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

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Non-structural protein 2 (nsp2) of porcine reproductive and respiratory syndrome virus (PRRSV) is the largest protein of this virus. In addition to its crucial role in virus replication, recent studies have indicated its involvement in modulating host immunity. In this study, each of the six identified immunodominant nsp2 B-cell epitopes (ES2–ES7) was deleted using a type I PRRSV cDNA infectious clone. Deletion of ES3, ES4 or ES7 allowed the generation of viable virus. In comparison with the parental virus, the Δ ES3 mutant showed increased cytolytic activity and more vigorous growth kinetics, whilst the Δ ES4 and Δ ES7 mutants displayed decreased cytolytic activity and slower growth kinetics in MARC-145 cells. These nsp2 mutants were characterized further in a nursery pig disease model. The results showed that the Δ ES4 and Δ ES7 mutants exhibited attenuated phenotypes, whereas the Δ ES3 mutant produced a higher peak viral load in pigs. The antibody response reached similar levels, as measured by IDEXX ELISA at 21 days post-infection, and slightly higher levels of mean virus neutralizing titres were observed from pigs infected by the Δ ES4 and Δ ES7 mutants. The expression of innate and T-helper 1 cytokines was measured in peripheral blood mononuclear cells or virus-infected macrophages. The results consistently showed that interleukin-1 β and tumour necrosis factor alpha expression levels were downregulated in cells that were stimulated (or infected) with the Δ ES3 mutant compared with parental virus and the other nsp2 deletion mutants. These results suggest that certain regions in nsp2 are non-essential for PRRSV replication but may play an important role in modulation of host immunity *in vivo*.

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INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) causes late-term reproductive failure in sows and severe pneumonia in neonatal pigs. Since its emergence in the 1980s, PRRSV has been estimated to cost at least US\$600 million annually in the USA alone (Neumann *et al.*, 2005). The recent outbreak of porcine high-fever disease in China, caused by a highly pathogenic PRRSV (HP-PRRSV), has increased the threat to the swine industry worldwide (Tian *et al.*, 2007).

PRRSV is a small, enveloped virus containing a single, positive-sense RNA genome. Genomic sequence compar-

isons have shown that PRRSV consists of two genotypes: European (type I) and North American (type II). These two genotypes share only about 60% sequence identity (Allende *et al.*, 1999; Nelsen *et al.*, 1999). The PRRSV genome is about 15 kb in length and contains nine open reading frames (ORFs). The replicase-associated genes, ORF1a and ORF1b, are situated at the 5' end of the genome and represent nearly 75% of the viral RNA. From studies of the related equine arteritis virus, PRRSV ORF1a and ORF1ab encode replicase polyproteins, pp1a and pp1ab, which are predicted to be cleaved into 13 non-structural protein (nsp) products: nsp1 α , nsp1 β and nsp2–nsp12 (Snijder *et al.*, 1994; den Boon *et al.*, 1995; van Dinten *et al.*, 1996; Ziebuhr *et al.*, 2000). The nsp2 is the largest viral protein of PRRSV and has been determined to be critical in proteolytic processing for virus replication. The N-terminal region of nsp2 contains a cysteine protease

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A supplementary table showing sequences of the primers used in this study is available with the online version of this paper.

(CP) domain, which induces nsp2/3 cleavage (Ziebuhr *et al.*, 2000; Han *et al.*, 2009). Nsp2 functions as a co-factor with nsp4 serine protease to process the other cleavage products (Snijder *et al.*, 1994; den Boon *et al.*, 1995; Snijder *et al.*, 1995; Wassenaar *et al.*, 1997; Snijder & Meulenberg, 1998). nsp2 has also been implicated in the formation of double-lipid-membrane vesicles, as well as acting as a membrane anchor for the assembly of multi-protein virus replication complexes (Pedersen *et al.*, 1999; Snijder *et al.*, 2001).

In addition to its critical roles in virus replication, recent studies from our laboratories and others have shown that PRRSV nsp2 may also participate in modulation of the host immune response. Frias-Staheli *et al.* (2007) reported that the CP domain of PRRSV nsp2 belongs to the ovarian tumour (OTU) protease superfamily. The OTU protease is capable of deconjugating both ubiquitin (Ub) and interferon (IFN)-stimulated gene (ISG) 15 from cellular proteins, which inhibits Ub- and ISG15-dependent innate immune responses. Several laboratories have also demonstrated that nsp2 is highly immunogenic, and it was shown to contain the highest frequency of immunodominant B-cell epitopes when compared with other non-structural and structural proteins (Oleksiewicz *et al.*, 2001; de Lima *et al.*, 2006). Most of these epitope regions are associated with natural deletions/insertions and peptide sequence hypervariability. Potential T-cell epitopes have also been mapped to these regions (Fang *et al.*, 2004, 2007). Virus isolates from the recent outbreaks of HP-PRRSV in Southeast Asia were found to contain a discontinuous 30 aa deletion in the nsp2 region (Tian *et al.*, 2007), and this deletion region contained previously identified B-cell epitopes (de Lima *et al.*, 2006) and potential T-cell epitopes.

