficiently but induced protein levels minimally in infected macrophages when compared to the infection with wild type virus. In infected swine, the Nsp1β mutant virus exhibited growth-attenuated phenotype as compared to the wild type virus. Overall, these results suggest the possibility of generating attenuated PRRSVs for vaccine development through mutations in Nsp1β.

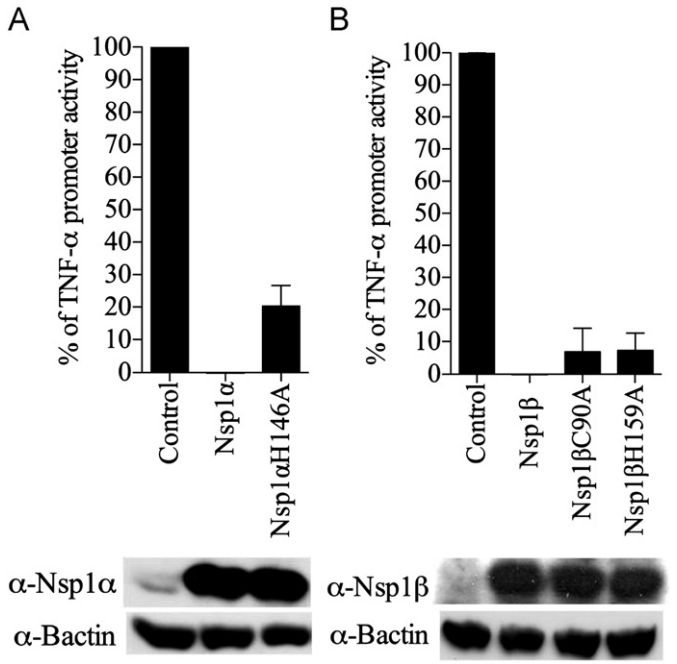

Fig. 1. PRRSV Nsp1α and Nsp1β suppress TNF-α promoter activity independently of their cysteine protease functions. (A, B) RAW 264.7 cells were transfected with pswTNF-luc plasmid (0.2 μg) along with indicated viral protein expressing plasmid (1 μg) and a renilla luciferase-expressing vector (10 ng). After 24 h, cells were stimulated with LPS (1 μg) for 6 h. In cell lysates, firefly luciferase activities were measured and normalized with renilla luciferase activities. 100% TNF-α promoter activity represents the activity in control vector transfected cells and 0% promoter activity represents the activity in wild type Nsp1α or Nsp1β transfected cells. Each bar represents mean ± standard error ($n=3$). Bottom panels depict the amount of corresponding viral proteins in transfected cells. RAW 264.7 cells were transfected with viral protein expressing plasmid (2 μg). After 24 h post-transfection, the viral protein in cell lysates was detected by western blotting using anti-Nsp1 antibodies. β-actin was used as loading control.

Results

Cysteine protease activities of Nsp1 are not necessary for TNF-α promoter suppression

Nsp1α and Nsp1β cysteine protease activities are mediated by PCPα and PCPβ, respectively (Kroese et al., 2008). The histidine residue at amino acid position 146 in vFL12-Nsp1α is a part of PCPα active site as deduced by pairwise sequence alignment with Nsp1α of Lelystad strain (Kroese et al., 2008). Similarly, the cysteine residue at amino acid position 90 and the histidine residue at amino acid position 159 of vFL12-Nsp1β are part of the PCPβ active site, as deduced by pairwise sequence alignment with Nsp1β of Lelystad strain (Kroese et al., 2008). Mutation of these residues to alanine in the expression constructs did not affect their respective protein levels (Fig. 1A and B, bottom panels). Transient reporter assays were carried out with a TNF-α promoter-luciferase construct and lipopolysaccharide (LPS) was used to stimulate the promoter. Both Nsp1α and Nsp1β mutated in their respective cysteine protease active sites (PCPα and PCPβ, respectively) efficiently reduced the TNF-α promoter activity in those assays when compared to their wild type counterparts (Fig. 1A, B). The results suggest that Nsp1α and Nsp1β reduce the TNF-α promoter activity independently of their cysteine protease activities.

Identification of Nsp1α amino acid residues critical for TNF-α promoter suppression

Nsp1α has two distinct domains: the ZF domain (1–65 amino acids) and the PCPα domain (66–166 amino acids) (Sun et al., 2009). To identify which of these domains is necessary for reducing TNF-α promoter activity, we performed alanine-scanning mutagenesis in randomly selected blocks of 4–6 amino acid length spanning the entire Nsp1α protein. In transient reporter assays, none of the PCPα domain mutants were able to reduce TNF-α promoter activity (Fig. 2A). Certain PCPα domain mutants such as Nsp1α122-6A, Nsp1α139-5A, and Nsp1α155-5A exhibited reduced protein levels when compared to the wild type protein (Fig. 2A). Three out of four ZF domain mutants (Nsp1α20-4A, Nsp1α55-5A, and Nsp1α63-5A) reduced the TNF-α promoter activity to similar extent as the wild type protein (Fig. 2A). The remaining ZF domain mutant (Nsp1α41-5A) suppressed the TNF-α promoter less efficiently than did the wild type protein, which may be due to reduced protein expression (Fig. 2A). Three amino acid scanning mutations in PCPα domain but not in the ZF domain also relieved TNF-α promoter suppression (data not shown). These results suggest that the PCPα domain but not ZF

