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TNF-α induced by porcine reproductive and respiratory syndrome virus inhibits the replication of classical swine fever virus C-strain

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ABSTRACT

Porcine productive and respiratory syndrome virus (PRRSV) and classical swine fever virus (CSFV) both are major pathogens of swine that pose a great threat to the Chinese pig industry. It has been found that PRRSV infection can lead to vaccination failure of CSFV C strain-derived modified live vaccine (CSFV-C) by interfering with the immune responses to the latter. To investigate whether PRRSV can suppress CSFV-C replication, we created a 3D4/21-based cell line PAM39 that is susceptible to both viruses by expressing PRRSV receptors CD163 and CD169, and then investigated their interplay under the condition of either sequential or simultaneous co-infection. The most significant suppressive effect came from the sequential infection when the cells were first infected by PRRSV and then followed by CSFV-C at an interval of 6 h. In addition, this effect was independent of PRRSV strains. Mechanistically, PRRSV induced an elevated level of a subset of pro-inflammatory cytokines, especially tumor necrosis factor (TNF-α), through the nuclear factor κB (NF-κB) signaling pathway to inhibit the replication of CSFV-C *in vitro*. Thus, our studies provide an alternative explanation on PRRSV-induced CSFV vaccination failure, and this has an important implication in CSF vaccination and control.

1. Introduction

Classical swine fever (CSF) is unarguably one of the most important viral diseases of domestic pigs and wild boars and causes significant economic losses to the worldwide pig industry. In most swine farms, systematic prophylactic vaccination is the major practice for CSF control and prevention (Huang et al., 2014). Modified live vaccines (MLVs) are currently widely used in most endemic areas because of their inexpensiveness and high protection efficacy. In China, the CSF MLVs are based on the classical swine fever virus C strain (CSFV-C), a lapinized attenuated vaccine strain that was attenuated from a highly virulent strain (Shimen strain) after at least 480 passages in rabbits (Luo et al., 2014). This vaccine has been found to be very safe for pigs of any age and any breed and provides complete clinical protection against either homologous or heterologous CSFV strains (Luo et al., 2017). However, vaccination failure can occur in the field when piglets have high levels of maternal antibodies to CSFV at the time of vaccination (Suradhat and Damrongwatanapokin, 2003), or when the pigs are already infected by some immunosuppressive pathogens, such as porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type II (PCV2) (Wang et al., 2016).

PRRSV is an enveloped positive-stranded RNA virus and belongs to the family *Arteriviridae* of the order *Nidovirales* (Kuhn et al., 2016). Since the first emergence in the late 1980s, PRRSV has remained one of the most economically important pathogens of swine. Infections by this agent often lead to reproductive failure of the sows and respiratory diseases in young pigs (Han et al., 2017). In addition, PRRSV can deregulate host immunity, leading to potential immunosuppression in pigs. Studies have shown that PRRSV has evolved several strategies to escape from host immune responses, including innate and adaptive immune system (Du et al., 2016; Jung et al., 2009), and results in delayed, low-level neutralizing antibodies and cytotoxic T lymphocyte (CTL) responses. This kind of immunity deregulation may exert a negative effect on the host immune response to other pathogens following vaccination. In the case of CSFV, it has been documented that PRRSV infection prior to CSFV vaccination can significantly reduce the antibody responses to CSFV vaccine in vaccinated pigs, resulting in CSFV vaccination failure (Li and Yang, 2003; Suradhat et al., 2006). However, this can arise from either immune suppression or inhibition of the replication of CSFV vaccine strain. Host immunosuppression may occur

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due to the induction of cytokines, including IL-10. A previous study has shown that PRRSV infection could induce higher levels of IL-10 expression in the first week, which suppresses the host immune response, and it may dampen the effect of CSFV vaccination (Wang et al., 2016). However, little is known about whether PRRSV infection can influence CSFV-C replication.

CD163 has been shown to be a critical determinant of PRRSV tropism to porcine alveolar macrophages (PAMs), and co-expression with CD169 can promote efficient PRRSV infection (Calvert et al., 2007; Delrue et al., 2010; Van Gorp et al., 2008). Porcine alveolar macrophage cell line 3D4/21 (Weingartl et al., 2002) is susceptible to CSFV (Ning et al., 2017) but refractory to PRRSV infection due to lack of CD163 (Lee et al., 2010). To investigate the interplay between PRRSV and CSFV-C *in vitro*, we first constructed a PAM39 cell line expressing the PRRSV receptors CD163 and CD169. By taking advantage of this cell line, we found that PRRSV could inhibit the replication of CSFV-C, and this suppressive effect was likely attributed to the high-level expression of TNF-α induced by PRRSV infection.

2. Materials and methods

2.1. Cells, plasmids, and viruses

MARC-145, HEK 293 T, ST, PK-15, and SK-6 cells were all cultured in Dulbecco's modified Eagle medium (DMEM) with high glucose (Gibco) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Beyotime Biotechnology). PAMs were prepared as previously described (Zhang et al., 2009). 3D4/21 cells (CRL-2843) were cultivated in RPMI 1640 medium (Gibco) supplemented with 10% FBS (Gibco), 1% penicillin-streptomycin (Beyotime Biotechnology), 10 mM of HEPES (Amresco), and non-essential amino acids (Gibco). All cells were maintained at 37℃ with 5% CO_2. PRRSV strains (8th passage) JXwn06, HB-1/3.9, and CHsx1401 were isolated and described previously (Zhou et al., 2015a, b; Zhou et al., 2009). JXwn06 (GenBank accession no. EF641008), a highly pathogenic PRRSV strain, was isolated from an intensive pig farm with an atypical PRRS outbreak in Jiangxi province of China in 2006 and caused high fatality for pigs. HB-1/3.9 (GenBank accession no. EU360130) is a low-virulent strain and induces no fatality for pigs. CHsx1401 (GenBank accession no. KP861625) is a NADC30-like PRRSV strain, which can induce abortion and stillbirth in pregnant sows and clinical respiratory disorders in the weaning pigs. CSFV-C was kindly provided by Dr. Qin Wang (China Institute of Veterinary Drug Control). The viral titers were measured by the endpoint dilution assay (50% tissue culture infective dose, $TCID_{50}$) according to the Reed-Muench method (Reed and Muench, 1938; Zhou et al., 2009). The plasmids of pLVX-IRES-ZsGreen1 and pLVX-IRES-Puro were bought from Clontech. Antibodies against CD163 (Catalog no. MCA2311) and CD169 (Catalog no. MCA2316) were bought from Bio-Rad. Antibodies against nuclear factor κB (NF-κB) P65 (Catalog no. 6956S) and phosphorylated NF-κB P65 (p-NF-κB P65) (Catalog no. 3033S) were bought from Cell Signaling Technology. Antibody against PRRSV N protein was produced by our lab at China Agricultural University.

