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Identification of viral genes associated with the interferon-inducing phenotype of a synthetic porcine reproductive and respiratory syndrome virus strain

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ABSTRACT

We recently generated a fully synthetic porcine reproductive and respiratory syndrome virus strain (designated as PRRSV-CON), which confers unprecedented levels of heterologous protection. We report herein that the synthetic PRRSV-CON possesses a unique phenotype in that it induces type-I interferons (IFNs) instead of suppressing these cytokines as most of the naturally occurring PRRSV isolates do. Through gain- and loss- of-function studies, the IFN-inducing phenotype of PRRSV-CON was mapped to the 3.3 kb genomic fragment encoding three viral nonstructural proteins: nsp1α, nsp1β and the N-terminal part of nsp2. Further studies indicated that a cooperation among these 3 proteins was required for effective induction of IFNs. Collectively, this study constitutes the first step toward understanding the mechanisms by which the synthetic PRRSV-CON confers heterologous protection.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the etiological agent of an important swine disease characterized by late-term reproductive failure in sows and pneumonia in young pigs (Albina, 1997; Rossow, 1998). The virus is widespread in most swine producing countries and causes significant economic losses to swine producers. The annual losses caused by PRRSV to the U.S. swine industry is estimated to reach at least $664 million (Holtkamp DJ et al., 2013). The recent emergence of the highly pathogenic variant of PRRSV, which causes unusually high mortality to pigs of all ages in China and Southeast Asia, further underlines the significance of this virus (Tian et al., 2007). PRRSV is an enveloped, positive sense, single stranded RNA virus that belongs to the family *Arteriviridae,* within the order *Nidovirales* (Cavanagh, 1997). The PRRSV genome is approximately 15 kb in size, encoding at least 11 open reading frames (ORFs). ORF1a and ORF1b comprise ~80% of the viral genome and encode 2 polyproteins which are cleaved by viral proteases to produce at least14 nonstructural proteins (nsps) (Conzelmann et al., 1993; Johnson et al., 2011; Li et al., 2012; Music and Gagnon, 2010; Snijder et al., 1994; van Dinten et al., 1996). In addition to their roles in replication and transcription of the viral genome, the nsps are involved in modulating the host immunity (reviewed in Fang and Snijder, 2010).

Most PRRSV strains or isolates can actively suppress the induction of type-I IFNs (reviewed in Han and Yoo, 2014; Wang and Zhang, 2014). *In vivo* studies demonstrated that IFN-α was barely detected in serum and lung secretion of pigs infected with type 1 PRRSV strains SDPR I (Albina et al., 1998) or 2982 (Garcia-Nicolas et al., 2014). *In vitro* studies indicated that the expression levels of IFN-α and IFN-β mRNA were not changed in MARC-145 cells infected with a type 2 PRRSV strain 16244B (Buddaert et al., 1998; Miller et al., 2004). Further studies involved 7 wild-type and 1 attenuated type-II PRRSV strains revealed that these 8 PRRSV strains can actively inhibit IFN-α production by porcine peripheral blood monocytes (PBMCs) exposed to a TLR9 agonist (Calzada-Nova et al., 2011). In addition to the ability to suppress type-I IFNs, PRRSV can subvert the adaptive immunity. Pigs infected with PRRSV often do not develop virus-neutralizing antibodies and virus-specific T-cells until a month after infection (reviewed in Lopez and Osorio, 2004). It has been hypothesized that the substandard development of adaptive immune responses against PRRSV infection is in part due to the suboptimal levels of innate response (reviewed in Kimman et al., 2009; Murtaugh et al., 2002). Significant efforts have been made to uncover the mechanisms by which PRRSV suppresses the type-I IFN responses and to generate recombinant PRRSV mutants capable of inducing type-I IFNs. Multiple viral proteins have been reported to be involved in the suppression of the type-I IFN signaling including nsp1α, nsp1β, nsp2, nsp4, nsp11 and structural protein N (Beura et al., 2010; Chen et al., 2014; Li et al.,

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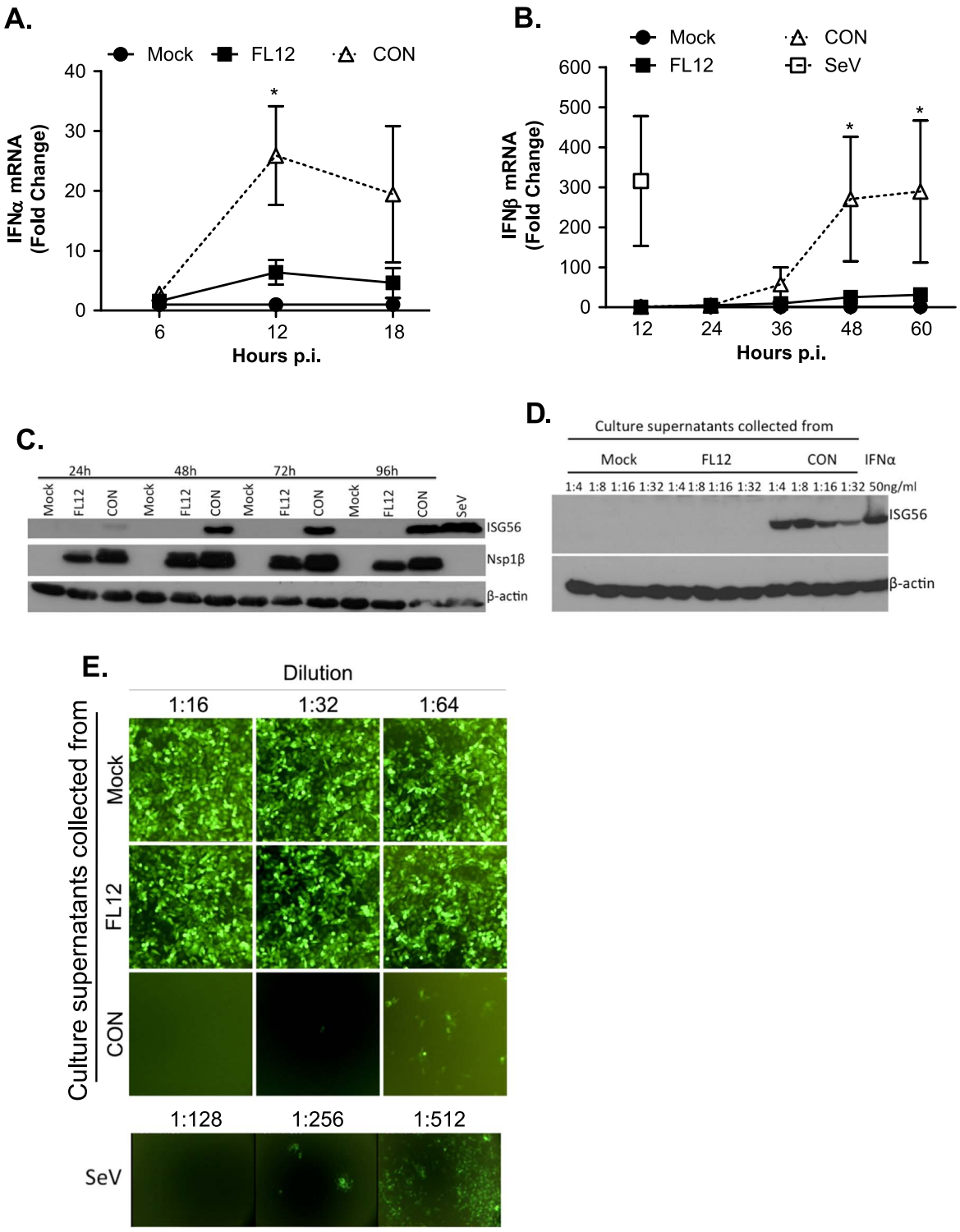