To elucidate further the biological role of the nsp2 immunodominant epitopes, in this study we deleted each of the identified epitopes on nsp2 using a type I PRRSV infectious clone. The effect of nsp2 epitope deletion on the host immune response was investigated in a young pig respiratory disease model. The results suggested that, although certain nsp2 epitope regions are not required for virus replication, they play an important role in the modulation of host immunity.

RESULTS

In vitro growth characteristics of nsp2 epitope deletion mutants

Six immunodominant B-cell linear epitopes, ES2–ES7, were identified previously on nsp2 of type I PRRSV (Oleksiewicz *et al.*, 2001). In this study, each epitope site was deleted from our type I PRRSV full-length cDNA infectious clone, pSD01-08 (Fang *et al.*, 2006) (Fig. 1). The plasmids containing the full-length genome with nsp2 deletions were linearized and transcribed *in vitro* as described previously (Fang *et al.*, 2006). The *in vitro*-transcribed capped RNA was transfected into BHK-21 cells. At 48 h post-transfection, cells were examined for expres-

sion of nsp2 and nucleocapsid (N) protein by fluorescent antibody staining. The results showed that about 10% of transfected cells expressed nsp2 and N protein for the ES3 (aa 691–722 of pp1a), ES4 (aa 736–790 of pp1a), ES5 (aa 822–832 of pp1a), ES6 (aa 895–923 of pp1a) and ES7 (aa 1015–1040 of pp1a) deletion constructs. For the ES2 (aa 456–469 of pp1a) deletion construct, we detected the expression of nsp2 only in transfected cells, and no N protein expression was detected. As ES2 is located in the CP region, deletion of this epitope may affect the protease activity and subsequently block the transcription step of virus replication. Supernatants from the transfected BHK cells were passaged onto MARC-145 cells. After 72 h, MARC-145 cells were stained with the N protein-specific monoclonal antibody SDOW17. For ES5 and ES6 deletion constructs, only a few single cells were stained. Deletion of the ES5 or ES6 epitope appeared to affect the cell-to-cell spread of the virus to form clusters of infected cells. We failed to detect any viral proteins on the second passage, suggesting that these deletions may affect virion assembly or release of infectious virions. The ES3, ES4 and ES7 deletion constructs stained as bright clusters of cells, and viable virus was recovered from the cell culture. The cell culture supernatant from transfected BHK-21 cells was also passaged onto porcine alveolar macrophages (PAMs), and results were obtained similar to those for MARC-145 cells. To confirm the stability of the deletion region, we sequenced the corresponding region from passage 3 of each mutant in cell culture. The results showed that the corresponding deletion region remained present in each mutant. These results indicated that the ES3, ES4 and ES7 epitope sites are non-essential for virus replication *in vitro*. However, growth kinetics analysis showed that the Δ ES3 mutant seemed to grow more rapidly and had a higher mean peak viral titre compared with that of the parental viruses. In contrast, the Δ ES4 and Δ ES7 mutants had peak viral titres that were about 0.5–1 log lower (Fig. 2a, b). The growth phenotype of the deletion mutants was further determined by plaque assay (Fig. 2c). In comparison with the parental virus, the Δ ES4 and Δ ES7 mutants showed a reduced plaque size. Interestingly, the Δ ES3 mutant displayed increased cytolysis activity, with a larger plaque size.

nsp2 epitope deletion mutants Δ ES3, Δ ES4 and Δ ES7 replicate *in vivo*

The *in vivo* characteristics of the nsp2 deletion mutants were studied in a nursery pig disease model. Five groups of 4-week-old, PRRSV-negative pigs ($n=6$) were inoculated with the Δ ES3, Δ ES4 or Δ ES7 mutant (groups 1–3, respectively) or with parental virus (group 4), or were mock-infected with cell culture medium (group 5). To determine whether these nsp2 epitope deletion mutants replicated *in vivo*, serum samples taken at 7–28 days post-infection (p.i.) were used for virus isolation on MARC-145 cells. Viruses were recovered from the serum samples of pigs from groups 1–4 collected at 7, 14 and 21 days p.i. To confirm the presence of deletions in the nsp2 region, the