2.2. Establishment of a stable cell line expressing CD163 and CD169

To make the 3D4/21 cell line permissive to PRRSV, the swine CD163 and CD169 cDNAs were stably integrated into the cell genome. Briefly, the primer pairs for swine CD163 and CD169 amplification (Table 1) were designed according to the known sequences EU016226.1 and NM_214346.1, respectively. Total RNAs were extracted from PAMs with TRIzol reagent (Biomed) following the manufacturer's instructions. First-strand cDNA of CD163 and CD169 were synthesized using FastQuant RT Kit (With gDNase) (TianGen) with specific primers of CD163-R and CD169-R (Table 1). Then they were amplified with KOD-Fx-Neo DNA polymerase (TOYOBO) using the primer pairs of CD163 and CD169, respectively. Finally, PCR products were inserted into the vectors of pLVX-IRES-ZsGreen1 and pLVX-IRES-Puro and named as pLVX-IRES-ZsGreen1-CD163 and pLVX-IRES-Puro-CD169 correspondingly. The packaging of lentiviral vectors was performed according to the manufacturer's instructions (Clontech) in HEK 293 T cells. The culture supernatants were collected and concentrated at 48 h post-transfection. Viral titers were determined using the limited dilution method. For the selection of a stable monoclonal cell line, lentiviruses-transduced 3D4/21 cells were cultured in 100-mm dishes at ten-fold serial dilutions in the presence of 800 ng/ml puromycine (CalBiochem). Antibiotic-resistant cell clones were isolated using a cloning cylinder (Sigma) and transferred into 48-well plates. The selected cells were propagated and further purified by flow cytometry for the enhanced green fluorescent protein (EGFP) expression. The resulted cell line was named as PAM39.

2.3. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Two μg of total RNAs extracted from each cell sample with the TRIzol reagent were reverse transcribed into cDNAs using FastQuant RT Kit with random primers. All specific primers used in this assay were shown in Table 1. The abundance of CSFV-C genome was quantified with the absolute qRT-PCR assay, and the mRNA levels of IL-6, IL-8, IL-12, TNF-α, IFN-α, IFN-γ, and peptide prolyl isomerase A (PPIA) were measured with relative qRT-PCR using cytokine gene-specific primers respectively as described previously (Gudmundsdottir and Risatti, 2009). The cDNAs of RNA samples were quantitated in triplicate with SYBR® Select Master Mix (Applied Biosystems), and all qRT-PCR reactions were conducted with an ABI7500 real-time PCR system (Applied Biosystems) according to the manufacturer's instructions.

2.4. Immunofluorescence assay

The immunofluorescence assay was carried out as previously described (Du et al., 2016; Jin et al., 2017). Briefly, PAM39 monolayer cells grown on coverslips were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.1% Triton X-100 in PBS/2% bovine serum albumin (BSA). After being blocked in PBS/2% BSA for 30 min, cells were incubated with the proper primary antibodies at room temperature (RT) for 1 h in a humidity chamber. Briefly, the PAM39 cells from co-infection experiment were stained with mouse monoclonal antibody to P65 or mouse monoclonal antibody to PRRSV N at a dilution of 1: 1000; for the identification of CD163 and CD169, PAM39 cells were incubated with mouse monoclonal antibody to CD163 (dilution 1: 200) or with mouse monoclonal antibody to CD169 (dilution 1: 200). Then, the coverslips were washed with PBS 3 times for 5 min each, followed by incubation with Alexa-Fluor-568-conjugated goat anti-mouse immunoglobulin G (IgG) F (ab') fragment (Molecular Probes) at a dilution of 1: 1000 for 1 h at RT in a humidity chamber. Nuclear DNA was stained with 4′, 6′-diamidino-2-phenylindole (DAPI) (Molecular Probes) for 10 min and washed with PBS 5 times for 5 min each. The images were taken with a Nikon A1 microscope.

2.5. Western blot assay

Cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime) supplemented with protease inhibitor (Beyotime). The concentration of protein in samples was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce), and 2 μg of total protein for each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Membranes were blocked in 5% skimmed-milk-PBS for 2 h, followed by incubation with appropriate primary antibodies for 1 h at RT. The membranes were

Table 1
Primers used in this study.