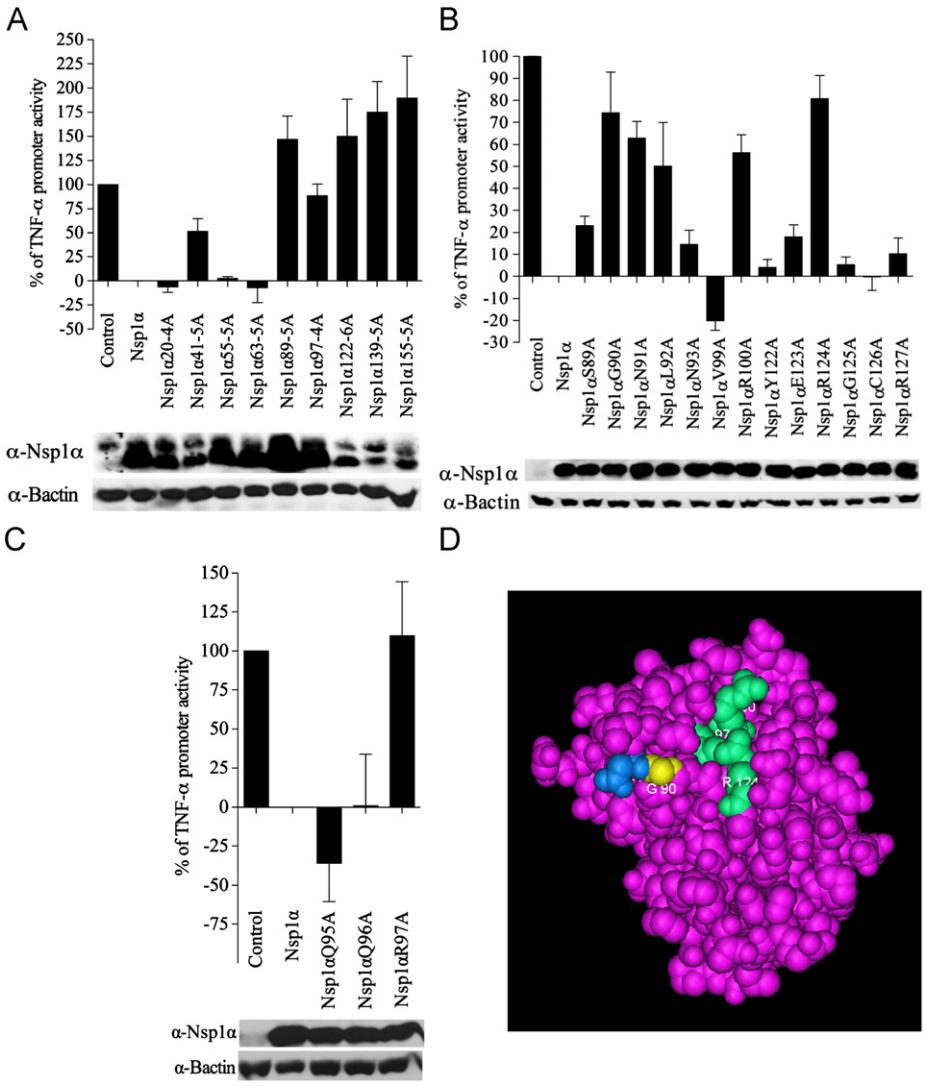

Fig. 2. Characterization of Nsp1α amino acid residues necessary for reducing TNF-α promoter activity. (A–C) RAW 264.7 cells were transfected with pswTNF-luc (0.2 μg) along with Nsp1α wild type or mutant protein expressing plasmid (1 μg) and a renilla luciferase-expressing vector (10 ng). After 24 h, cells were stimulated with LPS (1 μg/mL) for 6 h. Firefly luciferase activities were measured in cell lysates and normalized with renilla luciferase activities. 100% TNF-α promoter activity represents the activity in control vector transfected cells and 0% promoter activity represents the activity in wild type Nsp1α transfected cells. Each bar represents mean ± standard error ($n=3$). The immunoblot panels depict the amount of corresponding viral proteins in transfected cells. RAW 264.7 cells were transfected with Nsp1α wild type or mutant protein expressing plasmid (2 μg). After 24 h post-transfection, the viral protein in cell lysates was detected by western blotting using anti-Nsp1 antibodies. β-actin was used as loading control. (D) Tertiary structure of Nsp1α. Residues necessary for reducing TNF-α promoter activity are highlighted: Three Arginine residues (in green), the Glycine residue (in yellow) and the Asparagine residue (in blue). The figure was adopted from NCBI structure database (PDB ID: 3IFU) using Cn3D software. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

domain appears to be primarily responsible for suppression of TNF-α promoter activity.

Additional studies were performed to identify individual amino acid residues in PCPα domain that are necessary for reducing TNF-α promoter activity. Point mutations were introduced in selected amino acid stretches 89–93, 95–97, and 122–127 of PCPα domain. Mutation of PCPα domain residues, Gly90, Asn91, Arg97, Arg100, and Arg124 significantly up regulated TNF-α promoter activity when compared to the wild type protein (Fig. 2B, C). Mutations of these amino acid residues did not affect protein levels (Fig. 2B and C) or protease activities of Nsp1α (data not shown). Examination of the Nsp1α tertiary structure revealed that the amino acid residues, Gly90, Asn91, Arg97, Arg100, and Arg124 are closely positioned on PCPα domain surface (Fig. 2D). These studies suggest that the five amino acid residues in PCPα domain, Gly90, Asn91, Arg97, Arg100, and Arg124 are important for suppressing the TNF-α promoter activity.

Mutations in several Nsp1β amino acid stretches relieved TNF-α promoter suppression

Nsp1β has three domains: an N-terminal nuclease domain (1–48 amino acids), a flexible linker domain (49–84 amino acids) and a C-terminal PCPβ domain (85–181 amino acids) (Xue et al., 2010). To identify the domains involved in suppressing the TNF-α promoter activity, alanine-scanning mutations of 4–6 amino acids in all Nsp1β domains were introduced. In transient reporter assays, most mutant proteins demonstrated relief of TNF-α promoter suppression relative to the wild type protein (Fig. 3). The mutant, Nsp1β101-5A suppressed TNF-α promoter moderately when compared to the wild type protein (Fig. 3). Although the mutants, Nsp1β124-4A and Nsp1β136-5A, were expressed at reduced protein levels when compared to the wild type protein (Fig. 3), the relief of TNF-α promoter suppression activity was significant. The results suggest that all Nsp1β domains may be

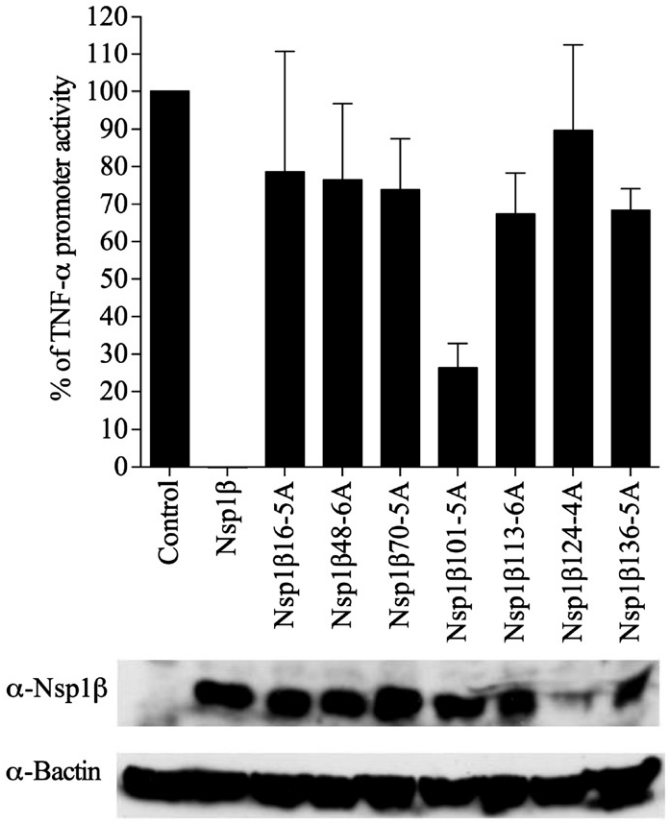

Fig. 3. All domains of Nsp1β contribute to TNF-α promoter suppression. RAW 264.7 cells were transfected with pswTNF-luc (0.2 μg) along with Nsp1β wild type or mutant expressing plasmid (1 μg) and a renilla luciferase vector (10 ng). After 24 h, cells were stimulated with LPS (1 μg/mL) for 6 h. Firefly luciferase activities were measured in cell lysates and normalized with renilla luciferase activities. 100% TNF-α promoter activity represents the activity in control vector transfected cells and 0% promoter activity represents the activity in wild type Nsp1β transfected cells. Each bar represents mean ± standard error ($n=3$). Bottom panels depict the amount of corresponding viral proteins in transfected cells. RAW 264.7 cells were transfected with Nsp1β wild type or mutant expressing plasmid (2 μg). After 24 h post-transfection, the viral protein in cell lysates was detected by western blotting using anti-Nsp1 antibodies. β-actin was used as loading control.

involved in inhibiting TNF-α promoter activity. Alternatively, it is possible that mutations in some of these domains may have affected the overall structure of the protein, thus rendering it nonfunctional in its ability to suppress TNF-α promoter activity.