Fig. 1. PRRSV-CON induces high levels of type-I IFNs. (A) Evaluate the induction of type-I IFNs in PAMs. PAMs were infected with the indicated PRRSV strains at an MOI of 1. At various time points p.i., total RNA was extracted from cell lysates and relative IFN-α mRNA levels were quantitated by qRT-PCR. Data are expressed as the fold change relative to mock infection. Statistical difference as compared with FL12 is indicated by asterisk (*$p < 0.05$). (B-E) Evaluate the induction of type-I IFNs in MARC-145 cells. (B) MARC-145 cells were mock-infected or infected with the indicated PRRSV strains at an MOI of 0.01. Sendai virus (SeV) was used as a positive control. At various time points p.i., total RNA was extracted from cell lysates and relative IFN-β mRNA levels were quantitated by qRT-PCR. Data are expressed as the fold change of IFN-β mRNA relative to mock infection. Error bars represent standard error of mean (SEM) calculated from at least three independent experiments. Statistical difference as compared with FL12 is indicated by asterisk (*$p < 0.05$). (C) MARC-145 cells were mock-infected or infected with the indicated PRRSV strains at an MOI of 0.01. At 72 h p.i., infected cells were collected and lysed in RIPA buffer. The expression of ISG-56 was detected by western blotting using antibody to ISG-56. β-actin serves as a loading control. (D) MARC-145 cells were mock-infected or infected with the indicated PRRSV strains at an MOI of 0.01. At 72 h p.i., culture supernatants were collected and treated with UV to inactivate the virus. The UV-treated culture supernatants were serially diluted in fresh DMEM and inoculated onto naïve MACR-145 cells for 18 h. The expression of ISG-56 was analyzed by western blotting. (E) Interferon bioassay. Different dilutions of UV-treated culture supernatants were inoculated onto naïve MARC-145 cells for 18 h. The medium was then removed and the cells were subsequently infected with vesicular stomatitis virus expressing GFP (VSV-eGFP) that is highly sensitive to type I IFNs. VSV-eGFP replication was examined by using a fluorescent microscope.

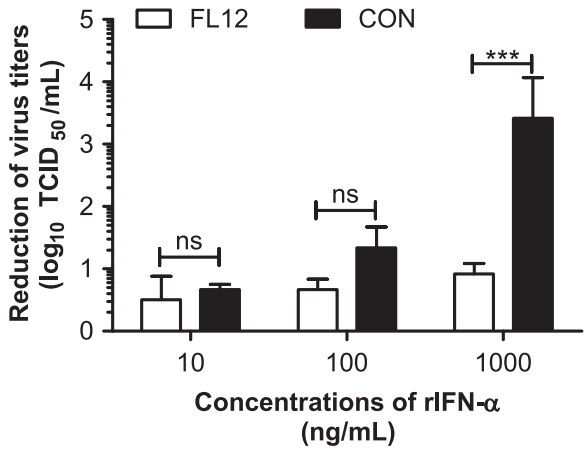

Fig. 2. PRRSV-CON is sensitive to IFN-α. MARC-145 cells were incubated with increasing concentrations of recombinant IFN-α (rIFN-α) for 18 h. The medium containing rIFN-α was removed and the cells were separately infected with FL12 or PRRSV-CON at an MOI of 0.01. At 48 h p.i, culture supernatants from infected cells were collected. Virus titers were measured by titration in MARC-145 cells and expressed TCID50 per ml. Statistical difference is indicated by asterisk (***$p < 0.001$).

2010; Patel et al., 2010; Sagong and Lee, 2011; Song et al., 2010; Sun et al., 2014, 2010; Wang et al., 2013a). These viral proteins interfere with different steps of the type-I IFN signaling pathway including inhibition of IRF-3 phosphorylation and nuclear translocation (Beura et al., 2010; Kim et al., 2010) and impediment of NF-κB activation (Sun et al., 2010). In addition, PRRSV infection also inhibits the cellular response to type-I IFN stimulation through its ability to block nuclear translocation of STAT1/2; thus, inhibiting the expression of interferon-stimulated genes such as ISG-15 and ISG-56 (Patel et al., 2010). Several PRRSV mutants capable of inducing type-I IFNs have been generated through the use of reverse genetics to disturb the anti-IFN activity of selected viral proteins (Beura et al., 2012; Huang et al., 2014; Li et al., 2016). It has been reported that the PRRSV mutants capable of inducing type-I IFNs elicit better levels of innate and adaptive immune responses than the parental PRRSV strain that suppresses type-I IFNs (Li et al., 2016). Thus far, there is only one naturally occurring PRRSV strain that is reported to induce type-I IFNs (Nan et al., 2012). This type-I IFN-inducing PRRSV strain has been reported to elicit enhanced levels of virus-neutralizing antibodies (Wang et al., 2013b). However, the linkage between the capability of inducing type-I IFNs of a PRRSV strain and the virus ability to confer protection against re-infection with heterologous PRRSV strains has not been determined.

