The N-N non-covalent domain of the nucleocapsid protein of type 2 porcine reproductive and respiratory syndrome virus enhances induction of IL-10 expression

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Porcine reproductive and respiratory syndrome virus (PRRSV) usually establishes a prolonged infection and causes an immunosuppressive state. It has been proposed that IL-10 plays an important role in PRRSV-induced immunosuppression. However, this mechanism has not been completely elucidated. In this study, we found that transfection of 3D4/2 macrophages with the N protein gene of type 2 PRRSV significantly upregulated IL-10 expression at the transcriptional level. Moreover, alanine substitution mutation analysis revealed that the N protein residues 33–37, 65–68 and 112–123 were related to the upregulation of IL-10 promoter activity. Recombinant PRRSV with mutations at residues 33–37 in the N protein (rQ33-5A and rS36A) recovered from corresponding infectious cDNA clones and induced significantly lower levels of IL-10 production in infected monocyte-derived dendritic cells, as compared with their revertants rQ33-5A(R) and rS36A(R), and the wild-type recombinant PRRSV strain rNT/wt. These data indicate that the type 2 PRRSV N protein plays an important role in IL-10 induction and the N-N non-covalent domain is associated with this activity.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS), an economically important disease worldwide, is caused by the porcine reproductive and respiratory syndrome virus (PRRSV), an enveloped, single-stranded, positive-sense, RNA virus containing 10 ORFs that encode 14 non-structural proteins (Nsps) and eight structural proteins. Among them, ORF7 encodes the nucleocapsid (N) protein (Firth et al., 2011; Johnson et al., 2011; Snijder et al., 2013). PRRSV is divided into two genotypes: European (type 1 virus) and American (type 2 virus) (Meng et al., 1995). The N protein in type 2 PRRSV is a small basic protein composed of 123 aa (Snijder & Meulenberg, 1998; Wootton et al., 2002), which is highly immunogenic in pigs and believed to be multifunctional. The N protein has five important antigenic regions at residues 30–52, 37–52, 52–69, 69–112 and 112–123 (Rodriguez et al., 1997; Wootton et al., 1998). The non-covalent interactions involving residues 31–37 contribute to N protein polymerization (Rowland & Yoo, 2003). The N protein residues (112–123) of the N protein are crucial to maintain structural conformation (Doan & Dokland, 2003). A cryptic nuclear localization signal (NLS), called NLS-1, a functional NLS (NLS-2) and a nucleolar localization sequence (NoLS) are, respectively, located at residues 10–13, 41–47 and 41–72 (Rowland et al., 2003). Three conserved cysteine residues at positions 23, 75 and 90, respectively, participate in covalent interactions through disulfide linkages (Fu et al., 2012; Lee et al., 2005).

IL-10 is an immunoregulatory cytokine that plays an important role in protecting the host from infection-associated immunopathology, autoimmunity and allergy (Ng et al., 2013). Since its discovery in the 1980s, many reports have led to the recognition of IL-10 as a pleiotropic cytokine that modulates both the innate and adaptive immune responses. IL-10 was originally named ‘cytokine synthesis inhibiting factor’, since it can inhibit the transcription and translation of a variety of inflammatory cytokines (Donnelly et al., 2004). IL-10 is expressed by dendritic cells (DCs), macrophages, natural killer cells and T-cells (Magatti et al., 2014; Roquilly et al., 2014; Xie et al.,...
and induced by many viruses, such as human immunodeficiency virus (HIV)-1 and foot-and-mouth disease virus (Díaz-San Segundo et al., 2009; Liu et al., 2014).