Recovery, growth kinetics and plaque morphology of Nsp1α and Nsp1β mutant viruses

In the case of Nsp1α, attempts were made to recover viruses harboring 3–6 amino acid block mutations from infectious cDNA clones containing the mutations. Viruses with mutations at the Nsp1α positions, 89–93, 95–97, 97–100, and 122–127 were non-viable as judged by the absence of anti-N (PRRSV nucleocapsid) immunofluorescence (Table 1) in cells infected with supernatants from full-length viral RNA transfected cells. However, two viruses (vFL12Nsp1αG90A and vFL12Nsp1αG90S) were recovered with single point mutations at Nsp1α amino acid residue, Gly90 (Table 1). Viruses harboring other single point mutations at Nsp1α amino acid residues, Asn91, Arg97, Arg100, and Arg124 were not viable (Table 1).

In the case of Nsp1β, attempts were made to generate viruses with mutations at Nsp1β positions, 70–74 and 113–118. The infectious virus was successfully recovered with mutations at residues 70–74 (vFL12Nsp1β70-5A) but a viable virus could not

Table 1
Recovery of PRRSV mutant viruses by reverse genetics.

Amino acid position[a]	Anti-N immunofluorescence	Virus recovery
Nsp1α (89–93)	−	−
Nsp1α (95–97)	−	−
Nsp1α (97–100)	−	−
Nsp1α (122–127)	−	−
Nsp1α Gly90Ala	+	+
Nsp1α Gly90Ser	+	+
Nsp1α Asn91Ala	−	−
Nsp1α Arg97Ala	−	−
Nsp1α Arg100Ala	−	−
Nsp1α Arg124Ala	−	−
Nsp1β (70–74)	+	+
Nsp1β (113–118)	−	−

[a] Number in the parentheses indicate the position of amino acids mutated to alanines.

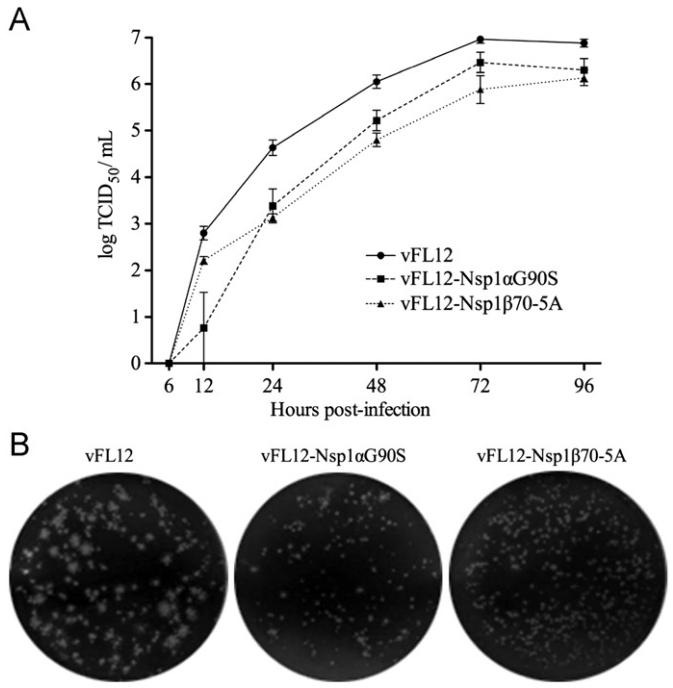

Fig. 4. Nsp1 mutant viruses have reduced growth when compared to wild type vFL12 virus. (A) Multi-step growth kinetics in MARC-145 cells after infection with 0.1 m.o.i. of indicated viruses. Supernatant of infected cells was collected at indicated time points and viral titration was performed in MARC-145 cells. Viral titers are expressed as tissue culture infectivity dose$_{50}$ (TCID$_{50}$). Viral titer values represent the mean ± standard error ($n=3$). (B) Plaque morphology of viruses in MARC-145 cells. Cells were infected with FL12 wild type or mutant viruses, covered with growth medium agar and incubated for 4 day. Cells were fixed with glutaraldehyde followed by crystal violet staining.

be recovered with mutation at residues 113–118 (Table 1). In an attempt to recover a virus containing mutations in both Nsp1α G90 and Nsp1β positions 70–74, we generated cDNA clones with these mutations. However, repeated attempts to recover such a virus were unsuccessful. Overall, we recovered three mutant PRRSVs, two presenting mutations in Nsp1α at G90 (G90A, G90S) and one with a mutation in Nsp1β at positions, 70–74. Of the two Nsp1α mutant viruses, we used the virus with G90S substitution for further studies, as this mutant virus contains two nucleotide substitutions in this codon and is therefore less likely to readily revert to the wild type sequence.

Multi-step growth kinetic analysis revealed that the mutant viruses (vFL12Nsp1αG90S and vFL12Nsp1β70-5A) exhibited overall similar growth kinetics as the parental wild type vFL12

in MARC-145 cells. The vFL12Nsp1β70-5A virus grew slightly slower than vFL12 virus exhibiting approximately a ten-fold difference in titer (Fig. 4A). The vFL12Nsp1αG90S virus grew to a titer that is approximately 5–7 fold less compared to vFL12 (Fig. 4A). Both mutant viruses also exhibited reduced plaque sizes as compared to the wild type virus (Fig. 4B). Overall, these results suggest that the mutant viruses, vFL12Nsp1αG90S and vFL12Nsp1β70-5A, exhibit somewhat reduced growth in cultured MARC-145 cells.

Expression of TNF-α in macrophages infected by Nsp1 mutant PRRSVs is up regulated

Studies were performed to examine TNF-α responses after infecting swine macrophages with vFL12 wild type or the mutant viruses. Infections with Nsp1 mutant viruses induced higher levels of TNF-α mRNAs only at 12 hpi when compared to the infection with the wild type virus (Fig. 5A). Importantly, the mutant virus vFL12Nsp1β70-5A induced significant levels of TNF-α mRNA at the early (12 hpi) time of infection (Fig. 5A). However, there were no differences observed in TNF-α mRNA levels between wild type and mutant virus infected macrophage cultures at 24 hpi (Fig. 5A). Our additional experiments also showed that the Nsp1β70-5A mutant protein did not affect Sp1-dependent transcriptional activities in transient transfection assays (data not shown), which are necessary for activating the TNF-α promoter (Falvo et al., 2000). The mutant vFL12Nsp1αG90S virus induced higher levels of TNF-α protein in the supernatant of infected macrophage cultures both at 12 and 24 hpi (Fig. 5B). However, both wild type vFL12 and mutant vFL12Nsp1β70-5A viruses induced negligible levels of TNF-α protein in the supernatant of infected macrophage cultures (Fig. 5B). Furthermore, the mutant viruses, replicated with low viral titers in macrophage cultures after infection when compared to wild type vFL12 (Fig. 5C). In particular, the mutant vFL12Nsp1β70-5A showed an approximately 100-fold reduction in viral titers in macrophage culture supernatants when compared to vFL12 (Fig. 5C).