Fig. 1. Schematic diagram of the nsp2 epitope deletion mutants. (a) The amino acid sequences of nsp2 B-cell epitopes (ES2–ES7) of different type I PRRSV strains were aligned using CLUSTAL W of the MEGALIGN program. Amino acid sequences identical to the Lelystad virus (LV) are indicated by dashes. The positions of the start and end amino acids of the ES2–ES7 epitopes in SD01-08 pp1a are indicated above the boxes. The nsp2 sequences used for comparison have been described by us previously (Fang *et al.*, 2004, 2007). (b) Construction of the SD01-08 nsp2 ES2–ES7 epitope deletion mutants. In the SD01-08 nsp2 protein, the ES2, ES5 and ES6 epitope regions are indicated by grey boxes, whilst the ES3, ES4 and ES7 epitope regions are indicated by black boxes. The putative CP domain and predicted transmembrane (TM) regions (aa 1126–1256 of pp1a) are indicated by hatched boxes. Predicated cleavage sites are annotated at the N and C termini, based on the prediction from Ziebuhr *et al.* (2000). The deleted nsp2 regions are shown as a grey line (ES2, ES5 and ES6) or a black line (ES3, ES4 and ES7). The viability of each deletion mutant is shown on the right: +, viable; –, non-viable.

Fig. 2. *In vitro* characterization of nsp2 epitope deletion mutants. (a, b) Growth kinetics of nsp2 epitope deletion mutants. MARC-145 cells (a) or PAMs (b) were infected in parallel at an m.o.i. of 0.1 with passage 3 deletion mutants (■, ΔES3; ○, ΔES4; ▲, ΔES7) or parental virus (□). At 6, 12, 24, 36, 48, 60 and 72 h p.i., cells were harvested and virus titres were determined by an immunofluorescence assay on MARC-145 cells. The results shown are mean values from three replications of the experiment, and viral titres are expressed as fluorescent focus units (f.f.u.) ml⁻¹ (a) or 50 % tissue culture infective dose (TCID₅₀) ml⁻¹ (b). (c) Plaque morphology of nsp2 epitope deletion mutants and parental viruses. Confluent cell culture monolayers were infected with virus at an m.o.i. of 0.1. After 2 h, the cell culture supernatant was removed and an agar overlay was applied. Plaques were detected after 5 days' incubation at 37 °C and stained with 0.1 % crystal violet.

corresponding regions containing the ES3, ES4 or ES7 deletion were sequenced using viruses isolated from serum samples taken at 14 days p.i., which were collected from three pigs each from groups 1–3. The results confirmed that the ES3, ES4 or ES7 deletion mutant recovered from pigs retained its corresponding deletions in the nsp2 region. These results indicated active replication of nsp2 epitope deletion mutant viruses in pigs, and that the ES3, ES4 and ES7 regions of nsp2 are dispensable for PRRSV replication *in vivo*.

The duration and peak of viraemia was further determined by real-time RT-PCR. Viral RNA was detected from 3 to 42 days p.i. (Fig. 3). Pigs that were infected with the ΔES7 mutant had the lowest peak viral load (mean peak viral load, 1.2×10^5 copies ml⁻¹). Interestingly, similar to the *in vitro* growth characteristics, pigs that were infected with the ΔES3 mutant had a higher peak viral load (mean peak viral load, 3.6×10^7 copies ml⁻¹) compared with pigs infected with parental virus (4.3×10^6 copies ml⁻¹).

Antibody response in pigs infected with nsp2 epitope deletion mutants

By 14 days p.i., all pigs in the infected groups had seroconverted. Although there were differences in the peak

viral loads between groups of pigs infected with different nsp2 deletion mutants and parental virus, the antibody responses reached similar levels, as measured by IDEXX ELISA at 21 days p.i. (Fig. 4a). Further measurement of the

Fig. 3. Comparison of viral load in pigs infected with nsp2 epitope deletion mutants (■, ΔES3; ○, ΔES4; ▲, ΔES7) or parental virus (□). △, Negative control. Viral load was quantified by real-time RT-PCR, and the result was interpreted as RNA copy number ml⁻¹. Each data point shown represents a mean value from six pigs in each group.

Fig. 4. Comparison of antibody response in pigs infected with nsp2 epitope deletion mutants (■, ΔES3; ○, ΔES4; ▲, ΔES7) or parental virus (□). △, Negative control. (a) PRRSV-specific serum antibodies measured using an IDEXX HerdChek PRRSV ELISA 2XR kit. Each data point shown represents a mean value ($n=6$) of sample:positive (S:P) ratios. S:P ratios greater than 0.4 were considered positive. (b) Serum neutralizing-antibody response determined by fluorescent focus neutralization assay. Results were determined by a 90% reduction in the viral infection, and the neutralizing-antibody titres are presented as mean values ($n=6$) and expressed on a log₂ scale. The parental SD01-08 virus was used for the virus neutralizing assay.

virus neutralizing (VN) antibody levels showed that a VN antibody response was first detected at 21 days p.i. In groups of pigs infected with the parental virus or with the ΔES4 or ΔES7 mutant, VN antibody was detected in 1/6 pigs from each group. At 35 days p.i., most of the pigs infected with the ΔES7 mutant (4/6 pigs) developed a detectable VN antibody response, and the mean VN titre for the ΔES7 mutant-infected group of pigs was higher than those of other groups. However, the difference between groups was not statistically significant due to large variation among individual pigs and the limited number of pigs in each group (Fig. 4b).