IL-10 is thought to play an important role in the immunopathogenesis of PRRSV infection (Mateu & Diaz, 2008). PRRSV infection can upregulate IL-10 expression (Charerntantanakul et al., 2006; Suradhat & Thanawongnuwech, 2003), which causes an imbalance between proinflammatory cytokines, such as TNF-α, and impairment of the immune response in the lungs (Gómez-Laguna et al., 2013). Transcriptional analysis of PRRSV-infected pulmonary alveolar macrophages (PAMs) revealed IL-10 upregulation at 12 h post-infection (p.i.) (Genini et al., 2008). IL-10 induction was also observed in PRRSV-infected DCs (Chang et al., 2008). However, some conflicting results showed that IL-10 expression was not correlated with PRRSV infection (Klinge et al., 2009; Thanawongnuwech et al., 2001; Wang et al., 2007). Thus, further exploration of PRRSV proteins or even the aa residues of these proteins that are correlated with IL-10 expression is warranted. A recent study showed that transfection of porcine monocyte-derived DCs (MoDCs) and PAMs with a plasmid encoding the N protein resulted in significant upregulation of IL-10 expression (Wongyanin et al., 2012). In this study, we investigated whether the N protein of PRRSV strain NT0801 had the ability to induce IL-10 expression. In order to elucidate the relationship between the N protein and IL-10 expression, we performed alanine-scanning mutagenesis to identify key aa that influence IL-10 induction. Our results indicate that aa 33–37 of the PRRSV N protein play important roles in IL-10 induction.

RESULTS

PRRSV upregulates IL-10 protein level

MoDCs were incubated with PRRSV or UV-inactivated PRRSV at an m.o.i. of 1, while lipopolysaccharide (LPS; 1 μg ml⁻¹) was used as a positive control. As shown in Fig. 1(a), secreted IL-10 protein levels were measured in supernatants containing PRRSV at the indicated time points p.i., which showed that the ability of the virus to induce IL-10 production was dose-dependent (Fig. 1b). The kinetics of PRRSV infection showed a peak viral titre at 24 h p.i. (Fig. 1c), indicating the IL-10 response induced by PRRSV coincided with viral replication. UV-inactivated PRRSV failed to induce an IL-10 response, as compared with that of the live virus.

PRRSV N protein upregulates IL-10 promoter activity

The 3D4/2 macrophage is a porcine PAM cell line that is frequently used for transfection of PRRSV genes to explore
immunomodulatory functions in response to infection (Fu et al., 2012; Wongyanin et al., 2012). After transfection of 3D4/2 macrophages with the wt pVAX-NP plasmid, PRRSV N protein expression was determined by immunoblotting. Meanwhile, a pVAX-GST construct expressing the Flag-GST marker was used as a negative control (Fig. 2a). The 3D4/2 macrophages were co-transfected with pIL-10 or the pGL3 control vector and pRL-TK along with pVAX-NP. Luciferase assay results indicated that N protein expression was significantly correlated with upregulation of IL-10 promoter activity ($P$, 0.01) (Fig. 2b). The activity of the IL-10 promoter was maintained at 48 and 72 h p.i. ($P$, 0.01) (Fig. 2c), and was dose-dependent with the level of the N protein ($P$, 0.01) (Fig. 2d).

**Effect of truncated and mutant N protein constructs on IL-10 promoter activity**

To determine whether the loss of protein conformation reduces IL-10 expression at the transcriptional level, a truncated N protein gene (aa 1–112; ORF7t) was constructed and the relationship between ORF7t and pIL-10 was explored. The results showed that the ability to upregulate IL-10 promoter activity was significantly decreased with the ORF7t construct compared with the wt N protein (Fig. 3a).

To determine whether the disulfide linkages mediating N protein dimerization actually influenced the effect on IL-10 promoter activity, three cysteine residues in the N protein were separately substituted with alanine residues (C23A, C75A and C90A) by site-directed mutagenesis. According to the luciferase assay results, none of the three cysteine residues influenced the ability of the N protein to stimulate IL-10 promoter activity (Fig. 3b). Furthermore, luciferase assay results of constructs of eight multi-point mutations in functional regions [K10-4A (e.g. aa 10–13 mutated as alanine), Q33-5A, K43-5A, K52-4A, P56-4A, E60-5A, H65-4A and V112-4A] revealed that alanine substitutions at Q33-5A, H65-4A and V112-4A significantly decreased IL-10 promoter activity, compared with the wt N protein ($P$, 0.01) (Fig. 3c).