Name	5'-3'
pLVX-cd163-F	CCG*CTCGAG*AGGGCCATGGACAAACTCAGAATGGTGCTACATGAA
pLVX-cd163-R	CGC*GGATCC*TCATTGTACTTCAGAGTGGTCTCCT
pLVX-cd169-F	C*CTCGAG*GTCACCATGGACTTCCTGCTCCTGCTCCTCCTCC
pLVX-cd169-R	*GGATCC*CGTTCAGACTGTGCTTTTCACAGACTGT
CSFV-C-F	AGCCCACCTCGAGATGCTA
CSFV-C-R	CTATCAGGTCGTACTCCCATCAC
PPIA -F	AAGGTTCCTGCTTTCACAGAATAAT
PPIA-R	AATTTCTCTCCATAGATGGACTTGC
TNF-α-F	AACCTCAGATAAGCCCGTCG
TNF-α-R	ACCACCAGCTGGTTGTCTTT
IL-6-F	CTGGCAGAAAACAACCTGAACC
IL-6-R	TGATTCTCATCAAGCAGGTCTCC
IL-8-F	GGCAGTTTTCCTGCTTTCT
IL-8-R	CAGTGGGGTCCACTCTCAAT
IL-12-P40-F	AACTCTTCACGGACCAAATCTCA
IL-12-P40-R	GGTCCCGGGCTTGCAC
IFN-α-F	GCCTCCTGCACCAGTTCTACA
IFN-α-R	TGCATGACACAGGCTTCCA
IFN-γ-F	AATGGTAGCTCTGGGAAACTG
IFN-γ-R	ACTTCTCTTCCGCTTTCTTAGG
P65-F	*CTCGAG*AACCCGGCGCATTGCTGTGCC
P65-R	*AAGCTT*TTAGGAGCTGATCTGACTCAGAAG

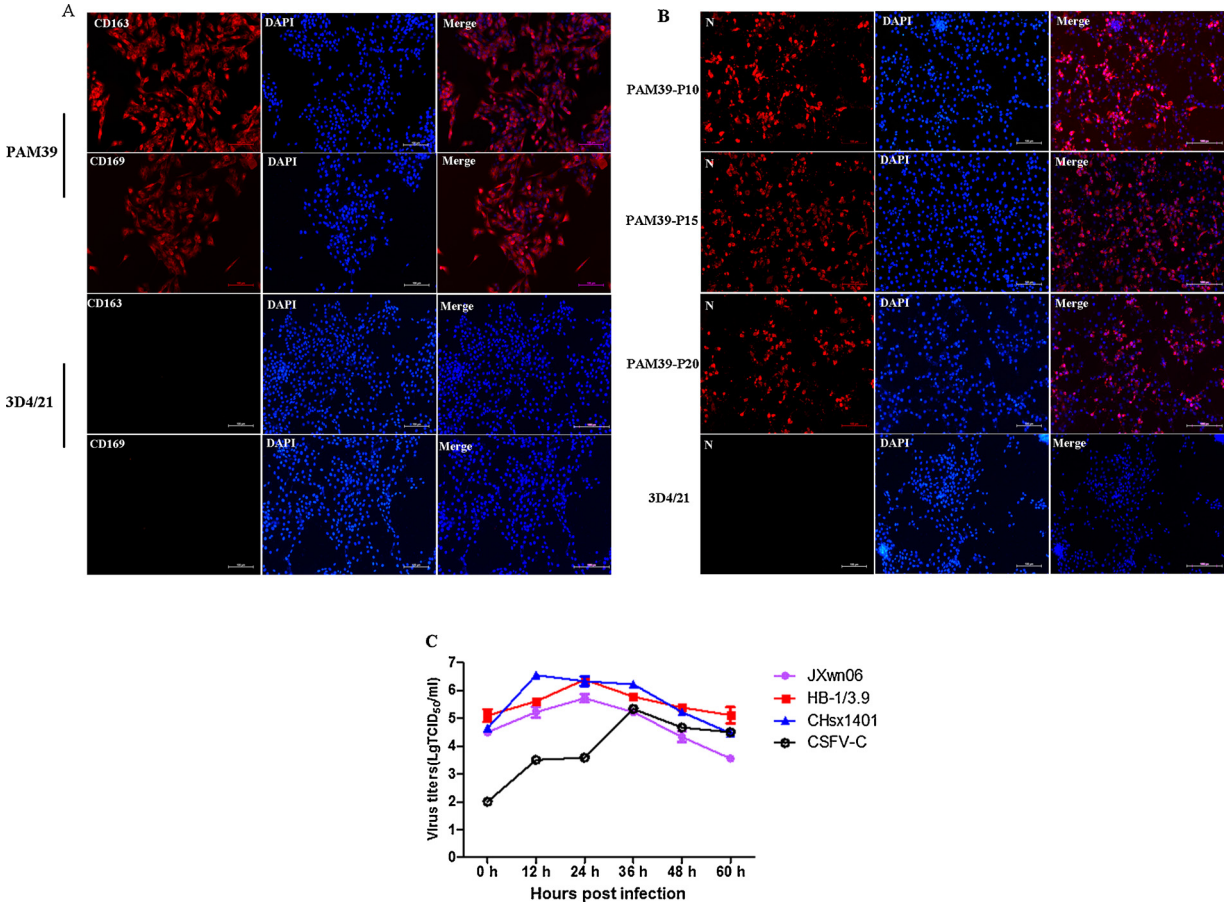

Fig. 1. Generation of PAM39 cell line and its susceptibility to PRRSV and CSFV-C. (A) Detection of CD163 and CD169 in PAM39 and 3D4/21 cells (40 × magnification). CD163 was stained with a mouse anti-pig CD163 monoclonal antibody (dilution 1:200) coupled by Alexa Fluor-568-conjugated anti-mouse secondary antibody (dilution 1:1000), and CD169 was stained with a mouse anti-pig CD169 monoclonal antibody (dilution 1:200) coupled by Alexa Fluor-568-conjugated anti-mouse secondary antibody (dilution 1:1000). **(B) Immunofluorescence assay (IFA) analysis of PRRSV N protein expression in PAM39 cells infected with PRRSV (40 × magnification).** Various generations of transgenic cells (F10, F15, and F20) were infected with PRRSV JXwn06 (MOI = 0.1), and expression of the N protein was examined at 36 hpi using the PRRSV N protein monoclonal antibody, and a Alexa Fluor-568-conjugated anti-mouse secondary antibody, 3D4/21 cells were used as control. Nuclei were stained with DAPI. **(C) The Growth kinetics of PRRSV and CSFV-C in PAM39 cells.** PRRSV various strains (JXwn06, HB-1/3.9, and CHs × 1401, 0.1 MOI) and CSFV-C (0.1 MOI) were inoculated in PAM39 cells. The virus titers were measured by a microtitration infectivity assay and expressed as $TCID_{50}$ per milliliter at the indicated time points.