Innate and cellular immune response in pigs infected with nsp2 epitope deletion mutant viruses

To determine whether the nsp2 epitope deletion had altered the effect on the innate and cellular immune responses, we

monitored the temporal expression of selected innate and cellular immune genes in peripheral blood mononuclear cells (PBMCs) from virus-infected pigs. Initially, cytokine real-time RT-PCR was performed to measure the mRNA expression of targeted immune marker genes, including the genes for proteins involved in innate immunity: IFN- α , IFN- β , interleukin (IL)-1 β , IL-6 and IL-8; T helper 1 (Th1) immunity: IFN- γ , IL-12 β , IL-15, IL-18 and tumour necrosis factor alpha (TNF- α); regulatory T-cell responses: IL-10; and the control gene *RPL32*. PBMCs were prepared from whole blood collected from control or virus-infected pigs at 3, 7, 14, 21, 28, 35 and 42 days p.i. and stimulated *in vitro* with individual deletion mutants (ΔES3, ΔES4 and ΔES7), parental virus or medium for 24 h. The RT-PCR results showed weak restimulation responses as expected for PBMCs. However, from PBMCs at 3 and 7 days p.i., ΔES3-stimulated cells produced a different mRNA expression pattern compared with that from ΔES4, ΔES7 and parental virus-stimulated cells, with notable differences in IL-1 β and TNF- α mRNA expression (data not shown). We further performed IL-1 β and TNF- α cytokine ELISAs to confirm the RT-PCR results. As shown in Fig. 5, the IL-1 β and TNF- α protein expression levels were much lower in cells stimulated with the ΔES3 mutant than with the parental virus, and there was no significant difference for the IL-1 β and TNF- α expression levels among ΔES4, ΔES7 and parental virus-stimulated cells. This result suggested that deletion of the ES3 epitope may alter the IL-1 β and TNF- α response compared with the response to wild-type virus.

To confirm these results further, we tested immune marker mRNA expression in mutant virus-infected PAMs using RT-PCR and cytokine ELISAs. These assays were repeated three times with three different lots of infected PAMs. The RT-PCR results showed that IL-1 β and TNF- α mRNA expression levels were downregulated ($P<0.05$) in cells infected by the ΔES3 mutant in comparison with mRNA expression levels in cells infected by parental virus (Fig. 6a). Further comparison of cytokine protein expression levels consistently showed that IL-1 β and TNF- α protein expression was also downregulated in macrophages infected with the ΔES3 mutant compared with those infected with parental virus (Fig. 6b). When cytokine mRNA expression was tested in PAMs using real-time RT-PCR, the IL-10 mRNA expression level was shown to be downregulated in cells infected by the ΔES3 mutant (Fig. 6a). However, this result was not consistent among different assays. No difference was observed in IL-10 levels from supernatants of different nsp2 mutant-infected macrophages by ELISA. In addition, no difference in IL-10 expression levels was observed when tested in virus-stimulated PBMCs. Whether epitope deletion in nsp2 alters the IL-10 response needs to be further determined.

DISCUSSION

The principal reasons for incomplete immune protection of PRRSV vaccine are the inability of current vaccine

Fig. 5. Comparison of immune marker gene expression in activated PBMCs from pigs infected with nsp2 deletion mutants or parental virus. Neg., Negative control. PBMCs were harvested at 3 and 7 days p.i. and stimulated in culture with nsp2 deletion mutant virus, parental virus or medium. Cell culture supernatants and pellets were harvested at 24 h after stimulation. Cytokine protein (IL-1 β and TNF- α) expression was determined as described in Methods. ELISA tests were repeated three times.

technologies to overcome antigenic variation and the lack of knowledge on mechanisms employed by the virus to survive despite the ongoing host immune response. PRRSV infection typically induces poor antiviral innate and cell-

mediated immune responses. Anti-PRRSV neutralizing antibodies are generated slowly, and their titres remain low. In contrast, initial viral infection induces a robust host humoral antibody response that comprises predominantly

Fig. 6. Comparison of immune marker gene expression in macrophages infected by nsp2 deletion mutants or parental virus. PAMs were infected with each nsp2 deletion mutant virus or parental virus at an m.o.i. of 0.1. At 24 h p.i., supernatants were collected for determination of cytokine protein expression. Macrophage cell pellets were collected and stored in TRIzol reagent to determine the cytokine mRNA expression. The experiment was repeated three times. (a) Immune gene expression profiles from virus-infected macrophage cell pellets. Real-time RT-PCR was performed using RNA prepared from TRIzol-solubilized macrophages. Results (Ct values) are shown as mean values for each group. The Ct value for each gene was compared with the value for the negative control. The upregulation (red) or downregulation (green) of immune gene expression is shown. (b) Cytokine protein (IL-1 β and TNF- α) expression was determined as described in Methods.