To identify the key aa in the regions of residues 33–37, 65–68 and 112–115, each residue was mutated to alanine individually (Fig. 3d–f). The results showed that serine at
position 36 and arginine at position 113 played important roles (Fig. 3d, e). Meanwhile, no significant differences were observed with the mutants harbouring residues H65–68 (Fig. 3f).

Recovery of PRRSV harbouring mutations in the N protein and revertant viruses

To confirm the role of residues/domains in the PRRSV N protein in the regulation of IL-10 expression, a standard reverse genetics approach was used to recover mutant viruses (Fig. S1, available in the online Supplementary Material). But only two, rQ33-5A (aa 33–37 mutated as alanine) and rS36A (aa 36 mutated as alanine), containing mutations Q33-5A and S36A in the N protein, were successfully rescued. In order to further confirm the role of these residues in the regulation of IL-10 expression, the corresponding revertants, rQ33-5A(R) and rS36A(R), were also rescued. As shown in Fig. 4(a), plaque formation by the viruses in MARC-145 cells was similar to that of the wt recombinant rNT/wt. After passage five times, the nucleotides of all viruses used in the experiment were confirmed by sequencing. The genetic markers of all recombinant PRRSV were detected and the sequences of the N protein mutations in the viruses were stable, with no other aa substitutions observed, as compared with the rNT/wt strain (data not shown). Besides, the results of multi-step growth kinetics analysis showed no significant differences between recombinant viruses and the rNT/wt strain (P>0.05) (Fig. 4b).

Effect of recombinant PRRSV on IL-10 promoter activity

As shown in Fig. 4(c), the abilities of rQ33-5A and rS36A to activate the IL-10 promoter were significantly weaker than that of rNT/wt (P<0.01), while those of rQ33-5A(R) and rS36A(R) were similar to that of rNT/wt. Meanwhile, virus infection was confirmed by immunoblotting using cell lysates with monoclonal antibodies against the PPRSV
N protein and the virus titres in harvested cell supernatants were accessed using a plaque assay (Fig. 4d).

**Effect of recombinant PRRSV on expression of IL-10 and other pro-inflammatory cytokines**

To investigate the role of the recombinant viruses to regulate expression of IL-10 and other pro-inflammatory cytokines (i.e. TNF-α, IL-1β and IL-6), MoDCs were infected with recombinant viruses or corresponding UV-inactivated viruses at an m.o.i. of 0.1. Culture supernatants were collected at the indicated time points after infection and virus titres were determined. Mean titres with SD (error bars) from three independent experiments are shown. (c) IL-10 promoter activity influenced by PRRSV rNT0801, rQ33-5A, rS36A, rQ33-5A(R) and rS36A(R) recombinants. Results are expressed as relative luciferase activity. The panel below the bar graph shows the virus infection detected by immunoblotting with anti-N antibody. All assays were repeated three times, with each experiment performed in triplicate. Bars represent means± SEMs from three independent experiments. *Significant difference between groups (P<0.01). (d) The cell supernatants from the luciferase assay were harvested to determine the titres of viruses by plaque assay. The virus titres were calculated as p.f.u. ml⁻¹. Error bars represent the SEM.