We recently generated a synthetic PRRSV strain (PRRSV-CON) containing a consensus genome sequence that can confer unprecedented levels of heterologous protection (Vu et al., 2015). While the immunological mechanisms of heterologous protection remain to be determined, we observed that this synthetic PRRSV strain induces strong type-I IFN response in cell cultures. We report here the identification of 3 genes of the PRRSV-CON (e.g. nsp1α, nsp1β and the N-terminal part of nsp2) that are associated with the virus' ability to induce type-I IFNs.

2. Results

2.1. PRRSV-CON induces atypical levels of type I IFN response

The synthetic PRRSV-CON was rescued from the infectious cDNA clone (Vu et al., 2015). We observed that culture supernatant harvested from MARC-145 cells transfected with the PRRSV-CON full genome RNA transcripts did not infect fresh cells unless the supernatant was diluted over 100 times. This observation suggested to us that the culture supernatant containing rescued PRRSV-CON might also contain antiviral substance such as type-I IFNs. To test this hypothesis, we compared PRRSV-CON and FL12 on the ability to induce type-I IFNs. The PRRSV strain FL12 is a well-characterized reference strain that can actively suppress the production of type-I IFNs (Beura et al., 2010, 2012). Sendai virus (SeV), a strong inducer of type-I IFNs (Ito and Hosaka, 1983; Strahle et al., 2006), was used as a positive control. First, we compared the levels of IFN-α mRNA expression in PAMs after viral infection. As shown in Fig. 1A, the expression of IFN-α mRNA in PAMs infected with PRRSV-CON virus was significantly greater than in cells infected with FL12. It is not convenient to work with PAMs as it requires repeated harvest of the cells from pigs. Therefore, we conducted subsequent experiments in MARC-145 cells, a continuous cell line that is widely used to propagate PRRSV (Kim et al., 1993). The results showed that MARC-145 cells infected with PRRSV-CON expressed much greater levels of IFN-β mRNA than cells infected with FL12 (Fig. 1B). Once produced, the secreted IFNs bind to their cellular receptors and stimulate the expression of hundreds of interferon-stimulated genes (ISGs), including ISG-56 (reviewed in Gonzalez-Navajas et al., 2012). Therefore, we used ISG-56 expression as an indicator of the induction of type-I IFNs. We observed the high levels of ISG-56 expression in cells infected with PRRSV-CON, but not in cells infected with FL12 (Fig. 1C). Next, we used the ISG-56 induction and IFN-bioassay to detect the presence of type-I IFNs in culture supernatant collected from cells infected with PRRSV-CON virus. As shown in Fig. 1D, ISG-56 was detected in cells incubated with UV-treated culture supernatant collected from PRRSV-CON infection but not in cells incubated with UV-treated culture supernatant collected from FL12 infection. Additionally, culture supernatant collected from PRRSV-CON infected cells subsequently protected naïve MARC-145 cells from being infected with VSV-eGFP while the supernatant collected from FL12 infected cells did not (Fig. 1E). Together, these data indicate that PRRSV-CON infection induces strong type-I IFN response in PAMs and in MARC-145 cells.

2.2. PRRSV-CON is sensitive to IFN-α

Because the PRRSV-CON virus can induce type-I IFNs, we sought to evaluate the sensitivity of this synthetic PRRSV strain to IFN. MARC-145 cells were treated with increasing concentrations of recombinant IFN-α (rIFN-α) before infection with PRRSV-CON or FL12. As shown in Fig. 2, prior treatment of cells with rIFN-α significantly reduced the titers of both PRRSV-CON and FL12. However, the titers of PRRSV-CON reduced more dramatically than that of FL12, indicating that PRRSV-CON was more sensitive to rIFN-α treatment than FL12 (Fig. 2).

2.3. IFN-inducing phenotype of PRRSV-CON maps to the 3.3 kb region at the 5′end of the genome

Our next experimental aim was to identify the genomic sequence of PRRSV-CON responsible for the virus' ability to induce type-I IFNs. We first conducted a gain-of-function study in which we divided the PRRSV-CON genomes into 3 major fragments, based on the availability of the unique restriction enzyme sites, and separately exchanged these fragments into the FL12 infectious cDNA clone (Fig. 3A). The resulting FL12 chimeras carrying the PRRSV-CON genomic fragments replicated efficiently in cell cultures (Fig. 3B). Of these 3 chimeras constructed on the FL12 backbone, only the FL12/ConD acquired the ability to induce type-I IFNs. MARC-145 cells infected with the chimeric virus FL12/ConD expressed high levels of IFN-β mRNA and ISG-56 protein, which were comparable to the levels of IFN-β mRNA and ISG-56 protein detected in cells infected with the PRRSV-CON (Fig. 3C & D). Likewise, only the culture supernatant from cells infected with chimeric FL12/ConD could induce ISG-56 expression and protect cells from being infected with VSV-eGFP (Fig. 3E and F). Together, the results indicated that exchanging the fragment D of PRRSV-CON into the FL12 genome