Additionally, the macrophages infected with the mutant viruses also induced higher mRNA levels of two pro-inflammatory chemokines: CCL2 and CXCL10 at 24 and 36 h post-infection when compared to macrophages infected with vFL12 (data not shown). This is not unexpected considering that these two chemokines are induced by TNF-α (Murao et al., 2000; Qi et al., 2009). In summary, the two mutant viruses, vFL12Nsp1αG90S and vFL12Nsp1β70-5A, stimulated the production of TNF-α mRNA and protein more strongly than the wild type vFL12 in infected macrophage cultures.

Growth attenuation and TNF-α induction phenotypes of PRRSV mutant viruses in infected pigs

To examine the growth of the Nsp1 mutant viruses and TNF-α responses in swine, pigs were infected with wild type vFL12 or the mutant viruses. By 14 day post-infection (dpi), all infected pigs seroconverted (data not shown). In addition, all infected pigs exhibited their peak viremia between 3 and 7 day post-infection (Fig. 6A, B). Sequencing of viral RNAs isolated from serum until 14 day post-infection confirmed the presence of Nsp1αG90S and Nsp1β70-5A mutations in respective mutant viruses. When compared to wild type vFL12, the mutant vFL12Nsp1β70-5A exhibited nearly two $\log_{10}$ reduction in viral titers and viral RNA copies in serum at 3 dpi (Fig. 6A, B). By 7 dpi, two out of three animals infected with the mutant vFL12Nsp1β70-5A had no detectable infectious virus in

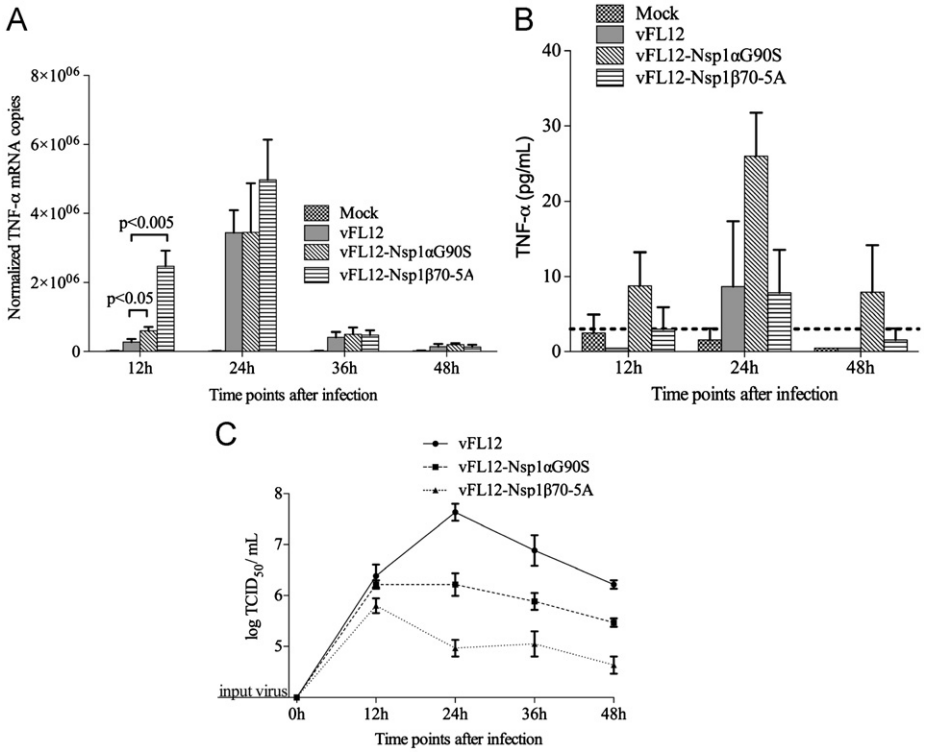

Fig. 5. TNF-α response against Nsp1 mutant viruses in infected primary macrophages *in vitro*. (A) Quantitative PCR analysis. PBMC-derived macrophages were mock-infected or infected with 0.1 m.o.i. of indicated viruses. At different time points, total RNAs were prepared from infected cells and cDNAs were synthesized. Using cDNAs as template, the amount of TNF-α mRNAs was quantified by qPCR. TNF-α copy numbers were calculated using standard curve prepared with known amount of templates and normalized with β-actin copy numbers. Bars represent mean ± standard error ($n=3$). (B) TNF-α protein measurement by ELISA. PBMC-derived macrophages were infected with vFL12 wild type or indicated mutant viruses at 0.1 m.o.i. TNF-α protein levels in the supernatant were measured by ELISA. Bars represent mean ± standard error ($n=3$). Dotted line in *y*-axis represents the detection limit of assay. (C) Viral titers were measured in MARC-145 cells and expressed as $TCID_{50}$/mL. Mean ± standard error ($n=3$) values were shown.