**DISCUSSION**

Increased IL-10 expression has been reported to be a strategy of several viral pathogens, such as HIV-1, rhinovirus and cytomegalovirus, to suppress host immune responses (Koponen et al., 2013; Liu et al., 2014; Loebermann et al., 2012; Mason et al., 2013; Redpath et al., 2001). Upregulation of IL-10 could lead to the suppression of antigen-presenting-cell functions, including the production of pro-inflammatory cytokines (Redpath et al., 2001). Ultimately, reduced host immune responses facilitate infection and replication of viruses. However, in PRRSV infection, the issue as to whether IL-10 plays a critical role in PRRSV-induced immunosuppression remains controversial (Klinge et al., 2009), with most studies demonstrating that IL-10 upregulation plays an important role in immunopathogenesis in vitro (Charerntantanakul & Kasinrerk, 2010, 2012). PRRSV infection has been shown to upregulate IL-10 expression.
in peripheral blood mononuclear cells (PBMCs), leukocytes and mature MoDCs both in vitro and in vivo (Flores-Mendoza et al., 2008; Suradhat & Thanawongnuwech, 2003; Suradhat et al., 2003). However, the components of PRRSV responsible for IL-10 induction have not been clearly identified. Recently, it was reported that the N protein of type 2 PRRSV was associated with the induction of IL-10 responses (Wongyanin et al., 2012). The results of the present study also showed that the N protein of PRRSV strain NT0801 increased IL-10 promoter activity in 3D4/2 macrophages. The regions of aa 33–37, 65–68 and 112–123 in the N protein were found to play important roles in the upregulation of IL-10 gene promoter activity. Next, five recombinant PRRSVs [rNT/wt, rQ33-5A, rQ33-5A(R), rS36A and rS36A(R)] were constructed for infection studies of which the results showed that the region of aa 33–37 (N-N non-covalent domain) of the PRRSV N protein is important for induction of IL-10.

PRRSV can induce IL-10 and TNF-α response during in vitro and in vivo infection (López-Fuertes et al., 2000). Knockdown of IL-10 transcription was found to enhance TNF-α expression following PRRSV infection (Charerntananakul & Kasinrerk, 2012). Different molecular mechanisms resulting in IL-10 expression are thought to be activated by different stimuli. For example, PRRSV infection was found to activate IL-10 production through the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B-cells) and p38/mitogen-activated protein kinase (MAPK) pathways in porcine alveolar macrophages (Song et al., 2013) and the N protein was involved in NF-κB activation (Luo et al., 2011). Moreover, PRRSV Nsp1 was found to suppress activation of the TNF-α promoter by inhibition of NF-κB and Sp1 (Subramaniam et al., 2010). These studies confirmed that induction of inflammatory cytokines was involved in the activation of the NF-κB pathway. Consequently, activated NF-κB acts as a transcription factor in the regulation and induction of several inflammatory cytokines (Kawai & Akira, 2007; Lupfer & Kanneganti, 2013). Also, the PRRSV N protein reportedly plays a role in the function of activation of NF-κB (Fu et al., 2012; Luo et al., 2011). In our study, the IL-10 promoter contained elements of the transcription factors NF-κB, Sp1, activator protein 1, interferon regulatory factor, CCAAT enhancer binding protein (C/EBP), signal transducer and activator of transcription, and TATA-binding protein (Fig. S2) (Quan et al., 2012). Since the transcription factors NF-κB and C/EBP are involved in the NF-κB pathway, and Sp1 is involved in the p38 MAPK pathway, we speculated that the PRRSV N protein may have an effect on the NF-κB, C/EBP or Sp1 elements of the IL-10 promoter. However, this issue will have to be investigated in future studies (Song et al., 2013).