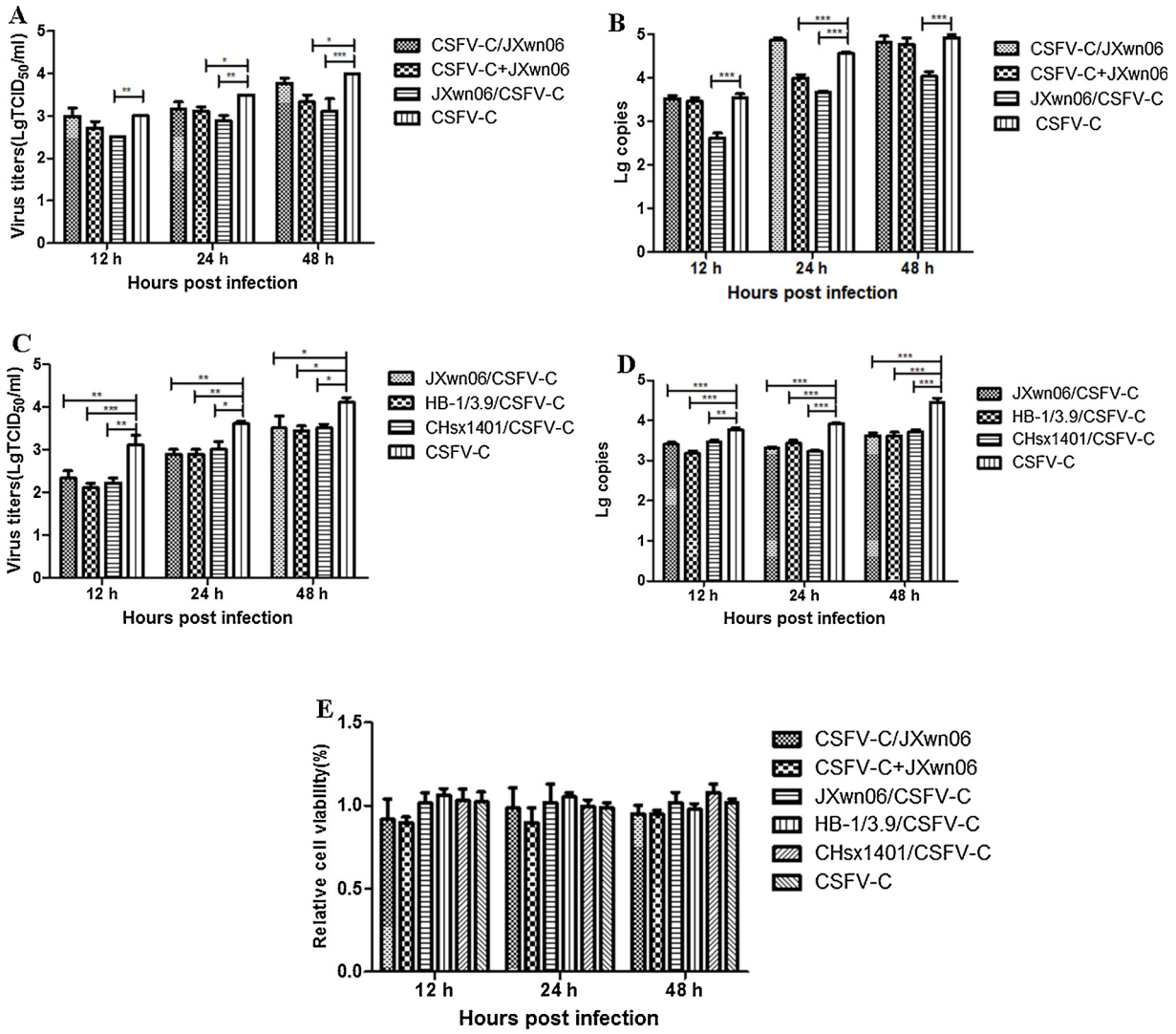

Fig. 2. Effect of JXwn06 and CSFV-C sequential co-infection on the CSFV-C proliferation (A) CSFV-C titers in the PAM39 cells sequentially co-infected with JXwn06 and CSFV-C. The PAM39 cells were sequentially infected with JXwn06 (0.1 MOI) and CSFV-C (0.1 MOI) or with CSFV-C alone, and the virus titers were determined by a microtitration infectivity assay in ST cells and expressed as $TCID_{50}$ per milliliter at the indicated time points. **(B) Genomic copies of CSFV-C in the PAM39 cells sequentially co-infected with JXwn06 and CSFV-C. (C) CSFV-C titers in the PAM39 cells co-infected with various PRRSV strains and CSFV-C. (D) Genomic copies of CSFV-C in the PAM39 cells co-infected with various PRRSV strains and CSFV-C. (E) The viability of PRRSV and CSFV-C infected PAM39 cells.**

then washed with 0.05% PBS-Tween (PBST) three times and incubated with the proper horseradish peroxidase (HRP)-conjugated secondary antibodies. The membranes were washed using the same procedure described above, then detected by a enhanced chemiluminescence (ECL) detection kit (Pierce) and finally, the image was acquired with a chemiluminescence apparatus (ProteinSimple).