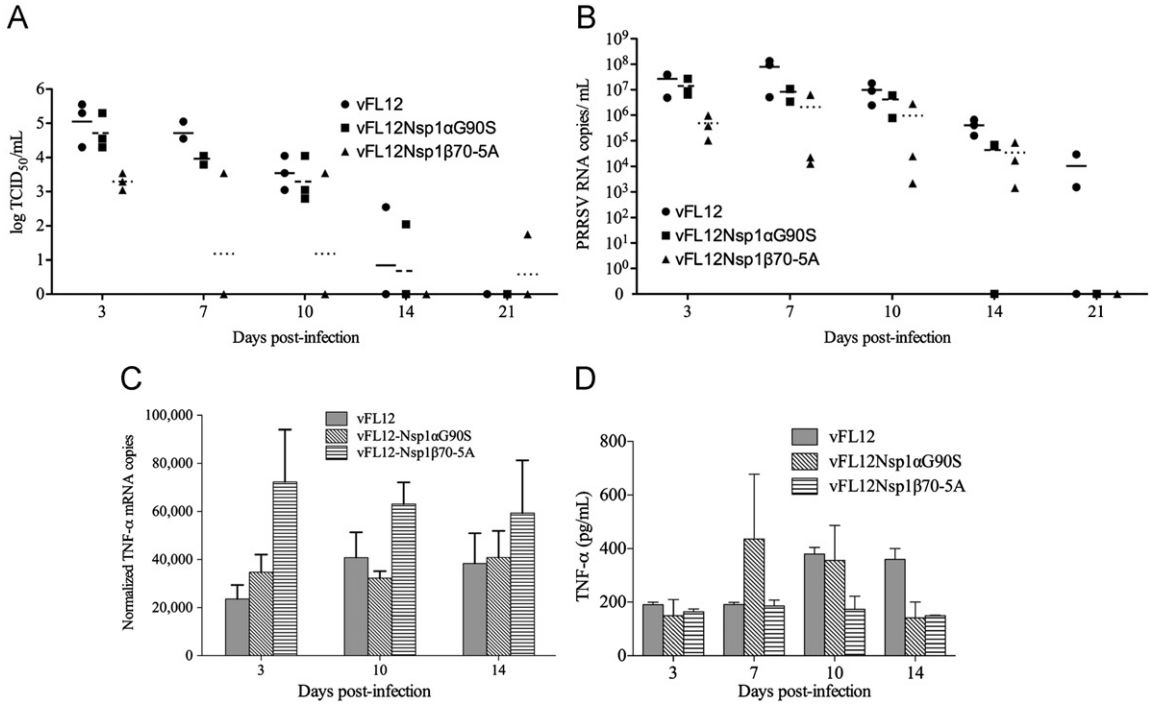

Fig. 6. Comparison of viremia, TNF-α expression levels between pigs infected with vFL12 wild type and pigs infected with mutant viruses. Four-week old piglets ($n=3$ each group) were infected intra-muscularly with $10^{5.1}$TCID$_{50}$ of vFL12 wild type or indicated mutant viruses. (A) Viral titers were evaluated in serum of infected pigs at indicated time points after infection. Viral titration was done in MARC-145 cells. The viral titers were calculated by Reed and Muench method, and expressed as tissue culture infectivity dose$_{50}$ (TCID$_{50}$/mL). Data indicate mean ± standard error ($n=3$). (B) PRRSV-specific quantitative RT-PCR analysis. Viral RNAs were isolated from serum of infected pigs at indicated time points after infection. They were quantified by qRT-PCR specific to vFL12 3'UTR as described in methods section. Viral RNA copies were calculated by using a standard curve prepared with known amount of templates. Data indicate mean ± standard error ($n=3$). (C) TNF-α-specific qRT-PCR. Total RNAs were isolated from 1×10^6 PBMCs collected from pigs at indicated time points after infection. TNF-α mRNA levels were quantified by qRT-PCR as described in methods section. TNF-α mRNA copies were normalized with β-actin mRNA copies. Bars represent mean ± standard error values ($n=3$). (D) TNF-α ELISA. TNF-α protein levels in serum of infected pigs were measured by ELISA at indicated time points after infection. Values are expressed as mean ± standard error ($n=3$).

serum (Fig. 6A). However, viral RNAs were detected at lower levels in pigs infected with the mutant vFL12Nsp1β70-5A when compared to pigs infected with vFL12 for 14 dpi (Fig. 6B). In contrast, pigs infected with the mutant vFL12Nsp1αG90S had similar viral titers as well as viral RNA copies in serum when compared to the pigs infected with vFL12 (Fig. 6A, B).

The TNF-α mRNA levels in PBMCs were enhanced in pigs infected with vFL12Nsp1β70-5A virus at 3 dpi when compared to the wild type vFL12 infection (Fig. 6C). Pigs infected with the mutant vFL12Nsp1αG90S showed increased TNF-α protein levels in serum at 7 dpi when compared to pigs infected with wild type vFL12 (Fig. 6D). However, at 10 dpi, both vFL12 and vFL12Nsp1αG90S groups had similar elevated levels of TNF-α (Fig. 6D). In summary, the mutant vFL12Nsp1β70-5A virus replicated with reduced viral titers and the virus also up-regulated TNF-α mRNA levels at 3 dpi in infected pigs.

Discussion

PRRSV decreases the production of TNF-α during infection *in vitro* and *in vivo* (Calzada-Nova et al., 2011; Labarque et al., 2003; Lopez-Fuertes et al., 2000; Subramaniam et al., 2010; van Gucht, van Reeth, and Pensaert, 2003). Nsp1α and Nsp1β are important viral mediators that reduce TNF-α promoter activity in transient transfection assays (Subramaniam et al., 2010). In this study, we identified critical amino acid residues in Nsp1α and Nsp1β that are important for TNF-α down-regulation. Subsequently, through reverse genetics, we generated two mutant PRRSV viruses that up-regulated TNF-α expression, particularly at mRNA level in infected macrophage cultures. These two mutant viruses replicated in cell culture with reduced viral titers. In infected pigs, one mutant virus with mutation in Nsp1β replicated with reduced viral titers in serum.

Several laboratories demonstrated that PRRSV infection leads to poor TNF-α response in serum and broncho-alveolar lavage fluid from infected pigs (Labarque et al., 2003; Thanawongnuwech et al., 2004; van Gucht, van Reeth, and Pensaert, 2003). Similarly, PRRSV infection actively inhibits TNF-α expression in macrophages and dendritic cells *in vitro* (Calzada-Nova et al. 2011; Lopez-Fuertes et al., 2000). Previous results from our laboratory indicated that PRRSV cysteine proteases, Nsp1α and Nsp1β suppress TNF-α promoter by modulating the activity of specific transcription factors, NF-κB and Sp1, respectively (Subramaniam et al., 2010). In this study, we sought to further characterize the down-regulatory effect by identifying the amino acid residues in Nsp1α and Nsp1β that reduce TNF-α production. Upon such identification, we pursued recovering PRRSV strains with mutations in those positions by reverse genetics and examining the mutants in infected animals for their ability to relieve TNF-α suppression.

A previous study had shown that active site mutations in Nsp1 cysteine proteases lead to failure of virus recovery by reverse genetics (Kroese et al., 2008). Our result showed that the Nsp1 cysteine protease activities were not necessary for suppressing TNF-α promoter activity. This observation is consistent with previous findings that the Nsp1 cysteine proteases cleave only cis-substrates present at the end of respective proteins (Sun et al., 2009; Xue et al., 2010). Therefore, it is less likely that Nsp1 cysteine protease activities degrade signaling molecules required for the TNF-α-induction pathway.

Screening of domain-specific mutations in Nsp1α revealed that the PCPα domain, but not the ZF domain, was primarily

important for inhibiting TNF-α promoter activity. Five amino acid residues (Gly90, Asn91, Arg97, Arg100, and Arg124) on the surface of the PCPα domain were important for inhibiting the TNF-α promoter. Many mutations in the ZF domain of Nsp1α did not significantly relieve TNF-α suppression, which suggested that the ZF domain may not directly participate in inhibiting the TNF-α promoter activity. We were only able to recover mutant viruses with mutations at Nsp1α Gly90 residue. However, viruses with mutations at Nsp1α residues Asn91, Arg97, Arg100, and Arg124, were not viable, though they were found to be dispensable for PCPα protease activity. These amino acid residues may be important for other Nsp1α functions, such as viral transcription, replication and/or virion biogenesis (Fang and Snijder, 2010).