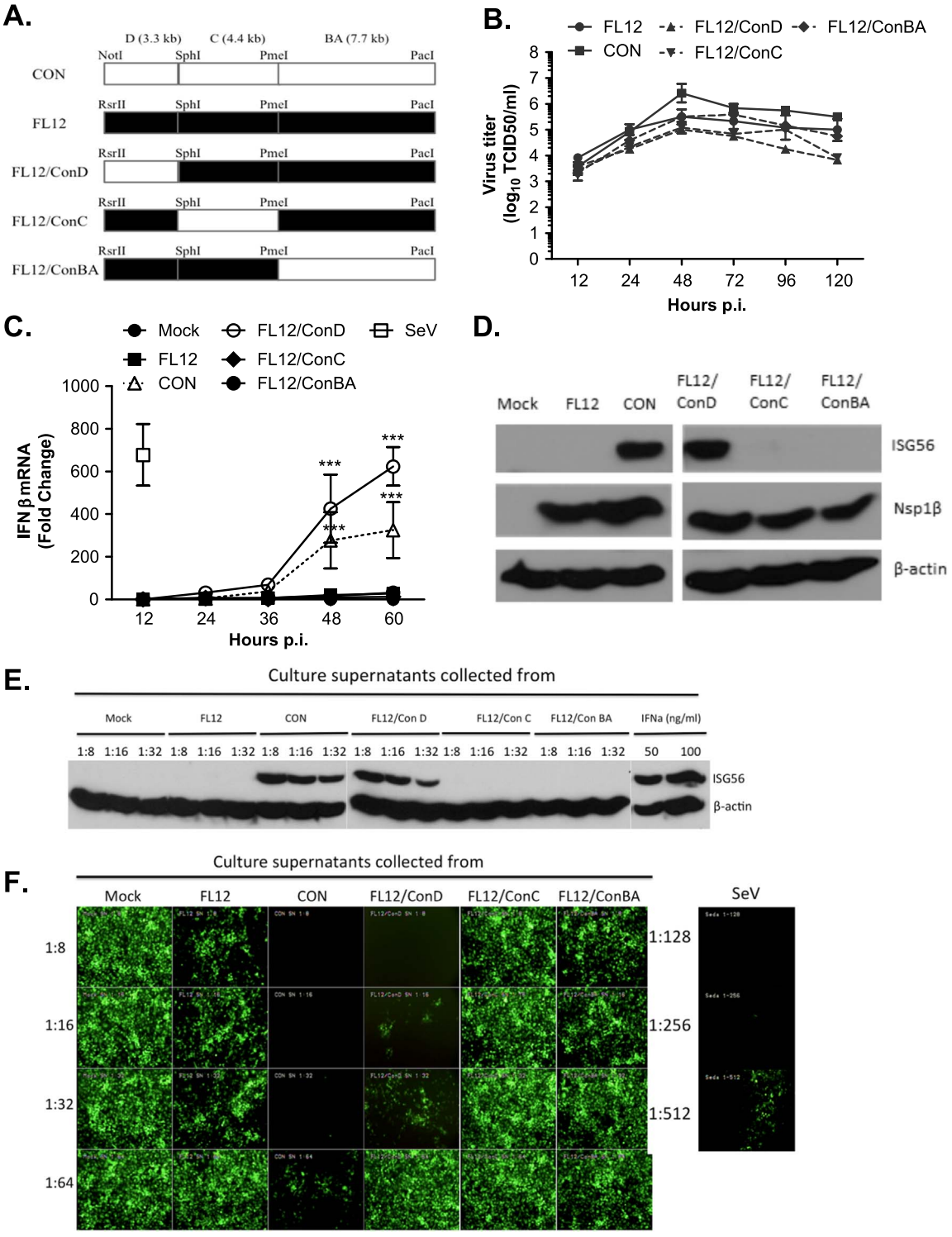

Fig. 3. Gain-of-function studies. (A) Schematic representation of the chimeric PRRSVs constructed by exchanging the genomic fragments between PRRSV-CON and FL12. The restriction enzymes used for exchanging the genomic fragments are shown on top. The size of each fragment is shown. (B) Multiple-step growth curves of the indicated PRRSV strains in MARC-145 cells. Data are expressed as the mean of virus titer and SEM calculated from 3 independent experiments. (C–F) Evaluate the induction of type-I IFNs in MARC-145 cells infected with the indicated PRRSV strains. These experiments were conducted in the same manner as described in the legend to Fig. 1. (C) Relative mRNA levels of IFN-β quantitated by qRT-PCR. Statistical difference as compared with FL12 is indicated by asterisk (***$p < 0.001$). (D) Expression of ISG-56 in cells infected with the indicated PRRSV strains. (E) Expression of ISG-56 protein in cells incubated with UV-treated cell culture supernatants collected from the indicated virus cultures. (F) Interferon- bioassay.

conferred the virus ability to induce type-I IFNs.

We next confirmed the above finding by a loss-of-function study in which we exchanged fragment D of the FL12 into the PRRSV-CON backbone (Fig. 4A). The resulting chimeric virus CON/FL12D replicated efficiently in MARC-145 cells (Fig. 4B). The CON/FL12D virus was no longer able to induce type-I IFNs. MARC-145 cells infected with the chimeric virus CON/FL12D did not express significant levels of IFN-β mRNA or ISG-56 protein (Fig. 4C and D). Additionally, culture supernatant collected from the CON/FL12D infected cells neither induced ISG-56 expression nor protected cells from being infected