2.6. Small interfering RNA (siRNA) assay

The siRNAs against P65 were designed and synthesized by Gene Pharma, and siNC was designed as a negative control. The sequences of siRNAs were as follows: siP65-848 sense: 5′−CCAUGGAAUUCCAGUACUUTT, siP65-848 antisense: AAGUACUGGAAUUCCAUGGTT, siP65-1042 sense: CCCUAUCCCUUUACGCCAUTT, siP65-1042 antisense: AUGGCGUAAAGGGAUAGGGTT, siNC sense: UUCUCCGAACGUGUCACGUTT, siNC antisense: ACGUGACACGUUCGGAGAATT. PAM39 cells were transfected with 10 pmol or 30 pmol of siRNAs against P65 with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol.

2.7. Cell viability assay

Cell viability was evaluated by Cell Titer 96 Aqueous One Solution Cell Proliferation Assay system (Promega) according to the manufacturer's instruction.

2.8. Statistical analysis

The difference between the control and treatment groups was analyzed with two-way ANOVA test of variance. A P value of < 0.05 was considered to be significant difference. All data analyses were performed with the GraphPad Prism (version 5.0) software.

3. Results

3.1. Establishment of PAM39 cell line permissive to both PRRSV and CSFV-C strains

To establish a cell line susceptible to both CSFV and PRRSV strains, the porcine alveolar macrophage cell line 3D4/21 was transfected with

CD163 and CD169 plasmids, and a new cell line PAM39 expressing both CD163 and CD169 was generate by puromycin and green fluorescence selection. As shown in Fig. 1A, both CD163 and CD169 were expressed at a high level in PAM39 cells, but undetectable in 3D4/21 cells. In addition, both molecules are genetically stable as different passages of PAM39 (P10, P15, and P20) can support efficient replication of PRRSV strain JXwn06 as monitored by the expression of N protein (Fig. 1B). To further test whether the replication efficiency is strain-dependent, the cells were infected with genetically distant PRRSV strains JXwn06, HB-1/3.9, and CHsx1401 as well as CSFV-C. At the indicated time points, the viruses were harvested and titrated. As shown in Fig. 1C, all four viruses could efficiently replicate in PAM39 cells (Fig. 1C). Of them, the titers of JXwn06 and HB-1/3.9 reached a plateau at 24 hpi, while that of CHsx1401 and CSFV-C reached at 12 hpi and 36 hpi, respectively. Thus, the PAM39 cell line can support efficient replication of various PRRSV strains and CSFV-C, and can be further used for investigation of the interplay between PRRSV and CSFV-C.

3.2. Prior infection of PRRSV can decrease CSFV-C replication in PAM39 cells

To investigate whether PRRSV infection can affect the replication of CSFV-C, PAM39 cells were infected with PRRSV JXwn06 and CSFV-C at an MOI of 0.1 either sequentially (CSFV-C/JXwn06 and JXwn06/CSFV-C) or simultaneously (CSFV-C + JXwn06). For sequential infection, we set up two different combinations. PAM39 cells in group CSFV-C/JXwn06 were first infected with CSFV-C and then followed by JXwn06 at 6 h interval, whereas cells in group JXwn06/CSFV-C were infected in an opposite order. For the control, the cells were infected only with CSFV-C only. At indicated time points, the viral titers and genomic copy numbers were determined by $TCID_{50}$ and qRT-PCR, respectively. As shown in Fig. 2A and B, the group JXwn06/CSFV-C showed significantly decreased replication level of CSFV-C at 12, 24, and 48 hpi ($P < 0.01$), while in the group CSFV-C + JXwn06, the reduction was mainly found at 24 hpi ($P < 0.05$). Interestingly, when the cells were infected with CSFV-C prior to the infection of PRRSV, there was no effect on the replication of CSFV-C. Thus, the order of infection appears critical for the replication efficiency of CSFV-C caused by co-infection of PRRSV.

To test whether the observation is PRRSV-strain dependent, we infected PAM39 cells with two other representative strains of PRRSV (HB-1/3.9 and CHsx1401) described above. As shown in Fig. 2C and D, these two PRRSV strains could also significantly inhibit the replication of CSFV-C at selected time points post infection ($P < 0.05$), whereas there was no significant difference in cell viability between co-infection groups and the CSFV-C group (Fig. 2E). Taken together, PRRSV infection prior to CSFV-C significantly decreased the replication of CSFV-C without reducing cell viability.

3.3. PRRSV-induced TNF-α can reduce the replication of CSFV-C

Cytokines are small hormone-like proteins that affect the behavior of themselves or other cells through autocrine, paracrine, and endocrine responses. They are important for cell-cell communication, and play an important role in the defense against viral infection. To determine whether cytokines play a role in the inhibition of CSFV-C replication, we examined the mRNA levels of various cytokines in PRRSV and CSFV co-infected cells by relative qRT-PCR. As shown in Fig. 3, the expression of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-8 were most significantly upregulated in the cells of JXwn06/CSFV-C group (Fig. 3A, B, and C) at 12 and 24 hpi. However, the expression of these three pro-inflammatory cytokines were only slightly affected in the cells of CSFV-C/JXwn06 group at 24 hpi. We also measured the mRNA levels of IL-10, IL-12, IFN-α, and IFN-γ in infected cell culture, and found that only a slight elevation of IL-10 and IL-12 in the groups PRRSV/CSFV-C and CSFV-C + PRRSV at 12 hpi (data not shown). In addition, the observed effect on pro-inflammatory cytokines (TNF-α, IL-6, and IL-8) was likely PRRSV strain-independent (Fig. 3D, E, and F).

The above result suggests a possible connection between pro-inflammatory cytokines production and the inhibition of CSFV-C replication. To further determine whether pro-inflammatory cytokines can have an effect on CSFV-C replication, PAM39 cells were treated with 20 ng/ml or 40 ng/ml of TNF-α for 12 h prior to the infection of CSFV-C. The genomic copies of CSFV-C in infected cells were measured by qRT-PCR. As shown in Fig. 4A, treatment of TNF-α significantly reduced the replication of CSFV-C at examined time points. To determine the influence of the secreted form of TNF-α on the replication of CSFV-C in various cells, we infected three other porcine cell lines (ST, SK-6 and PK-15) with CSFV-C following 12 h of treatment with the same dose of TNF-α. The results showed that the addition of TNF-α could also downregulate the quantity of CSFV-C at 12, 24, and 48 hpi in these three cell lines (Fig. 4B, C, and D), suggesting a cell-type independent effect, while the treatment of TNF-α had almost no effect on the viability of those cells (Fig. 4E).