Unlike Nsp1α, all three domains of Nsp1β seem to be important for down regulating TNF-α promoter activity. One of Nsp1β mutant (70-5A) exhibits protein levels equivalent to wild type but did not reduce TNF-α promoter activity efficiently. In agreement with this, 70-5A mutant protein did not also reduce transcription driven by Sp1, which bind TNF-α promoter to activate transcription (Falvo et al., 2000). The 70-5A mutation is located in a small linker domain of Nsp1β. The successful recovery of mutant virus in this position suggests that the amino acids at 70–74 position are not required for Nsp1β protease activity over Nsp1β–Nsp2 junction. It seems plausible that the Nsp1β linker domain may directly participate in the inhibition of Sp1 trans-activation or indirectly modulate the functions of the other Nsp1β domains to inhibit Sp1-dependent TNF-α transcription.

Previous studies demonstrated that PRRSV suppresses TNF-α expression at the transcriptional and post-transcriptional levels (Subramaniam et al., 2010; Thanawongnuwech et al., 2004). PRRSV particularly inhibited the TNF-α transcription during early time points after infection in macrophages (Subramaniam et al., 2010). We confirmed such previous finding in the present study. Both Nsp1 mutant virus infections showed enhanced TNF-α transcriptional activity at early times (12 h) post-infection. Particularly, the Nsp1β mutant virus significantly induced TNF-α mRNA levels at 12 h post-infection in infected macrophage cultures. In consistent with these *in vitro* observations, pigs infected with Nsp1β mutant virus also up-regulated TNF-α mRNA levels at 3 dpi in peripheral blood mononuclear cells.

When the growth characteristics of Nsp1 mutant viruses were examined, both viruses were moderately attenuated in growth in susceptible MARC-145 cells, which may be due to the effects in other Nsp1 functions such as transcription and replication. When we examined the growth of these mutant viruses in macrophages *in vitro* and in infected pigs, the growth of vFL12Nsp1β70-5A was severely compromised but the growth of vFL12Nsp1αG90S was affected to a minimal level. Even though vFL12Nsp1β70-5A mutant virus has ability to induce TNF-α mRNAs earlier than the wild type virus, it did not induce significant TNF-α protein at any time point after infection *in vitro* or *in vivo*. Hence, the crippled growth of vFL12Nsp1β70-5A in macrophages *in vitro* and in infected pigs is more likely due to loss of some other critical function of the protein important for viral progression.

The vFL12Nsp1αG90S mutant virus infection produced detectable levels of TNF-α protein in macrophages when compared to wild type virus infection. This suggests that in addition to its effect at the promoter level, Nsp1α also inhibits TNF-α production via a post-transcriptional mechanism. This is evident in animal studies in which vFL12Nsp1αG90S mutant virus induced TNF-α in serum of infected pigs at 7 dpi when compared to wild type vFL12 infected pigs. Additional experiments also revealed that, when over-expressed, Nsp1α reduces co-expressing TNF-α protein levels without affecting Internal Ribosome Entry Site (IRES)-dependent GFP expressed from a bi-cistronic mRNA. Further, in those experiments, Nsp1αG90S mutant protein did not efficiently reduce TNF-α protein levels expressed from the bi-cistronic construct when compared to the wild type protein.

In summary, the overall conclusions of this study are: (1) Nsp1α amino acid residue Gly90 is necessary for suppressing the TNF-α promoter activity and TNF-α protein levels during PRRSV infection; (2) Nsp1β amino acid residues at 70–74 are necessary for suppressing the TNF-α promoter activity; and (3) PRRSV-vFL12 strain with mutation at Nsp1β positions 70–74 produced significantly reduced viral titers in infected pigs, an information that may pave the way for new candidate attenuated live vaccines.

Materials and methods

Cells and viruses

RAW 264.7 cells, a murine macrophage cell line (ATCC) was maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). MARC-145 cells, an African green monkey kidney epithelial cell line (obtained from Dr. Will Laegreid, University of Illinois, Urbana-Champaign) was maintained in Dulbecco's modified eagle medium (DMEM)-low glucose supplemented with 10% FBS. BHK-21, baby hamster kidney epithelial cell line (ATCC) was maintained in DMEM-high glucose supplemented with 10% FBS. Primary swine macrophages were prepared from PBMCs as previously described (Subramaniam et al., 2010). PRRSV vFL12 strain (GenBank ID: AY545985) and its mutant viruses were propagated and titrated in MARC-145 cells. Viral titers were calculated using the Reed and Muench method (Reed and Muench, 1938). Viral plaque assays were performed in MARC-145 cells as previously described (Ansari et al., 2006).

TNF-α promoter-luciferase reporter assay

Swine TNF-α promoter-luciferase reporter assay was carried out as previously described (Subramaniam et al., 2010). RAW 264.7 cells were co-transfected with plasmids, pswTNF-luc (0.2 μg) (Subramaniam et al., 2010) and pRL-TK (10 ng) (Promega), and wild type or mutant Nsp1α/Nsp1β-expressing plasmid (1 μg) using DEAE-dextran (Sigma) by following a procedure described earlier (Subramaniam et al., 2010). After 24 h of transfection, the reporter genes were stimulated with LPS (1 μg/mL) for 6 h. Firefly and renilla luciferase activities were measured using dual luciferase reporter assay system in GloMax 20/20 luminometer (Promega). Firefly luciferase units were normalized with renilla luciferase units. Relative luciferase units were calculated by dividing normalized firefly luciferase units measured from stimulated cells with those measured from unstimulated cells.

Plasmids construction

pIHA-Nsp1Δ268–297 plasmid was constructed by deleting thirty amino acids from 268 to 297 amino acid positions in the whole Nsp1 sequence (Gene Bank Accession No. AY545985). Plasmids expressing Nsp1α or Nsp1β (pIHA-Nsp1α and pIHA-Nsp1β (Subramaniam et al., 2010)) were used to introduce 3–6 amino acid alanine-scanning mutations. pIHA-Nsp1Δ268–297 plasmid was used to introduce point mutations in Nsp1α. Mutations were introduced into Nsp1 genes by PCR mutagenesis following the mega primer method (Sarkar and Sommer, 1990).

In order to transfer Nsp1α and Nsp1β mutant sequences from pIHA constructs into pFL12 infectious clone (Truong et al., 2004), we constructed an intermediate transfer vector pIHA-2757. The plasmid was constructed by cloning *Rsr*II and *Spe*I restriction-digested fragment of pFL12 infectious clone into pIHA empty vector (Beura et al., 2010). Nsp1α mutant sequences were cloned

into pIHA-2757 using *Acc*I and *Stu*I restriction sites present within Nsp1α. Nsp1β mutant sequences were cloned into pIHA-2757 using *Avr*II and *Bsr*GI restriction sites present within Nsp1β. Finally, the *Rsr*II and *Spe*I digested fragment was transferred from the pIHA-2757 mutant plasmid into pFL12 infectious clone. The corresponding mutation in pFL12 plasmid was confirmed by sequencing.

Quantitative RT-PCR and ELISA

TNF-α-specific quantitative PCR (qPCR) was performed as previously described (Subramaniam et al., 2010). Briefly, total RNA fractions were prepared from mock-infected cells or cells infected with either vFL12 or mutant viruses using Trizol-LS reagent (Invitrogen). Complementary DNAs (cDNAs) were synthesized using oligo-dTs as primer. cDNAs were used as template in qPCR reactions to measure TNF-α or β-actin mRNAs using sequence-specific primers and probes (Subramaniam et al., 2010).