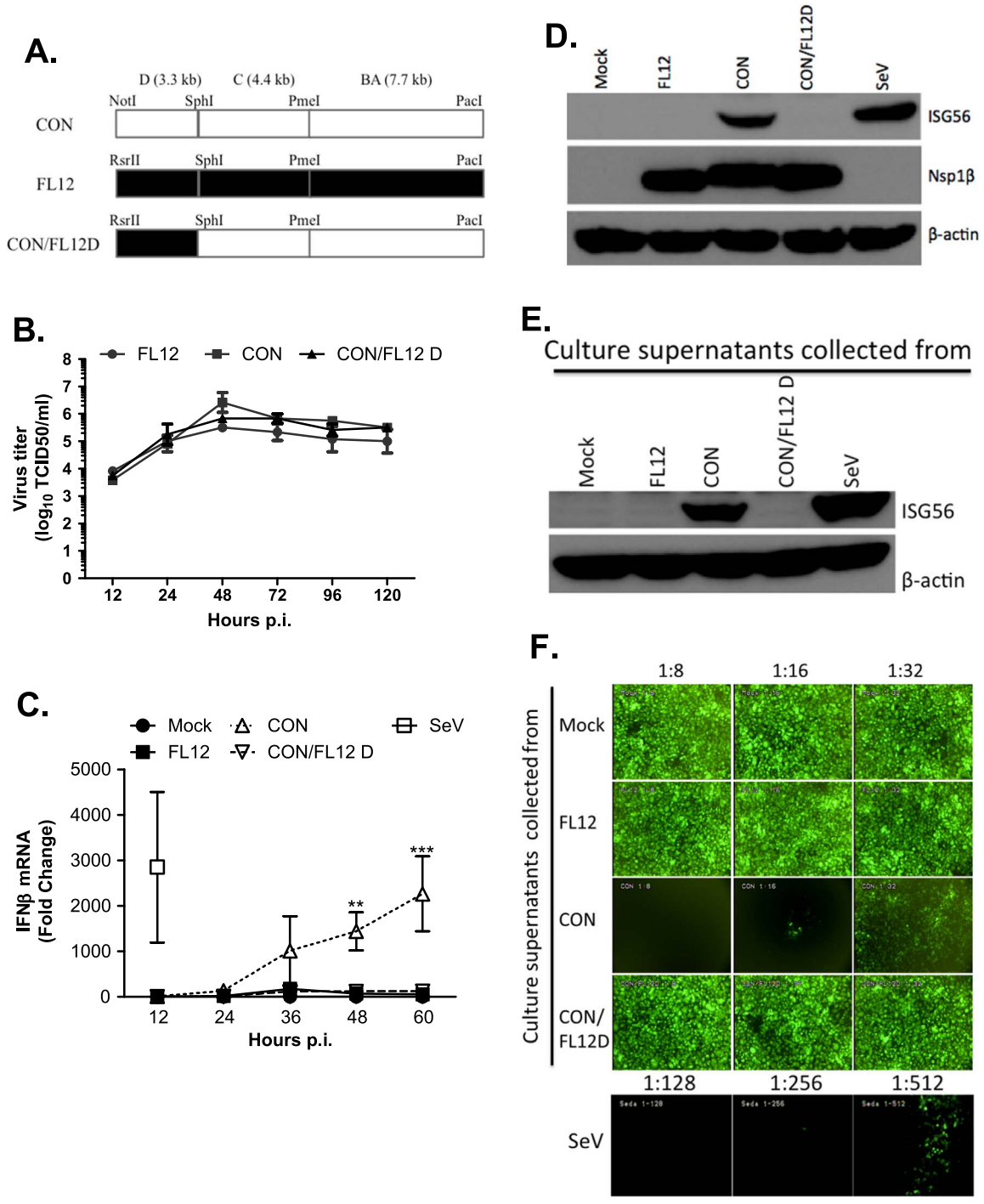

Fig. 4. Loss-of-function studies (A) Schematic representation of the chimeric virus CON/FL12D. The genomic fragment D of FL12 was swapped into the PRRSV-CON backbone. (B) Multiple-step growth curves of the indicated PRRSV strains in MARC-145 cells. Data are expressed as the mean of virus titer and SEM calculated from 3 independent experiments. (C-F) Evaluate the induction of type-I IFNs in MARC-145 cells infected with the indicated PRRSV strains. These experiments were conducted as described in the legend to Fig. 1. (C) Relative mRNA levels of IFN-β quantitated by qRT-PCR. Statistical difference as compared with FL12 is indicated by asterisk (**$p < 0.01$; ***$p < 0.001$). (D) Expression of ISG-56 in cells infected with the indicated PRRSV strains. (E) Expression of ISG-56 protein in cells incubated with UV-treated cell culture supernatants collected from the indicated virus cultures. (F) Interferon bioassay.

with VSV-eGFP (Fig. 4E and F). The results indicated that exchanging the fragment D of FL12 into the PRRSV-CON genome abolished the virus' ability to induce type-I IFNs. Collectively, the results obtained from both gain- and loss-of function studies clearly indicate that the 3.3 kb fragment located at the 5′ end of the PRRSV-CON genome (e.g. fragment D, from nucleotide 1 to 3315) is associated with the virus' capability of inducing type-I IFN response.

2.4. Individual genes encoded by fragment D of the PRRSV-CON are collectively required for the IFN-inducing phenotype

The genomic fragment D contains the 5′un-translated region (5′ UTR), nsp1α, nsp1β and the N-terminal part of nsp2 (nsp2p). The 5′ UTR of the PRRSV-CON and FL12 are identical in their nucleotide sequences (Vu et al., 2015). Between these 2 viruses, there are 182 nucleotide differences in the fragment D region which include 13 in

nsp1α, 45 in nsp1β and 124 in the N-terminal part of nsp2. These 182 nucleotide differences cause 77 amino acid changes including 1 in nsp1α, 19 in nsp1β and 57 in the N-terminal part of nsp2 (Table 1 and Table S1 in Supplementary Materials). We sought to determine which genes within the fragment D of PRRSV-CON are associated with the IFN-inducing phenotype. Three additional chimeric viruses were generated by exchanging nsp1α, nsp1β and nsp2p of the PRRSV-CON into the FL12 backbone (Fig. 5A). The resulting chimeric viruses grew efficiently in cell cultures except for the chimeric virus FL12/CON-nsp1α, which grew slightly less efficiently (Fig. 5B). However, none of these chimeras was able to induce type-I IFNs in MARC-145 cells. As shown in Fig. 5C & D, cells infected with these chimeras did not express significant levels of IFN-β mRNA or ISG-56 protein. Further, culture supernatants collected from cells infected with these chimeras neither induced ISG-56 expression nor protected cells from being infected with VSV (Fig. 5E and F). The results indicated that none of the individual genes within the fragment D of the PRRSV-CON is able to confer the ability to induce type-I IFN response.