non-neutralizing antibodies (Murtaugh *et al.*, 2002; Ostrowski *et al.*, 2002; Lopez & Osorio, 2004; Plagemann, 2004; Mateu & Diaz, 2008). In previous studies, nsp2 was identified as containing the highest frequency of B-cell epitopes, and swine mount an early non-neutralizing-antibody response to PRRSV nsp2 (Oleksiewicz *et al.*, 2001; de Lima *et al.*, 2006; Johnson *et al.*, 2007; Brown *et al.*, 2009). The antibody response to nsp2 is greater than towards any other PRRSV protein and lasts more than 202 days p.i., which indicates the immunodominant nature of this protein (Johnson *et al.*, 2007; Brown *et al.*, 2009). This strong, non-neutralizing humoral response induced by nsp2 suggests that nsp2 is involved in modulation of the host immune response.

In this study, we deleted each of the immunodominant epitopes (ES2–ES7) in nsp2 using a type I PRRSV cDNA infectious clone. Deletion of ES2, ES5 and ES6 did not result in recovery of viable virus. The ES2 epitope is located in the N-terminal CP domain region, which is highly conserved among different PRRSV strains. A recent report from Han *et al.* (2009) confirmed the important role of PRRSV nsp2 CP in virus replication. They introduced various mutations into the nsp2 CP region. Specifically, three mutations in the ES2 epitope region of nsp2 in VR2332 (D85N, W86G and D89N or D89E corresponding to D74, W75 and D78 of nsp2 in SD01-08) were determined to partially block the proteolytic processing of nsp2 in the *trans* condition. Reverse genetics revealed that these mutations were detrimental to virus viability. Consistent with the results generated from our study, deletion of the ES2 epitope may disrupt conformation of the CP domain and affect the nsp2/3 cleavage function of the protease, which is lethal to virus replication. Another study (Frias-Staheli *et al.*, 2007) showed that the CP domain of PRRSV nsp2 also belongs to the OTU protease superfamily and is capable of deconjugating Ub and ISG15 from cellular proteins and inhibiting Ub- and ISG15-dependent innate immune responses. Our results showed that a deletion in this OTU domain region generated non-viable virus. As the OTU domain region suppresses the innate immune response, this result raised concerns about its utility for modified live vaccines. We are currently determining whether point mutations in the OTU domain region could remove (or decrease) its Ub and ISG15 effect as well as generating viable virus. ES5 and ES6 are located in relatively conserved regions of nsp2 (Fig. 1). The specific function of these regions in virus replication is unknown. In cells transfected by the capped RNA transcripts from ES5- or ES6-deleted cDNA, we observed that levels of nsp2 and N protein expression were significantly reduced compared with that of parental virus. After passage from BHK-21 cells to MARC-145 cells, virus could not form the clusters typical of infected cells at 72 h p.i., suggesting that deletion of either the ES5 or ES6 epitope impaired the ability of the virus to spread from cell to cell.

In contrast to the ES2, ES5 and ES6 epitope deletions, deletions of the ES3, ES4 and ES7 epitope regions of nsp2

allowed recovery of viable viruses. A previous study showed that the ES4 epitope is located in the region with highest hydrophilicity and induced the highest antibody response compared with the other epitopes (ES3 and ES5–ES7) in nsp2 (Hopp & Woods, 1981; Oleksiewicz *et al.*, 2001). In our study, deletion of this epitope showed certain levels of attenuation in viral growth *in vitro* and *in vivo*. The ES7 epitope is located close to the transmembrane domain region in the C terminus of nsp2. Based on a study of equine arteritis virus, the C terminus of nsp2 interacts with nsp3 to function as a co-enzyme of nsp4 in the major proteolytic processing pathway (Wassenaar *et al.*, 1997). The interaction between nsp2 and nsp3 also plays an important role in the formation of double-membrane structures for assembling the membrane-anchored virus replication complex (Snijder *et al.*, 2001). We speculate that deletion of the ES7 region may affect the C-terminal structure of nsp2. Although the ES7 deletion generated viable virus, the deletion significantly affected virus replication *in vitro* and *in vivo*. In cell culture, the ES7 mutant had the lowest peak viral titre and smallest plaque size in comparison with parental virus and the other nsp2 epitope deletion mutants. In the *in vivo* study, the ES7 deletion virus was attenuated with significantly lower viral load in infected pigs. Interestingly, although the ES4 and ES7 deletion mutants did not replicate as well as parental virus, the antibody responses induced by ES4 and ES7 mutants reached levels similar to that of parental virus after 21 days p.i. as measured by IDEXX ELISA. The ES4 and ES7 mutant-infected pigs developed slightly higher levels of neutralizing antibody at 28 days p.i., although this response was not statistically significant relative to parental virus due to large variation among individual pigs. Whether the ES4 or ES7 epitope deletion could improve the VN antibody response needs to be analysed further.