![Fig. 5](http://vir.sgmjournals.org) Levels of IL-10 (a), TNF-α (b), IL-1β (c) and IL-6 (d) in the supernatant of MoDCs infected with PRRSV rNT0801, rQ33-5A, rS36A, rQ33-5A(R) and rS36A(R) recombinants and the UV-inactivated virus (internal control) were measured with commercial ELISA kits. LPS was used as a positive control. All assays were repeated three times, with each experiment performed in triplicate. Data are presented as means ± SEM generated from three independent experiments in all groups at 6, 12, 18, and 24 h p.i. Bars indicate mean secreted cytokine protein levels expressed in pg supernatant ml⁻¹ ± SEM from three independent experiments. *Significant difference between groups (P<0.05). **Significant difference (P<0.01) between groups.
The PRRSV N protein conveys immunomodulatory properties (Fu et al., 2012; Sagong & Lee, 2011). In order to explore the relationships between the functional domains of the N protein and IL-10 expression, both truncants and mutants were constructed and transfected into cells, and IL-10 promoter activity was assessed. The results showed that the Q33-5A, H65-4A and V112-4A mutants significantly decreased IL-10 promoter levels, compared with the wt N protein, demonstrating that these regions were associated with IL-10 expression (Wongyanin et al., 2012). Mutations of cysteine residues at positions 23, 75 and 90 had no influence on IL-10 promoter activity, which might be due to the lack of structural change to the N protein (Zhang et al., 2012). The aa at positions 30–73 of the PRRSV N protein were found to be essential for NF-κB activation (Luo et al., 2011). Likewise, residues 33–37 and 65–68 were important for IL-10 induction. Meanwhile, we noted that mutant K43-5A, located in the NLS-2 region of the N protein, had no significant impact on IL-10 promoter activity, although previous studies have reported that mutations to residues 43–47 in the NLS-2 could prevent the N protein form entering the nucleus (Pei et al., 2008; Rowland et al., 2003). These results imply that transcription factors in the cytoplasm might play more important roles in IL-10 induction. Another possible explanation is that another region of the N protein, such as the NoLS, may also have compensatory effects on IL-10 promoter activity. Of course, the nuclear localization of K43-5A mutant N proteins and the possible function of NLS-2 in IL-10 expression should be further studied.

Cytokines are among the first pro-inflammatory molecules produced by alveolar macrophages in response to viral infection (Gómez-Laguna et al., 2010; Thanawongnuwech et al., 2004). IL-10 is an immunomodulatory cytokine that is able to inhibit the synthesis and release of other cytokines (Iannone et al., 2001). In this study, our results showed that the rQ33-5A and rS36A recombinants could downregulate IL-10 induction, as compared with rNT/wt. Meanwhile, there were no significant differences in IL-10 levels between the rQ33-5A(R), rS36A(R) and rNT/wt infection groups. Along with the observations of the extent of IL-10 induction by rQ33-5A and rS36A, compared with that of rNT/wt, levels of TNF-α and IL-1β were significantly increased in the former groups. These results were in agreement with those of an earlier in vitro experiment, in which IL-10 knockdown enhanced TNF-α expression in response to PRRSV infection in naïve blood monocytes (Charerntananakul & Kasinrerk, 2012). In our study, the downregulation of IL-10 induction in vitro may have contributed to the upregulation of the pro-inflammatory cytokines TNF-α and IL-1β at initial time points. The innate cytokines TNF-α and IL-1β were shown to play vital roles in the modulation of innate immune responses of PRRSV-infected pigs, which may be helpful to clear the virus in vivo (Lawson et al., 2012; Subramaniam et al., 2012). Otherwise, upregulated IL-10 production may be responsible for maintenance of TNF-α and downregulation of IL-1β from 12 h p.i. onwards. The aa 33–37, 65–68 and 112–123 (except 117) of the N protein were conserved in different type 2 PRRSV strains (data not shown); however, the extents of IL-10 induction were distinct (Choi et al., 2014; Guo et al., 2013; Zhang et al., 2013). This finding suggested that other regions of the viral genome might also be related to IL-10 induction. Indeed, several non-structural proteins of PRRSV are also able to induce IL-10 (Burgara-Estrella et al., 2013). Thus, there may be other PRRSV proteins that influence IL-10 induction, which should be addressed in future studies.

In summary, PRRSV strain NT0801 was able to induce upregulation of IL-10 and the N protein was found to be one of the viral components responsible. The region of aa 33–37 (N-N non-covalent domain) of the PRRSV N protein is important for induction of IL-10 expression. These findings provide an important example of rationally designed modifications to abolish the host innate response without alterations to the replication potential of the virus.