3. Discussion

Currently available PRRS vaccines do not provide optimal levels of heterologous protection, largely due to the substantial variation of the viral genome (Shi et al., 2010). To overcome this challenge, we recently generated a fully synthetic PRRSV strain containing a consensus genome derived from a set of 59 full genome sequences of type 2 PRRSV isolates (Vu et al., 2015). We demonstrated that the synthetic PRRSV-CON confers superior levels of heterologous protection. The immunological mechanisms by which the synthetic PRRSV-CON confers heterologous protection remain unaddressed. In the present study, we observed that the synthetic PRRSV-CON possesses a unique phenotype in that it induces type-I IFNs instead of suppressing these cytokines as many naturally occurring PRRSV isolates do. Because type-I IFNs are important for innate resistance against viral infection as well as for the optimal development of adaptive immune responses (reviewed by Gonzalez-Navajas et al., 2012), we were interested in determining how the induction of type-I IFNs contributes to the levels of heterologous protection conferred by the synthetic PRRSV-CON. In this regard, the availability of a PRRSV-CON mutant that does not induce type-I IFNs would be valuable to determine the linkage between the virus capability of inducing type-I IFNs and its ability to confer heterologous protection. Our focus in this study was then to identify the genomic sequence of PRRSV-CON responsible for inducing type-I IFNs as this knowledge is critical for the generation of a PRRSV-CON mutant devoid of the ability to induce type-I IFNs.

Most of the experiments to identify PRRSV viral proteins involved in suppression of type-I IFNs are done by overexpression of individual viral proteins (Beura et al., 2010; Kim et al., 2010; Patel et al., 2010; Sagong and Lee, 2011; Sun et al., 2010). A potential limitation of such overexpression studies is that the proteins are forced to be expressed at atypical levels that might lead to abnormal protein functions (Beura et al., 2012). Therefore, in this study, we sought to identify the genomic regions of the PRRSV-CON responsible for inducing type-I IFNs in the context of virus infection. Taking advantage of the availability of the infectious cDNA clone of FL12 (Truong et al., 2004), our reference PRRSV strain that typically suppresses type I IFN response (Beura et al., 2010), we performed gain- and loss-of-function studies and identified that the fragment of 3.3 kb located at the 5′end of the PRRSV-CON virus (*so*-called fragment D) is solely responsible for the virus' ability to induce type I IFNs (Figs. 3 and 4). This genomic fragment encodes three viral nonstructural proteins: nsp1α, nsp1β, and the N-terminal part of nsp2. Our further studies indicate that none of these proteins of PRRSV-CON can individually confer the capability of inducing type I IFNs to FL12, the PRRSV strain that suppresses type-I IFN. At least five non-structural proteins of PRRSV (e.g. nsp1α, nsp1β, nsp2, nsp4 and nsp11) have been reported to be able to inhibit IFN-β gene expression (Beura et al., 2010; Chen et al., 2014; Song et al., 2010; Sun et al., 2010). Therefore, individual replacement of the PRRSV-CON nsp1α, nsp1β and nsp2p into the FL12 backbone may not be sufficient to confer the capability of inducing type-I IFNs to this PRRSV strain because of the presence of multiple other viral proteins with anti-IFN inhibitory function. It is noteworthy that several PRRSV mutants capable of inducing type-I IFNs have been generated by targeted mutations in individual nsps of PRRSV. For instance, alanine substitution of the R128 and R129 residues in the $_{123}$GKYLQRRLQ$_{131}$ motif of nsb1β of the PRRSV strain SD95-21 attenuated the viral suppression of type I IFNs (Li et al., 2016). The $_{123}$GKYLQRRLQ$_{131}$ is conserved in the nsp1β of PRRSV-CON (data not shown). Likewise, we have previously reported that alanine mutations of the 5 amino acid stretch $_{16}$KGKVS$_{20}$ in nsp1β of FL12 resulted in alleviation of the viral suppression of IFNs (Beura et al., 2012). Also in that study, we identified, through the use of alanine-scanning mutagenesis, several discontinuous amino acid stretches of FL12 nsp1β that are associated with its IFN-suppression function (Beura et al., 2012). Alanine-substitutions of these amino acid stretches resulted in a variable degree of alleviation of IFN-suppression. It is possible that the nsp1α, nsp1β and nsp2 of PRRSV-CON possess some degree of alleviation of IFN-suppression when compared to those of the naturally occurring PRRSV strains like FL12 or SD95-21, but such degree of alleviation might not be as potent as those of the $_{16}$KGKVS$_{20}$ mutations (in case of FL12) or the $_{123}$GKYLQRRLQ$_{131}$ mutations (in the case of SD95-21). When the 3 proteins were simultaneously replaced into the FL12 backbone, the additive effect of alleviation exerted by these 3 proteins might be enough for the induction of IFN.

In this study, we have generated a mutant form of the synthetic PRRSV-CON virus (designated as PRRSV CON/FL12D) that is devoid of the capability of inducing type-I IFNs (Fig. 4). The PRRSV CON/FL12D and PRRSV-CON differ from each other by only 77 amino acids. The availability of these two PRRSV strains provides us a powerful tool to study the relationship between the viral capability of inducing type-I IFNs and its ability to confer protection against heterologous PRRSV strains. We will conduct immunization/challenge experiments by infecting pigs with these two PRRSV strains separately and subsequently challenge them with genetically heterologous PRRSV strains. The results of these immunization/challenge experiments will ascertain the contribution of type-I IFNs on the virus' ability to confer heterologous protection.

4. Materials and methods

4.1. Cells, viruses and antibodies

MARC-145 cells (Kim et al., 1993) were cultured in Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. Porcine alveolar macrophage (PAM) cells were collected from pigs at 4 weeks of age and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium as described previously (Mengeling et al., 1996). The infectious cDNA clone FL12 was previously constructed based on the PRRSV stra-AY545985) (Truong et al., 2004). Likewise, the infectious cDNA clone PRRSV-CON was previously constructed based on a synthetic PRRSV genome (GenBank

Table 1
Genetic difference in the fragment D region between PRRSV-CON and FL12.