In comparison with the ES4 and ES7 epitopes, the results from this study indicated that the ES3 epitope region evolved a different mechanism in modulation of the host immune response. It is intriguing that the ES3 deletion mutant replicated faster *in vitro* and produced higher levels of viraemia *in vivo*. More importantly, expression of the innate cytokines IL-1 β and TNF- α was downregulated in Δ ES3 mutant-infected/stimulated cells. Sequence analysis showed that potential T-cell epitopes are also present in this epitope site, including the putative generalized T-cell epitope SGHKAVHSAL and the putative MHC-binding peptide motif QVQVVAGEQLK (Rothbard & Taylor, 1988; Sette *et al.*, 1989). Our results suggest that maintaining the ES3 epitope is a disadvantage to virus replication. As Pudupakam *et al.* (2009) indicated, sequences in the viral genome not required for virus infectivity are normally rapidly lost *in vivo*. In our previous study, we identified deletions of 17 aa (aa 734–750 of ORF1a) downstream of the ES3 epitope, which have been consistently identified in a group of emerging European-like type I PRRSV viruses in the USA (Fang *et al.*, 2004, 2007). In one isolate, SD02-11, we also found a 5 aa

deletion upstream of the ES3 epitope and a 1 aa deletion embedded in the ES3 epitope. In addition, the ES3 epitope is located at the most variable region in nsp2 among type I PRRSV isolates, and the corresponding region does not exist in type II PRRSV isolates. This suggests that this region is evolving rapidly, possibly due to the selective pressure of the host immune system. Deletions and hypervariability in this region may represent a mechanism of virus escape from host immune surveillance.

There are many reports about natural deletions/insertions in the central region of nsp2 that recognize nsp2 as the most variable region of the PRRSV genome (Shen *et al.*, 2000; Fang *et al.*, 2004; Gao *et al.*, 2004; Han *et al.*, 2006, 2007; Tian *et al.*, 2007). However, little is known about the mechanism of these deletions/insertions in viral pathogenesis. The HP-PRRSV associated with recent outbreaks in Southeast Asia was found to contain a discontinuous 30 aa deletion (29 and 1 aa) in the nsp2 region (Tian *et al.*, 2007). This deletion was suspected to be a potential determining factor that causes the fatal disease, but a recent study from Zhou *et al.* (2009) concluded that the 30 aa deletion in Chinese nsp2 (HP-PRRSV) was not related to its virulence. However, in their study, they did observe that replacement of the nsp2 deletion region of HP-PRRSV with a corresponding region from a low-virulence PRRSV resulted in slower growth kinetics *in vitro*, lower viral load *in vivo* and prolonged survival days of the infected animals when compared with the parental virus. Sequence analysis has revealed that the 29 aa region also contained identified B-cell epitopes (aa 536–560; de Lima *et al.*, 2006) and predicted T-cell epitopes (Falk *et al.*, 1991; Sette *et al.*, 1989). This region has been determined to be a major hydrophilic region (Hu *et al.*, 2009). In relation to our ES3 epitope deletion in type I PRRSV nsp2, the deletion mutant virus grew at higher titre *in vitro* and higher viral load *in vivo*. More interestingly, we observed downregulation of the innate cytokine (IL-1 β) and Th1 (TNF- α) immune marker gene mRNA expression in the Δ ES3 mutant-infected/stimulated cells in comparison with that of parental virus. These findings suggested that certain regions of nsp2 might contribute to the induction of a virus-specific host immune response and that deletion of such a region could produce a more virulent virus. In the case of HP-PRRSV, sequence comparison has shown that this group of viruses shares about 88.1–89.6% sequence identity with the type II prototypic strain VR-2332 (Li *et al.*, 2008; Hu *et al.*, 2009). We speculate that mutations in the other viral genomic regions might have a synergistic effect with the nsp2 deletion to trigger the highly pathogenic disease symptoms. As results from Zhou *et al.* (2009) and our study were based on limited numbers of pigs, future studies with larger number of animals are needed to fully evaluate the biological role of nsp2 immunodominant epitope regions in PRRSV replication and pathogenesis.

Taken together, the results from this study suggest that PRRSV nsp2 is a multi-functional protein, with different

regions involved in different functions in virus replication and pathogenesis. Our data show that certain regions in nsp2 are non-essential for PRRSV replication but may play important roles in modulation of the host immune response *in vivo*. Our results demonstrated that virus-specific host immunity can be enhanced by modifying certain nsp2 epitope regions. These findings provide important insights for future vaccine development.