METHODS

Cells, viruses and antibodies. Porcine PAM 3D4/2 cells were purchased from ATCC (CRL-2845) and cultured in Roswell Park Memorial Institute 1640 medium (Gibco, Invitrogen) supplemented with 10 % FBS (heat-inactivated; Gibco, Invitrogen). MARC-145 cells were incubated in Dulbecco’s modified essential medium (DMEM; Gibco, Invitrogen) supplemented with 10 % heat-inactivated FBS, stored in our lab, and used for transfection of full-length cDNA, viral infection, passage and titration.

Type 2 PRRSV strain NT0801 (GenBank accession no. HQ315836.1) and its infectious clones were propagated in MARC-145 cells to prepare virus stocks for this study. Recombinant viruses were also propagated in MARC-145 cells upon recovery and passage 5 (P5) viruses were used for experiments in vitro.

Monoclonal antibody to PPRSV N protein was made previously in our lab. Mouse anti-FLAG antibody (#M20008) was purchased from Abmart and mouse anti-β-actin antibody (Sc-47778) was obtained from Santa Cruz Biotechnology.

Preparation of MoDCs. Porcine MoDCs were generated using a previously described protocol for the introduction of human ‘fast’ DGs (Dauer et al., 2003, 2005; Guan et al., 2004) and used to determine IL-10 levels following PRRSV infection. Briefly, PBMCs were isolated from the blood of piglets (free of PRRSV, porcine circovirus type 2, classical swine fever virus, pseudorabies virus, swine influenza virus and Mycoplasma hyopneumoniae) and washed three times with complete medium. The cells were resuspended in Iscove’s modified Dulbecco’s media (Gibco, Invitrogen) supplemented with 10 % heat-inactivated FBS, seeded into wells of a six-well plate at a concentration of 2.5 × 10^6 cells ml^-1 per well, and incubated at 37 °C in a 5 % CO₂ incubator for 4 h. The adherent cells were washed four times with PBS. One millilitre of the complete media supplemented with 50 ng ml^-1 of recombinant human IL-4 and granulocyte-macrophage colony-stimulating factor (R&D Systems) was added to the culture. The monocytes were further cultured for 24 h to allow differentiation of the porcine MoDCs.

IL-10 protein level measurements using an ELISA. MoDCs were resuspended in complete medium and plated into 24-well plates at a concentration of 5 × 10^6 cells ml^-1 per well, then inoculated with
PRRSV or UV-inactivated PRRSV at an m.o.i. of 1.0 for 6, 12, 18 or 24 h. MoDCs were also treated with LPS (1 µg ml\(^{-1}\)) or MARC-145 cell lysate (the same volume as PRRSV) as positive and negative controls, respectively. The cell supernatants were harvested for measurement of IL-10 protein levels using a commercial swine IL-10-specific ELISA kit (R&D Systems). In addition, MoDCs were inoculated with PRRSV or UV-inactivated PRRSV at different doses (0.01, 0.1, 1.0 or 10 m.o.i.) to determine IL-10 protein levels.

Plasmid construction. The PRRSV strain NT0801 N protein gene was amplified by reverse-transcription PCR (RT-PCR) with primers (Table S1) containing HindIII or EcoRI restriction sites to facilitate directional cloning, as well as fused with an N-terminal FLAG tag. The PCR fragments were cloned into the pVAX1 vector (Invitrogen) to obtain plasmid pVAX-VP (wt). Further, using pVAX-VP (wt) as a template, alanine substitutions of the N protein gene were prepared by site-directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s recommendations. Briefly, oligonucleotide primers were designed in the way that the mutation sequence was incorporated into the middle of the primer, leaving at least 15 nt at the 5’ or 3’ end matching the template. The PCR was run according to the instructions of the QuikChange mutagenesis kit using the plasmid DNA as template. A truncated N protein gene (aa 1–112) was constructed, referred to as pVAX-ORF7m (ORF7m) (Wongyamin et al., 2012). All mutations to the N protein gene in recombinant plasmids are summarized in Fig. S3. Additionally, pVAX-GST containing glutathione S-transferase (GST) fused with an N-terminal FLAG tag was used as a negative control.