Gene	Number of nucleotide difference	Number of amino acid difference
5′ UTR	0	N/A
nsp1α	13	1
nsp1β	45	19
nsp2	124	57
Total	182	77

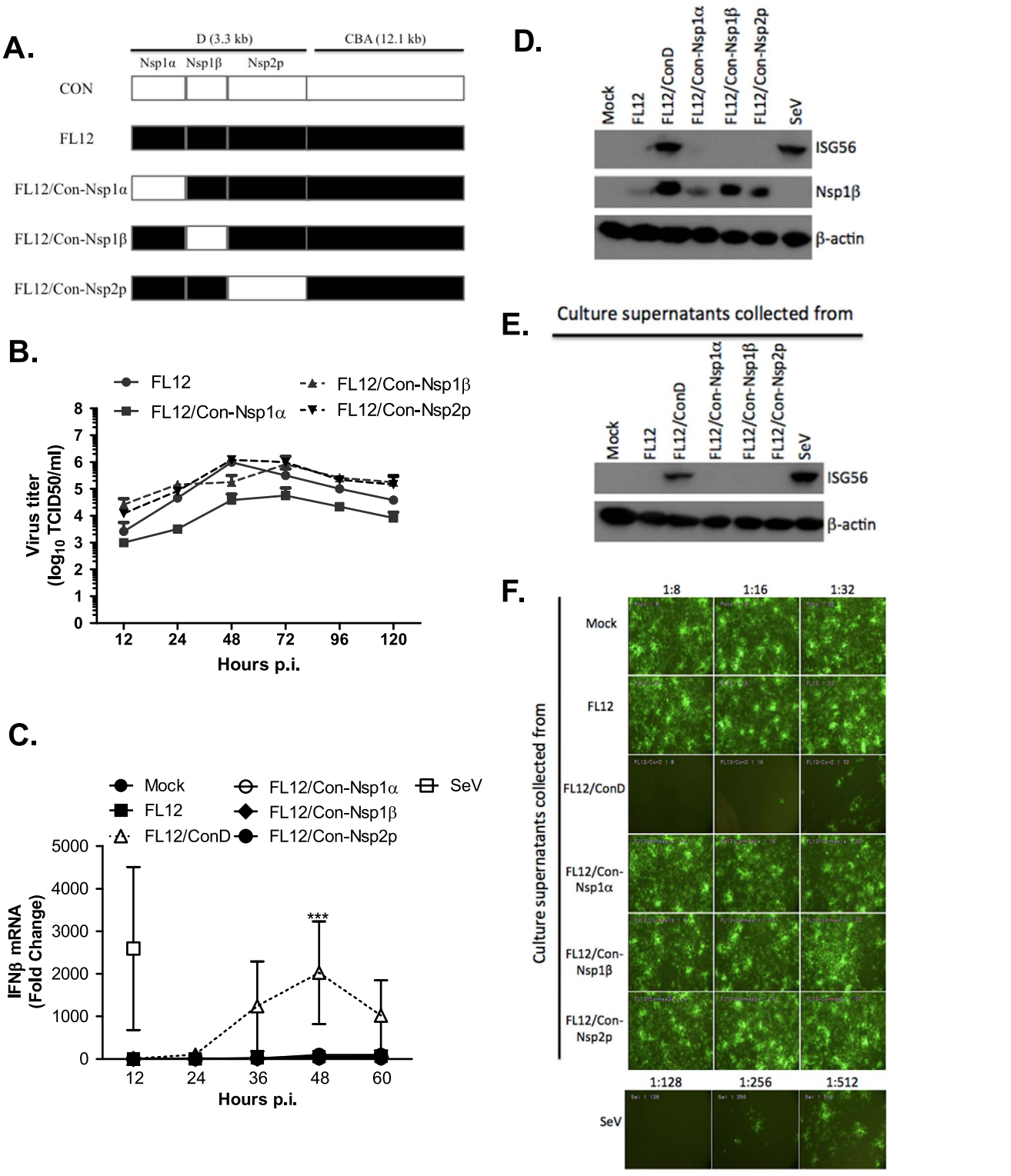

Fig. 5. PRRSV chimeric viruses FL12/Con-Nsp1α, FL12/Con-Nsp1β and FL12/Con-Nsp2p do not induce type I IFN response in MARC-145 cells. (A) Schematic representation of different PRRSV chimeras constructed by swapping individual genes of the PRRSV-CON into the FL12 backbone. (B) Multiple-step growth curves of the indicated PRRSV strains in MARC-145 cells. Data are expressed as the mean of virus titer and SEM calculated from 3 independent experiments. (C–F) Evaluate the induction of type-I IFNs in MARC-145 cells infected with the indicated PRRSV strains. These experiments were conducted as described in the legend to Fig. 1. (C) Relative mRNA levels of IFN-β quantitated by qRT-PCR. Statistical difference as compared with FL12 is indicated by asterisk (***$p < 0.001$). (D) Expression of ISG-56 in cells infected with the indicated PRRSV strains. (E) Expression of ISG-56 protein in cells incubated with UV-treated culture supernatants collected from the indicated virus cultures. (F) Interferon-bioassay.

accession no. KT894735) (Vu et al., 2015). Sendai virus (SeV, Cantell strain) was obtained from Charles River Laboratories and used as positive control in the experiments to evaluate the induction of type-I IFNs. Vesicular stomatitis virus expressing enhanced green fluorescent protein (VSV-eGFP) was reported previously (Das et al., 2006). The anti-ISG56 antibody was a generous gift from Dr. Saumendra Sarkar, University of Pittsburg. Rabbit polyclonal antibody against PRRSV nsp1 was previously developed by our laboratory (Subramaniam et al.,

2010). Mouse anti- β-Actin (Sc-47778) was obtained from Santa Cruz Biotech. HRP-conjugated secondary antibodies including goat anti-mouse (074-1807) and goat anti-rabbit (214-1516) antibodies were purchased from Kirkegaard & Perry Laboratories, Inc. (KPL).