METHODS

Cells. BHK-21 cells were used for initial transfection for recovery of virus from *in vitro*-transcribed RNA. MARC-145 cells, a continuous cell line that is permissive to PRRSV, were used for virus rescue and subsequent experiments. These cells were maintained in the appropriate medium and incubation conditions as described previously (Ropp *et al.*, 2004). PAM cells were obtained by lung lavage of 6-week-old PRRSV-naïve piglets using a method described previously (Zeman *et al.*, 1993). PAMs (1×10^6 cells ml $^{-1}$) were infected with nsp2 deletion mutants and parental virus at an m.o.i. of 0.1. At 24 and 48 h p.i., the cell culture supernatants were collected for evaluation of cytokine protein expression by ELISA, and PAMs were collected and stored in TRIzol reagent (Invitrogen) for further determination of cytokine mRNA expression by real-time RT-PCR.

PBMCs were isolated from infected and control pigs as described previously (Meier *et al.*, 2003) and plated in a 24-well plate (4×10^6 cells ml $^{-1}$). PBMCs were stimulated with 50 μ l UV-inactivated PRRSV (2×10^5 TCID $_{50}$); parallel cultures were stimulated with the mitogen concanavalin A or medium, and served as positive and negative controls, respectively. Stimulated cultures were incubated at 37 °C for 24 h. Cell culture supernatants were collected from stimulated cells and stored at –70 °C for cytokine ELISAs, and cells were harvested and stored in TRIzol reagent for further analysis by cytokine real-time RT-PCR.

Viruses. The nsp2 deletion mutants were constructed from a US type I PRRSV full-length cDNA infectious clone, pSD01-08 (GenBank accession no. DQ489311; Fang *et al.*, 2006). The parental virus SD01-08 was originally isolated from a group of 8-week-old pigs in 2001 in the USA. The SD01-08 virus grows well in both PAMs and MARC-145 cells. The third passage of the MARC-145 cell-recovered parental and recombinant viruses was used for *in vitro* and *in vivo* experiments. To eliminate the potential effect of cytokine protein residues in the virus-infected cell cultures, the *in vitro*-recovered recombinant viruses and parental virus were purified by sucrose cushion, as described previously by us (Wu *et al.*, 2001; Fang *et al.*, 2008).

Polyclonal antibody production. The antigen for production of polyclonal antibody specific to PRRSV nsp2 was expressed as a recombinant protein in *Escherichia coli*. The N-terminal portion of nsp2 (aa 386–1093 of pp1a, excluding the transmembrane region) was amplified by RT-PCR using primer pairs ET28a-EUnsp2PF/ET28a-EUnsp2PR (see Supplementary Table S1, available in JGV Online). The PCR product amplified from SD01-08 was cloned into the pET-28a(+) vector (Novagen). The recombinant protein was expressed and purified as described previously (Johnson *et al.*, 2007). Polyclonal antibodies were raised in New Zealand white rabbits using the recombinant nsp2 protein. For primary immunizations, 100 μ g antigen was mixed with an equal volume of Freund's incomplete adjuvant and injected subcutaneously at six different locations. Rabbits were boosted twice with the same dose at 2-week intervals.

nsp2 deletion mutant construction. Each individual B-cell epitope deletion was introduced by an overlapping extension PCR technique

(Hayashi *et al.*, 1994), and each deletion region is shown in Fig. 1 (see Supplementary Table S1 for primers used in the construction of each epitope deletion mutant). Briefly, in two separate PCRs, two separated fragments (A and B) of the targeted gene region were amplified in the first round. Each reaction used one flanking primer that hybridized at one end of the target sequence and one internal primer that hybridized at the site of the deletion. In the second-round of PCR, by using two internal primers that overlap, fragments A and B were fused by denaturing and annealing them in a subsequent primer-extension reaction. The overlap allowed one strand from each fragment to act as a primer on the other, and extension of these overlaps resulted in the deletion product. The PCR products were double digested and then ligated into the infectious clone plasmid pSD01-08 that had been digested with the same enzymes. The following primer sets (listed in Supplementary Table S1) were used to construct different nsp2 epitope deletion mutants: Δ ES2 (fragment A: T7-F/1566-R1, fragment B: 1629-F1/1978-R1), Δ ES3 (fragment A: 1972-F1/2271-R1, fragment B: 2388-F1/2919-R1), Δ ES4 (fragment A: 1972-F1/2403-R1, fragment B: 2592-F1/2919-R1), Δ ES5 (fragment A: 1972-F1/2661-R1, fragment B: 2718-F1/2919-R1), Δ ES6 (fragment A: 1972-F1/2883-R1, fragment B: 2988-F1/3355-R1), Δ ES7 (2592-F1/3243-R1).