The plasmid pIL-10, containing a partial porcine IL-10 promoter sequence from nt −1733 to +29 relative to the translation initiation site (+1) (GenBank accession no. K0P06513), was constructed in our lab using the pGL3 basic vector (Promega), which contains a modified coding region of firefly (Phoebys pyralis) luciferase. The pRL-TK plasmid (Promega), containing the Renilla luciferase reporter, served as an internal control.

Luciferase reporter assay. MARC-145 cells transfected with pIL-10 or pGL3 (negative control) were inoculated with PRRSV or MARC-145 cell lysate to test whether pIL-10 could be activated by PRRSV, as previously described (Fu et al., 2012; Quan et al., 2012). Cells in 12-well plates were transfected using Lipofectamine 2000 reagent (Invitrogen) with either 1 µg of pIL-10 or pGL3 together with 0.1 µg pRL-TK. After 24 h, the cells were mock infected (MARC-145 cell lysate) or infected with PRRSV at an m.o.i. of 1, and then harvested for luciferase activity analysis 24 h p.i. The contaminating LPS of the purified DNA plasmids from Escherichia coli was detected (<0.1 EU µg\(^{-1}\) before transfection, which guaranteed that the DNA plasmids used throughout the study had insufficient internal LPS to interfere with the results.

To determine the role of the N protein on IL-10 promoter activity, 3D4/2 macrophages seeded in 12-well plates at a concentration of 5 × 10\(^4\) cells per well were used for transfection. Each well contained 1 µg pIL-10 with 0.1 µg pRL-TK for normalization and 1 µg of various expression plasmids pVAX-VP (wt) or its mutants), pVAX-GST or empty control plasmid (pVAX1). As a positive control, the pVAX1-transfected group was treated with LPS at a concentration of 1 µg ml\(^{-1}\) 16 h prior to harvest for luciferase assay. Cell extracts were collected at the time points indicated.

Luciferase activity was monitored using a Dual-luciferase reporter assay system (Promega) following the manufacturer’s protocol. The values were normalized with respect to Renilla luciferase activity. The results are presented as fold-change of relative luciferase activities compared with the mock-treated group transfected with empty vectors. All assays were repeated at least three times with each experiment performed in triplicate.

Immunoblotting assay. The proteins isolated from the cell lysate were used as samples. The membranes were blocked in blocking buffer (Tris-buffered saline containing 10% non-fat dry milk and 0.1% Tween 20) for 1 h followed by incubation with the primary antibodies indicated. Afterwards, the membranes were washed and incubated with goat anti-mouse antibody labelled with horseradish peroxidase (Boster Bio-Tech). Finally, the proteins were detected using an ECL chemiluminescent kit (Bioyinique Technology) and imaged on X-ray films (Fujii Photo Film).

Construction of infectious cDNA clones of PRRSV. The full-length cDNA clone of PRRSV NT0801 was constructed and subcloned into modified pCMV vector using PacI and Spel as previously described (Lee et al., 2005) and shown in Fig. S1. The fragments were individually amplified by RT-PCR with the primers shown in Table S1 and cloned into a pMD18-T vector (TaKaRa Biotechnology). Finally, the fragments A, B, C and D in the vectors were cloned into the modified pCMV vector in the order D-C-B-A, and the full-length plasmid was obtained and named pNT/wt. Additionally, an Ncol restriction site as the genetic marker was introduced by replacement of the Xhol restriction site (bp 6992 in PRRSV NT0801 genome) with the primer pair GMF/GMR using a QuikChange XL site-directed mutagenesis kit as described above.