3.4. PRRSV induces TNF-α secretion through the NF-κB signaling pathway

Previous study has demonstrated that PRRSV infection could activate the NF-κB signaling pathway, which is the main pathway controlling the expression of pro-inflammatory cytokines in MARC-145 and PAMs (Luo et al., 2011). To further test whether PRRSV-induced production of TNF-α depends on NF-κB activation, we examined the NF-κB subunit P65 and its phosphorylation status (p-P65) in various infection groups. Cells treated with lipopolysaccharides (LPS) were used as the positive control. The results showed that the level of p-P65 in the JXwn06/CSFV-C group was significantly enhanced at both 12 and 24 hpi compared with CSFV-C group (Fig. 5A). However, no significant difference of the P65 level was observed among the groups. In addition, in the JXwn06/CSFV-C group, a higher level of P65 was translocated into the nucleus at both 12 and 24 hpi (Fig. 5B). To further understand whether the translocation of P65 is the key to induce the overexpression of pro-inflammatory cytokines, we designed siRNAs (siRNA-P65-848 and siRNA-P65-1042) to silence the expression of P65. SiNC was used as negative control. The Western blot results showed that siRNA-P65-848 and siRNA-P65-1042 could efficiently reduce the expression of P65 (Fig. 5C). In the RNAi transfection/infection assay, we found that the siRNAs against P65 significantly inhibited the expression of TNF-α induced by JXwn06/CSFV-C infection (Fig. 5F), and JXwn06 could no longer inhibit the replication of CSFV-C (Fig. 5D and E). To further test the effect of NF-κB signaling on the CSFV-C replication, we overexpressed P65 in PAM39 cells by transient transfection of pEGFP-N1-P65 (Fig. 5G), which was generated by cloning P65 gene into the pEGFP-N1 vector. The result showed that overexpression of P65 significantly inhibited the replication of CSFV-C and induced the secretion of TNF-α (Fig. 5H and I). It indicates that NF-κB signaling plays an important role in controlling CSFV-C replication.

3.5. Overexpression of P65 can inhibit the replication of CSFV-C in other cell types

We also overexpressed P65 in other cell lines including ST, SK-6, and PK-15 by transfection. The protein expression was confirmed by Western blot (Fig. 6A) and green fluorescence (Fig. 6B). Similarly, we found that the overexpression of P65 in all three cell lines significantly increased the secretion of TNF-α (Fig. 6D, F, and H) and inhibited the replication of CSFV-C (Fig. 6C, E, and G). These results suggest that NF-κB signaling regulates CSFV-C replication in a cell type-independent manner.

4. Discussion

Over the past half a century, vaccination with CSFV-C has been a

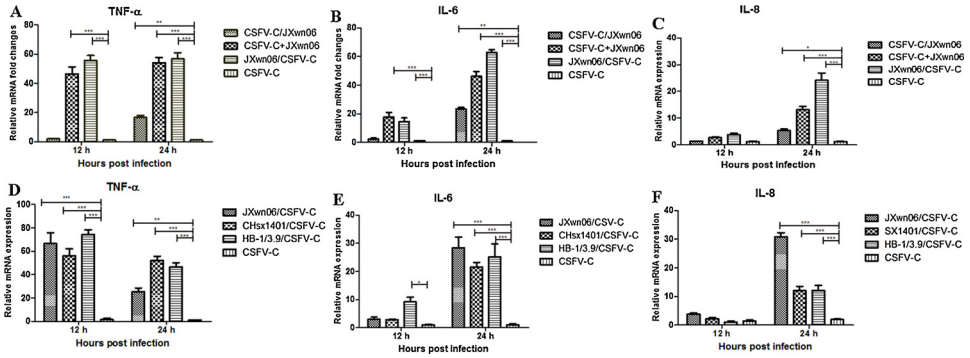

Fig. 3. Pro-inflammatory cytokine responses in the PAM39 cells co-infected with PRRSV and CSFV-C. PAM39 cells were infected with PRRSV and CSFV-C, and the expression of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-8 were examined by qRT-PCR at the indicated time points. The fold change was calculated using the $2^{-\triangle\triangle Ct}$ method. (mean ± SD; n = 3; ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. *P*-values were calculated using a two-way ANOVA test).