PRRSV 3′untranslated region (UTR)-specific qRT-PCR was performed to detect viral RNAs in serum of infected pigs. Briefly, viral RNAs were isolated from 140 μL of serum using QIAamp viral RNA mini kit (Qiagen). Viral RNAs (4 μL) were used as template in quantitative reverse-transcription (RT)-PCR reaction using hot start-IT Probe one step qRT-PCR kit (USB, 75772). The following primers and probe were used in the reaction: forward primer ATGTGTGGTGAATGGCACTG, reverse primer GCATGGTTCTCGCCAATTAAA, Taqman probe 6-FAM-TCACCTATTCAATTAGGGCGACCG-TAMRA. The cycling conditions employed were as follows: reverse transcription at 50 °C for 30 min, initial enzyme activation at 95 °C for 2 min, denaturation at 95 °C for 15 s followed by annealing/extension at 60 °C for 60 s. Denaturation and annealing/extension steps were repeated for total of 45 cycles.

To measure the TNF-α protein levels in the supernatant of mock-infected or virus-infected cells, a commercial swine TNF-α-specific ELISA was used (Pierce/Invitrogen).

Western blotting

Cellular protein lysates were prepared in either radio-immunoprecipitation assay buffer or cell-lysis buffer as described elsewhere (Alcaraz et al., 1990; Beura et al., 2010). Equal amounts of total protein were resolved in 12% Sodium dodecyl Sulfate (SDS)-polyacrylamide gel electrophoresis and transferred on Polyvinylidene fluoride (PVDF) membrane. The membrane was blotted with rabbit polyclonal antibodies specific for Nsp1 or mouse monoclonal antibodies specific for β-actin (Santacruz) for overnight at 4 °C. The membranes were treated with Horse Radish Peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG antibodies (KPL) for 1 h at RT and signals were obtained with chemiluminescence substrates (Thermo scientific).

In vitro transcription, electroporation and recovery of mutant viruses

Capped *in vitro* transcripts (IVTs) were prepared from wild type or mutant pFL12 infectious clones as previously described (Kwon et al., 2006; Truong et al., 2004). IVTs were electroporated into MARC-145 or BHK-21 cells by following a procedure described earlier (Ansari et al., 2006). After 1–2 day post-electroporation, the replication of mutant virus was confirmed by immunofluorescence using anti-N monoclonal antibodies (SDOW17, NVSL-USDA). The success of viral recovery was assessed by spread of cytopathic effect in electroporated MARC-145 cells. The supernatants from electroporated BHK-21 cells were transferred on to PBMC-derived macrophages to propagate recovered viruses.

Viral growth kinetics and in vitro infection studies

For multi-step growth kinetics, MARC-145 cells were cultured in 96-well plates two days before infection. On the third day, cells were infected with vFL12 wild type or mutant viruses at 0.1 multiplicity of infection (m.o.i.) in triplicates. At 6, 12, 24, 48, 72, 96 h post-infection, supernatants were collected and viruses were titrated in MARC-145 cells. The viral titers were calculated using Reed and Muench method (Reed and Muench, 1938). For measuring TNF-α mRNA and protein responses *in vitro*, PBMC-derived macrophages were infected with vFL12 wild type or mutant viruses at 0.1 m.o.i. At 12, 24, 36 and 48 h post-infection, cells were collected in Trizol-LS (Invitrogen) for mRNA quantification. Supernatants were collected to measure TNF-α protein and viral titers.

Animal experiments

Four-week old PRRSV-negative pigs ($n=3$ each group) were infected with vFL12 wild type or mutant strains at $10^{5.1}$ $TCID_{50}$/2 mL intra-muscularly. At 3, 7, 10, 14, 21 day post-infection (dpi), serum was collected to determine viremia and TNF-α protein levels by viral titration and ELISA, respectively. Similarly, PBMCs (1×10^6 cells/aliquot) were collected in Trizol-LS for mRNA quantification.

Statistical analysis

The significance in difference between means of two treatment groups was tested using one-tailed unpaired student's t-test. A '*p*' value of less than 0.05 was considered significant. Analyses were performed using Prism 5 (Graphpad).

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References

Alcaraz, C., De Diego, M., Pastor, M.J., Escribano, J.M., 1990. Comparison of a radioimmunoprecipitation assay to immunoblotting and ELISA for detection of antibody to African swine fever virus. J. Vet. Diagn. Invest. 2 (3), 191–196.

Allende, R., Laegreid, W.W., Kutish, G.F., Galeota, J.A., Wills, R.W., Osorio, F.A., 2000. Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. J. Virol. 74 (22), 10834–10837.

Ansari, I.H., Kwon, B., Osorio, F.A., Pattnaik, A.K., 2006. Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. J. Virol. 80 (8), 3994–4004.

Beura, L.K., Sarkar, S.N., Kwon, B., Subramaniam, S., Jones, C., Pattnaik, A.K., Osorio, F.A., 2010. Porcine reproductive and respiratory syndrome virus nonstructural protein 1beta modulates host innate immune response by antagonizing IRF3 activation. J. Virol. 84 (3), 1574–1584.

Calzada-Nova, G., Schnitzlein, W.M., Husmann, R.J., Zuckermann, F.A., 2011. North American porcine reproductive and respiratory syndrome viruses inhibit type I interferon production by plasmacytoid dendritic cells. J. Virol. 85 (6), 2703–2713.

Chen, Z., Zhou, X., Lunney, J.K., Lawson, S., Sun, Z., Brown, E., Christopher-Hennings, J., Knudsen, D., Nelson, E., Fang, Y., 2010. Immunodominant epitopes in nsp2 of porcine reproductive and respiratory syndrome virus are dispensable for replication, but play an important role in modulation of the host immune response. J. Gen. Virol. 91 (Pt 4), 1047–1057.

Christianson, W.T., Choi, C.S., Collins, J.E., Molitor, T.W., Morrison, R.B., Joo, H.S., 1993. Pathogenesis of porcine reproductive and respiratory syndrome virus infection in mid-gestation sows and fetuses. Can. J. Vet. Res. 57 (4), 262–268.

Christianson, W.T., Collins, J.E., Benfield, D.A., Harris, L., Gorcyca, D.E., Chladek, D.W., Morrison, R.B., Joo, H.S., 1992. Experimental reproduction of swine infertility and respiratory syndrome in pregnant sows. Am. J. Vet. Res. 53 (4), 485–488.

Costers, S., Lefebvre, D.J., Goddeeris, B., Delputte, P.L., Nauwynck, H.J., 2009. Functional impairment of PRRSV-specific peripheral CD3+CD8 high cells. Vet. Res. 40 (5), 46.

Darwich, L., Gimeno, M., Sibila, M., Diaz, I., de la Torre, E., Dotti, S., Kuzemtseva, L., Martin, M., Pujols, J., Mateu, E., 2011. Genetic and immunobiological diversities of porcine reproductive and respiratory syndrome genotype I strains. Vet. Microbiol. 150 (1-2), 49–62.