4.2. Plasmid construction

To generate the FL12/ConC and FL12/ConBA clones, DNA fragments C and BA were excised from the PRRSV-CON infectious cDNA clone by using 2 pairs of restriction enzymes SphI – PmeI and PmeI – PacI, respectively, followed by cloning into the FL12 cDNA clone (Fig. 3A). To generate the FL12/ConD chimeric clone, fragment D of PRRSV-CON was amplified by PCR using synthetic forward primers containing RsrII and SphI restriction enzyme sites. The PCR amplified fragment was then digested with RsrII and SphI enzymes and cloned into the FL12 cDNA clone. A Similar approach was used to construct the PRRSV-CON/FL12D chimeric clone. In this case, fragment D of the FL12 clone was amplified by PCR using synthetic primers containing NotI and SphI restriction enzyme sites. The PCR amplified fragment was then digested with NotI and SphI enzymes and cloned into the PRRSV-CON cDNA clone (Fig. 4A). To generate the FL12/Con- nsp1α, FL12/Con-nsp1β, and FL12/Con-nsp2p clones, chimeric fragments containing nsp1α, nsp1β or nsp2p from PRRSV-CON were constructed by using overlap-extension PCR with synthetic primers. Primers used this study are listed in Table S2 in Supplementary Materials. The PCR amplified fragment was then digested with RsrII and SphI enzymes and cloned into the FL12 cDNA clone. All the plasmids were verified by Sanger sequencing.

4.3. In vitro transcription and virus recovery

Plasmids carrying wild-type or chimeric PRRSV cDNA genomes were digested with AclI for linearization. After that, the purified, linear cDNA fragment was used as the template for an in vitro transcription reaction by using the mMESSAGE mMACHINE Ultra T7 kit (Ambion, Austin, TX) as described previously (Truong et al., 2004; Vu et al., 2013, 2011). Approximately 3 μg of the purified, full-genome RNA transcript was transfected into MARC-145 cells using TransIT-mRNA transfection kit (Mirus Bio, Madison, WI). When cytopathic effect (CPE) was apparent, culture supernatant containing the rescued viruses was harvested and transferred into naïve MARC-145 one more time to produce enough virus stock for future studies.

4.4. Multiple-step growth curve

MARC-145 cells were infected with different PRRSV strains at an MOI of 0.01. At various time points p.i., culture supernatants from infected cells were collected. Virus titers were measured by titration in MARC-145 cells and expressed as TCID50 per ml.

4.5. Measurement of relative mRNA levels of IFN-α and IFN-β

To quantify the levels of IFN-α mRNA, PAMs were infected with PRRSV strains at an MOI of 1. At various time-points p.i., cells were homogenized in TRIzol® Reagent (Life Technologies) and total RNA was isolated. After that, 0.5 μg of total RNA was reverse transcribed using random primers and the M-MLV reverse transcription (RT) kit (Invitrogen) as per the manufacturer's recommendation. The cDNA was used for quantitative real-time PCR (qRT-PCR) to quantitate the IFN-α mRNA using the swine IFNA1 primers and probe set (Life Technologies, Ss03394862_g1). Simultaneously, β-actin mRNA levels were quantitated by qRT-PCR using the swine ACTB primers and probe set (Life Technologies, Ss03376081-u1). To quantify the levels of IFN-β mRNA in MARC-145 cells, the cells were infected with different PRRSV strains at an MOI of 0.01. For positive control, MARC-145 cells cultured in a 24-well plate were infected with SeV at 16 HA unit per well. At various time points p.i., cells were homogenized in TRIzol® Reagent (Life Technologies) and total RNA was isolated using the Direct-zol RNA MiniPrep kit (Zymo Research), following the manufacturer's instructions. After that, 0.5 μg of total RNA was reverse transcribed using random primers and the M-MLV reverse transcription kit (Invitrogen) as per the manufacturer's recommendation. The cDNA was used for qRT-PCR to quantitate the IFN-β mRNA using the Rhesus monkey IFNB1 primers and probe set (Rh03648734_sl IFNB1, Life Technologies). Simultaneously, β-actin mRNA levels were quantitated by qRT-PCR using the Rhesus monkey ACTB primers and probe set (Rh03043380_gH ACTB, Life Technologies). The relative IFNβ mRNA levels were quantified by the 2 (-Delta Delta C (T)) method (Livak and Schmittgen, 2001) and shown as a relative fold change compared with mock control.

4.6. Detection of ISG-56 from MARC-145 cells infected with different PRRSV strains

MARC-145 cells were infected with different PRRSV strains at an MOI of 0.01. At 72 h p.i., infected cells were collected and lysed in RIPA buffer (10 mM Tris, 140 mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, 2 mM PMSF, 1 mM Leupeptin). The protein lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Beura et al., 2010; Subramaniam et al., 2012), followed by transferring onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked in a blocking buffer (Tris buffer saline containing 0.05% Tween 20 (TBS-T20) and 5% nonfat dry milk) for 2 h at room temperature, followed by incubation with anti-ISG-56 antibody at 4 °C overnight. After 3 washes with TBS-T20, the membrane was incubated with HRP-conjugated goat anti-rabbit antibody at room temperature for 2 h. After 3 washes with TBS-T20, protein bands were visualized by using an electrochemiluminescent (ECL) detection system (Pierce).

4.7. Detection of ISG-56 from MARC-145 cells incubated with UV-treated culture supernatants collected from cells infected with different PRRSV strains

Culture supernatants collected from PRRSV-infected cells were subjected to ultraviolet (UV) treatment at 1200 mJ/cm^2 for 120 s. The UV-treated culture supernatants were serially diluted in fresh DMEM and inoculated onto naïve MARC-145 cells. After 18 h incubation, cells were collected and the expression of ISG-56 was analyzed as described above.

4.8. Interferon bioassay

Culture supernatants collected from PRRSV-infected cells were subjected to ultraviolet (UV) treatment as described above. UV-treated culture supernatants were diluted 2-fold serially in DMEM and inoculated onto MARC-145 cells seeded in 96-well plates. After 18 h incubation, the culture supernatants were removed and the cells were subsequently infected with VSV-eGFP at 100 plaque forming unit per well. At 16 h post-infection, eGFP-positive cells were observed under fluorescence microscopy.

4.9. Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA) in GraphPad Prism 5.0 (GraphPad Software, Inc). A p value < 0.05 was considered significant.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2016.09.018.

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