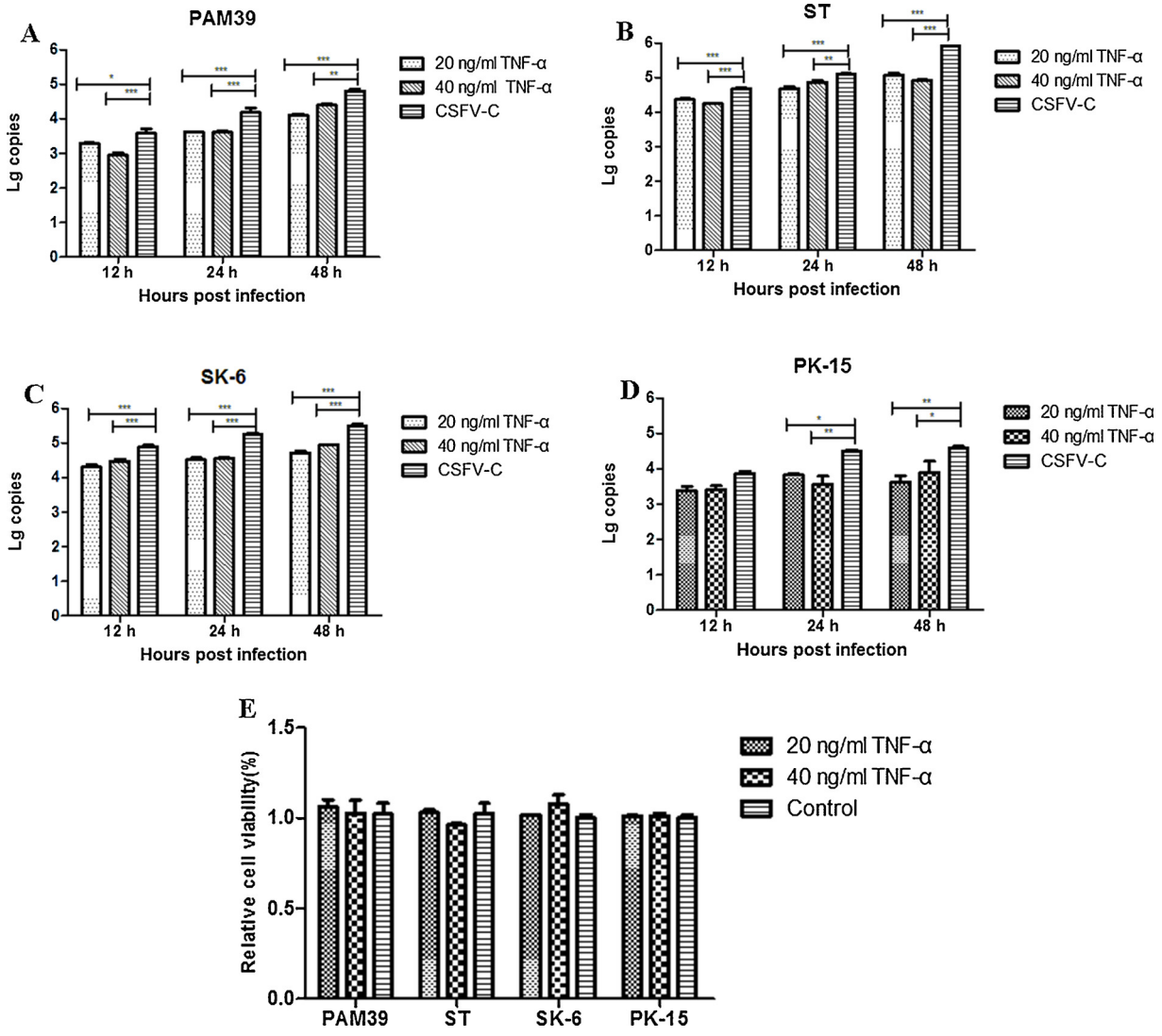

Fig. 4. TNF-α treatment inhibited the replication of CSFV-C *in vitro*. The effect of TNF-α on CSFV-C RNA copies in CSFV-infected PAM39 (A), ST (B), SK-6 (C), and PK-15 (D) cells. Various cells were treated with 20 ng/ml or 40 ng/ml of TNF-α for 12 h, followed by the infection of CSFV-C (MOI = 0.1). At 12, 24, and 48 hpi, the levels of CSFV RNA were measured using qRT-PCR (mean ± SD; n = 3 * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, *P*-values were calculated using a two-way ANOVA test). **(E) Viability of cells treated with TNF-α.** Various cells were treated with 20 ng/ml or 40 ng/ml of TNF-α for 12 h, and the viability of various cells was analyzed using the $2^{-\triangle\triangle Ct}$ method. (mean ± SD; n = 3; ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. *P*-values were calculated using a two-way ANOVA test).

common practice in intensive swine farms of epidemic areas (Qiu et al., 2005). CSFV-C mainly replicates in the secondary lymphoid tissues within immunized pigs, especially in the tonsils for 2–3 weeks, lasting up to 98 days post-vaccination (Tignon et al., 2010). Continuing CSFV replication in vaccinated pigs may produce sufficient amount of stimuli to activate the pig adaptive immune system, thus providing full protection against various CSFV infections in the field. In addition, vaccinated pigs rarely shed virus to the environment or contact pigs in secretions and excretions (Kojnok et al., 1980). The CSFV-C exists as a very safe and highly efficacious vaccine for decades. However, many