Duan, X., Nauwynck, H.J., Pensaert, M.B., 1997. Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). Vet. Microbiol. 56 (1-2), 9–19.

Falvo, J.V., Uglialoro, A.M., Brinkman, B.M., Merika, M., Parekh, B.S., Tsai, E.Y., King, H.C., Morielli, A.D., Peralta, E.G., Maniatis, T., Thanos, D., Goldfeld, A.E., 2000. Stimulus-specific assembly of enhancer complexes on the tumor necrosis factor alpha gene promoter. Mol. Cell. Biol. 20 (6), 2239–2247.

Fang, Y., Snijder, E.J., 2010. The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins. Virus Res. 154 (1–2), 61–76.

Gimeno, M., Darwich, L., Diaz, I., de la Torre, E., Pujols, J., Martin, M., Inumaru, S., Cano, E., Domingo, M., Montoya, M., Mateu, E., 2011. Cytokine profiles and phenotype regulation of antigen presenting cells by genotype-I porcine reproductive and respiratory syndrome virus isolates. Vet. Res. 42 (1), 9.

Kroese, M.V., Zevenhoven-Dobbe, J.C., Bos-de Ruijter, J.N., Peeters, B.P., Meulenberg, J.J., Cornelissen, L.A., Snijder, E.J., 2008. The nsp1alpha and nsp1 papain-like autoproteinases are essential for porcine reproductive and respiratory syndrome virus RNA synthesis. J. Gen. Virol. 89 (Pt 2), 494–499.

Kwon, B., Ansari, I.H., Osorio, F.A., Pattnaik, A.K., 2006. Infectious clone-derived viruses from virulent and vaccine strains of porcine reproductive and respiratory syndrome virus mimic biological properties of their parental viruses in a pregnant sow model. Vaccine 24 (49–50), 7071–7080.

Labarque, G., Van Gucht, S., Nauwynck, H., Van Reeth, K., Pensaert, M., 2003. Apoptosis in the lungs of pigs infected with porcine reproductive and respiratory syndrome virus and associations with the production of apoptogenic cytokines. Vet. Res. 34 (3), 249–260.

Lopez-Fuertes, L., Campos, E., Domenech, N., Ezquerra, A., Castro, J.M., Dominguez, J., Alonso, F., 2000. Porcine reproductive and respiratory syndrome (PRRS) virus down-modulates TNF-alpha production in infected macrophages. Virus Res. 69 (1), 41–46.

Lopez, O.J., Osorio, F.A., 2004. Role of neutralizing antibodies in PRRSV protective immunity. Vet. Immunol. Immunopathol. 102 (3), 155–163.

Meier, W.A., Galeota, J., Osorio, F.A., Husmann, R.J., Schnitzlein, W.M., Zuckermann, F.A., 2003. Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology 309 (1), 18–31.

Murao, K., Ohyama, T., Imachi, H., Ishida, T., Cao, W.M., Namihira, H., Sato, M., Wong, N.C., Takahara, J., 2000. TNF-alpha stimulation of MCP-1 expression is mediated by the Akt/PKB signal transduction pathway in vascular endothelial cells. Biochem. Biophys. Res. Commun. 276 (2), 791–796.

Ostrowski, M., Galeota, J.A., Jar, A.M., Platt, K.B., Osorio, F.A., Lopez, O.J., 2002. Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J. Virol. 76 (9), 4241–4250.

Qi, X.F., Kim, D.H., Yoon, Y.S., Jin, D., Huang, X.Z., Li, J.H., Deung, Y.K., Lee, K.J., 2009. Essential involvement of cross-talk between IFN-gamma and TNF-alpha in CXCL10 production in human THP-1 monocytes. J. Cell. Physiol. 220 (3), 690–697.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hygiene 27, 493–497.

Rossow, K.D., Collins, J.E., Goyal, S.M., Nelson, E.A., Christopher-Hennings, J., Benfield, D.A., 1995. Pathogenesis of porcine reproductive and respiratory syndrome virus infection in gnotobiotic pigs. Vet. Pathol. 32 (4), 361–373.

Rossow, K.D., Bautista, E.M., Goyal, S.M., Molitor, T.W., Murtaugh, M.P., Morrison, R.B., Benfield, D.A., Collins, J.E., 1994. Experimental porcine reproductive and respiratory syndrome virus infection in one-, four-, and 10-week-old pigs. J. Vet. Diagn. Invest. 6 (1), 3–12.

Sarkar, G., Sommer, S.S., 1990. The "megaprimer" method of site-directed mutagenesis. Biotechniques 8 (4), 404–407.

Subramaniam, S., Kwon, B., Beura, L.K., Kuszynski, C.A., Pattnaik, A.K., Osorio, F.A., 2010. Porcine reproductive and respiratory syndrome virus non-structural protein 1 suppresses tumor necrosis factor-alpha promoter activation by inhibiting NF-kappaB and Sp1. Virology 406 (2), 270–279.

Sun, Y., Xue, F., Guo, Y., Ma, M., Hao, N., Zhang, X.C., Lou, Z., Li, X., Rao, Z., 2009. Crystal structure of porcine reproductive and respiratory syndrome virus leader protease Nsp1alpha. J. Virol. 83 (21), 10931–10940.

Thanawongnuwech, R., Thacker, B., Halbur, P., Thacker, E.L., 2004. Increased production of proinflammatory cytokines following infection with porcine reproductive and respiratory syndrome virus and Mycoplasma hyopneumoniae. Clin. Diagn. Lab. Immunol. 11 (5), 901–908.

Tijms, M.A., Nedialkova, D.D., Zevenhoven-Dobbe, J.C., Gorbalenya, A.E., Snijder, E.J., 2007. Arterivirus subgenomic mRNA synthesis and virion biogenesis depend on the multifunctional nsp1 autoprotease. J. Virol. 81 (19), 10496–10505.

Truong, H.M., Lu, Z., Kutish, G.F., Galeota, J., Osorio, F.A., Pattnaik, A.K., 2004. A highly pathogenic porcine reproductive and respiratory syndrome virus generated from an infectious cDNA clone retains the in vivo virulence and transmissibility properties of the parental virus. Virology 325 (2), 308–319.

van Gucht, S., van Reeth, K., Pensaert, M., 2003. Interaction between porcine reproductive-respiratory syndrome virus and bacterial endotoxin in the lungs of pigs: potentiation of cytokine production and respiratory disease. J. Clin. Microbiol. 41 (3), 960–966.

Vu, H.L., Kwon, B., Yoon, K.J., Laegreid, W.W., Pattnaik, A.K., Osorio, F.A., 2011. Immune evasion of porcine reproductive and respiratory syndrome virus through glycan shielding involves both glycoprotein 5 as well as glycoprotein 3. J. Virol. 85 (11), 5555–5564.

Xue, F., Sun, Y., Yan, L., Zhao, C., Chen, J., Bartlam, M., Li, X., Lou, Z., Rao, Z., 2010. The crystal structure of porcine reproductive and respiratory syndrome virus nonstructural protein Nsp1beta reveals a novel metal-dependent nuclease. J. Virol. 84 (13), 6461–6471.