All full-length mutant clones were constructed with pNT/wt plasmid containing the N protein gene as the backbone (Fig. S1). Briefly, a 14767–15439 bp fragment was amplified using pNT/wt as template and cloned into pEASY-Blunt Simple vector (Beijing TransGen Biotech) with Mbal and SpeI restriction endonucleases (New England Biolabs), referred to as pORF6/7/3’UTR, which was used as the intermediate plasmid. Secondly, the splicing overlap extension PCR strategy was employed to construct the chimeric clones. Briefly, the intermediate PCR products were amplified using pORF6/7/3’UTR and mutants of N protein (ORF7m) as templates, and the resulting segments were spliced in a subsequent PCR. Thirdly, the Mbal/SpeI fragment of pNT/wt was replaced by the analogous fragments (ORF6/7m/3’UTR) derived from the final mutagenic PCR products; and the obtained full-length mutant clone of pNT/wt was referred to as pNT/ ORF7m. As for construction of revert mutants, ORFm(R) was first obtained by site-directed mutagenesis using ORFm as the template, and then the fragment ORF6/7m/3’UTR in pNT/ORF7m was substituted with ORF6/7m(R)/3’UTR (derived from the splicing overlap extension PCR using fragments ORF6, ORF7m(R) and 3’UTR) and resulted in pNT/ORF7m(R). All full-length mutant clones were verified by nucleotide sequencing. The plasmids of infectious cDNA clones pNT/wt, pNT/ORF7m and pNT/ORF7m(R) were isolated using the QIAprep spin miniprep kit (Qiagen).

MARC-145 cells were transfected at approximately 90% confluence in six-well plates with 2 µg per well of prepared DNA using 4 µl Lipofectamine 2000 reagent according to the manufacturer’s protocol. When about 80% cytopathic effects (CPEs) were observed, the cell culture supernatants were collected and designated passage 1 (P1). The P1 viruses were used to inoculate MARC-145 cells. The P2–P5 virus stocks were prepared in the same manner and the rescued viruses were referred to as rNT/wt, rORF7m (such as rQ33-5A and rS36A) and rORF7m(R) (such as rQ33-5A(R) and rS36A(R)).

RT-PCR and nucleotide sequencing. To verify the stability of mutations in the rescued viruses, RNA was isolated from the viruses (P5) using the QIAprep viral RNA minikit (Qiagen), according to the manufacturer’s instructions. To avoid possible carry-over DNA contamination from transfections (P1) during passage, the RNA of P5 recombinant viruses was treated with RNase-free DNase I (Promega). The fragments containing the genetic marker and the N protein gene were amplified using primer pairs GMDP6553/GMDRT766S and SF1476/SR15410 (Table S2). The PCR products were amplified using the following primer sets:
were purified using the AxyPrep DNA Mini Purification kit (Axygen Scientific) and cloned into the pEASY-Blunt Simple vector for sequencing.

**Viral plaque and growth kinetics assays.** Viral plaque and growth kinetics assays were conducted as previously described (Tian et al., 2011). The rescued viruses were serially diluted and used to infect MARC-145 cells cultured in 12-well plates. The cells were then overlaid with 2× DMEM (Gibco, Invitrogen) containing 4% heat-inactivated FBS and mixed with an equal volume of 2% low-melting-point agarose (Sigma-Aldrich). Finally, the plaques were stained with crystal violet. To construct growth curves, MARC-145 cells were infected at an m.o.i. of 0.1 with the P5 viruses indicated. Afterwards, culture supernatants were collected at the indicated time points p.i., and virus titres were determined using a plaque assay.

**Evaluation of cytokine production induced by recombination viruses.** MoDCs were prepared as described above and then inoculated with rNT/wt [rORF7m, rORF7m(R)] (P5) or corresponding UV-inactivated PRRSV as an m.o.i. of 1 and incubated for 24 h. LPS (1 µg ml⁻¹) was used as the positive control. The supernatants were then harvested at different time points p.i. (6, 12, 18 and 24 h) to measure the levels of IL-10 and other pro-inflammatory cytokines (i.e. TNF-α, IL-1β, IL-6) using relevant commercial ELISA kits (R&D Systems).

**Statistical analysis.** The significance of the variability among groups was determined with one-way or two-way ANOVA using GraphPad Prism software (version 5.0; GraphPad Software). Differences were deemed to be statistically significant at a probability (P) value of <0.05 or <0.01.

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