In vitro transcription and rescue of recombinant virus. Detailed methods for *in vitro* transcription and rescue of the cloned virus have been described by us previously (Fang *et al.*, 2006, 2008). Briefly, the full-length cDNA plasmid was linearized by digesting with *Xba*I restriction enzyme and was *in vitro*-transcribed using an mMessage Machine kit (Ambion). The full-length transcript was used for transfection of BHK-21 cells using a standard transfection reagent (DMR1E-C; Invitrogen) following the manufacturer's instructions. To rescue the virus, cell culture supernatant obtained 48 h post-transfection was passaged on MARC-145 cells or PAMs. Rescue of infectious virus was confirmed by an indirect immunofluorescence assay using PRRSV nucleocapsid (N) protein-specific monoclonal antibody SDOW17 (Nelson *et al.*, 1993) and PRRSV nsp2-specific rabbit antiserum.

Growth kinetics and plaque assay. Growth kinetics were examined by infecting MARC-145 cells with nsp2 deletion mutants and parental virus at an m.o.i. of 0.1. Infected cells were collected at 0, 6, 12, 24, 36, 48, 60 and 72 h p.i., and virus titres were determined by immunofluorescence assay on PAMs or MARC-145 cells and quantified as 50% tissue culture infective dose (TCID₅₀) ml⁻¹ or fluorescent focus units (f.f.u.) ml⁻¹. Plaque morphology between the recombinant virus and parental virus was compared by plaque assay on MARC-145 cells as described previously (Fang *et al.*, 2006). Confluent cell monolayers were infected with virus at an m.o.i. of 0.1. After 2 h, the cell culture supernatant was removed and an agar overlay was applied. Plaques were detected after 3–5 days incubation at 37 °C and stained with 0.1% crystal violet.

Sequencing of deletion regions. To determine the stability of each deletion mutant, cell lysate from virus-infected cells was harvested and RNA was extracted using a QiaAmp viral RNA kit (Qiagen) following the manufacturer's instruction. The corresponding deletion regions were amplified by RT-PCR using primer sets 2144-F1/2694-R2 (ES3-ES4) and 3125-F2/3421-R1 (ES7) (Supplementary Table S1). PCR products were sequenced at the Iowa State University DNA sequencing facility (Ames, IA, USA).

Animals/challenge groups. Thirty 4-week-old, PRRSV-naïve pigs obtained from a certified PRRSV-negative herd were divided randomly into five groups and housed separately in animal isolation facilities at South Dakota State University (SDSU). After a 4-day acclimation period, pigs from each group ($n=6$) were inoculated with 4 ml (5×10^5 TCID₅₀, given as 2 ml intranasally and 2 ml

intramuscularly) of each nsp2 deletion mutant (group 1: Δ ES3 mutant; group 2: Δ ES4 mutant; group 3: Δ ES7 mutant) or parental virus (group 4). The negative-control group (group 5) was mock infected with MARC-145 cell culture supernatant. Blood samples were obtained from all pigs on days 0, 3, 7, 14, 21, 28, 35 and 42.

Real-time RT-PCR quantification of viral load in infected animals. For the detection of viral RNA and determination of viral load, serum samples from 0, 7, 14, 21, 28, 35 and 42 days p.i. were examined using a real-time quantitative RT-PCR (Tetracore VetAlert PRRS), which is routinely performed at the South Dakota Animal Disease Research and Diagnostic Laboratory (SD-ADRDL) under strict quality assurance guidelines. The detailed method has been described in our previous publication (Wasilk *et al.*, 2004).

Determination of humoral immunity. To compare humoral antibody responses, all serum samples were evaluated using an IDEXX HerdChek PRRS 2XR ELISA and virus neutralization assay at SDSU-ADRDL. These tests are also routinely performed at SD-ADRDL under strict quality assurance guidelines.

Real-time PCR for immune marker gene expression. Real-time PCR for immune marker gene detection was performed as described previously (Royae *et al.*, 2004; Dawson *et al.*, 2005). Samples were evaluated for expression of selected immune genes encoding targeted immune markers for innate immunity: IFN- α , IFN- β , IL-1 β , IL-6, IL-8; Th1 immunity: IFN- γ , IL-12 β , IL-15, IL-18, TNF- α ; and regulatory T-cell responses: IL-10; and the control housekeeping L32 ribosomal protein gene (*RPL32*). Relative quantification of target gene expression was evaluated using cycle threshold (Ct) values, and each group's results were compared with the values for control pigs (or control cultures) using the JMP statistical package as published previously (Royae *et al.*, 2004; Dawson *et al.*, 2005). Least-squares means analyses were used to statistically evaluate group means for immune gene analyses.

Cytokine protein ELISA. Cell culture supernatant collected from virus-infected macrophages or virus-stimulated PBMCs was used for cytokine protein expression analysis. TNF- α and IL-1 β protein expression was measured by ELISA using a Swine TNF- α CytoSets TM kit (Invitrogen) and a porcine IL-1 β /IL-1F2 DuoSet kit (R&D Systems), respectively, following the manufacturers' instructions.

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