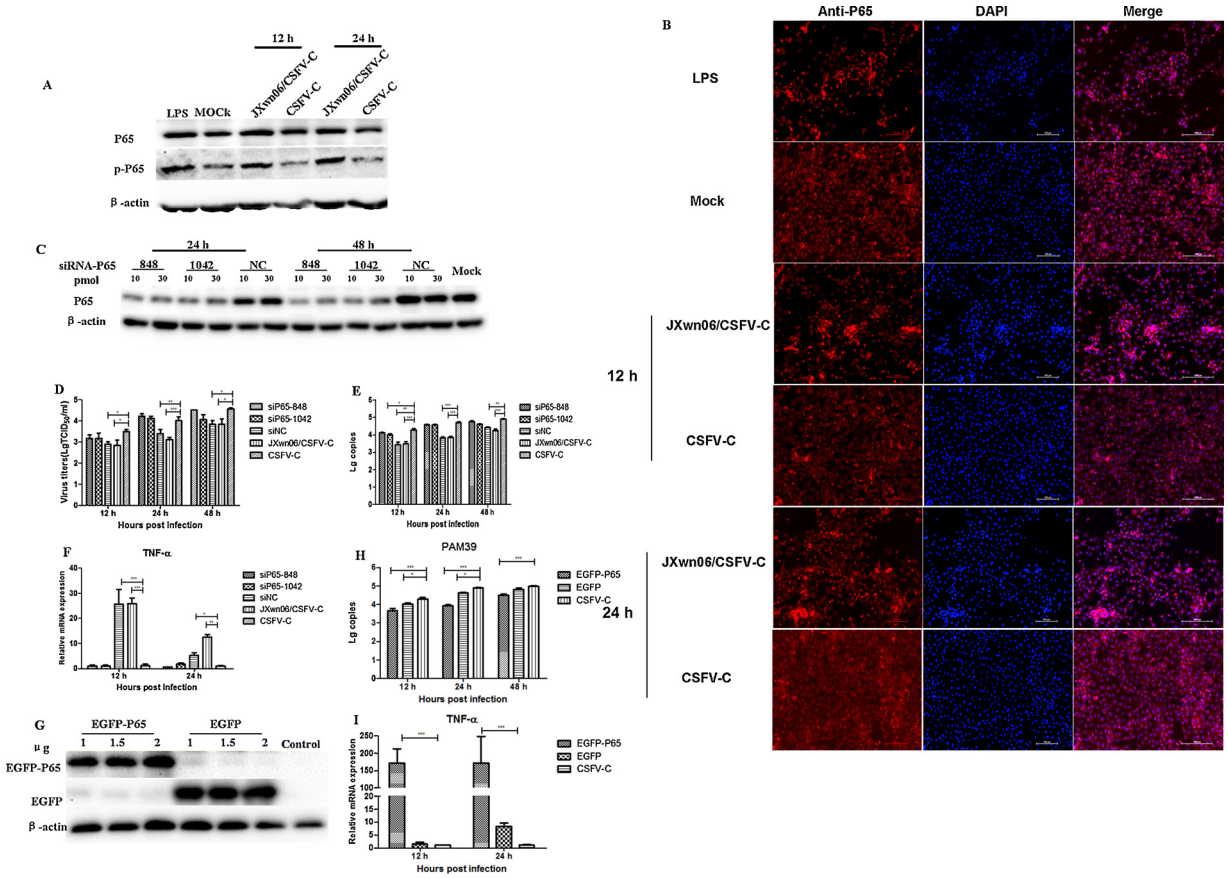

Fig. 5. PRRSV-induced pro-inflammatory cytokines inhibit CSFV-C replication through the NF-κB pathway. (A) Detection of p-P65 and P65 expression in PAM39 cells by Western blot. PAM39 cells were infected with 0.1 MOI PRRSV JXwn06/CSFV-C or CSFV-C alone and cells were collected at indicated time points to detect the expression of p-P65 and P65. LPS treatment (50 ng/ml) and Mock were used as positive and negative control, respectively. **(B) Detection of P65 expression in PAM39 cells by IFA (40 × magnification).** PAM39 cells were infected with 0.1 MOI JXwn06/CSFV-C or CSFV-C alone, and cells were fixed at indicated time points. **(C) P65 knockdown by siRNA 848 and siRNA 1042.** PAM39 cells transfected with 10 nM or 30 nM of siRNA 848, siRNA1042 or a non-targeting control (siNC) for 24 h, and then the expression of P65 was detected by Western blot. **(D and E) Determination of CSFV-C virus titers and RNA copies after siRNA treatment.** PAM39 cells were infected with JXwn06/CSFV-C after silence of P65 for 24 h, and then CSFV-C virus titers and virus copies were detected by TCID50 (C) and qRT-PCR (D) in various groups, respectively. **(F) TNF-α detection after the silence of P65 in various groups. (G) Overexpression of P65 in PAM39 cells.** PAM39 cells were transfected by pEGFP-N1-P65 or pEGFP-N1 vector for 24 h and P65 was detected by Western blot. **(H) The CSFV-C RNA copies in P65 overexpressed cells.** PAM39 cells were transfected by pEGFP-N1-P65 and pEGFP-N1 vector for 24 h and then infected with CSFV-C, and CSFV-C copies were detected by qRT-PCR at indicated time points. **(I) TNF-α detection in P65 overexpressed cells.**

factors contribute to the vaccination failure, such as maternal antibodies and immunosuppressive pathogens (e.g., PRRSV, PCV2, etc.). For the interplay with PCV2, a previous study has shown that CSFV does not affect the production of PCV2, but PCV2 can reduce CSFV replication in a dose-dependent manner in PK-15 cells (Zhou et al., 2015a, b). In this study, we established the PAM39 cell line that could support the infection and replication of both PRRSV and CSFV-C. With this cell line available, we showed that PRRSV infections prior to CSFV-C could significantly interfere with CSFV-C replication.

Inflammation plays an important role in combating infection or tissues damage and is a critical component of host innate immunity and adaptive immunity. To survive *in vivo*, pathogens always inhibit the activation of the host inflammation responses. Previous *in vivo* study has shown that, afollowing CSFV-C vaccination, some inflammatory cytokines, such as IL-1 and TNF-α, become downregulated in the blood, and IL-6 and IL-8 levels are not upregulated until 5 days post-vaccination (Graham et al., 2010; Xu et al., 2018). *In vitro*, although CSFV Shimen-strain and C-strain can upregulate the expression of IL-β and IL-8, the C-strain does not affect the production of TNF-α, even down-regulate the expression of IL-6 in porcine monocyte-derived macrophages (Cao et al., 2015). In addition, pro-inflammatory cytokine TNF-α activated by the NF-κB signaling pathway can significantly suppress the replication of CSFV in PK-15 cells (Li et al., 2015). During acute infection of high pathogenic PPRSV (HP-PRRSV), the production of pro-inflammatory cytokines (TNF-α, IL-6, and IL-8) in the lungs, serum, or even in infected-microglia is intensively elevated (Liu et al., 2010; Lunney et al., 2016; Senthilkumar et al., 2019; Wang et al., 2011). It has also been documented that PRRSV infection also make the pigs more susceptible to secondary infections by other pathogens such as porcine circovirus type 2 (PCV2), swine influenza virus (SIV), *Streptococcus suis*, *Haemophilus parasuis*, and *Mycoplasma hyopneumoniae* (Zhao et al., 2009; Han et al., 2017). Many of these secondary infection pathogens can induce inflammatory responses and may enhance PRRSV infection-mediated inflammatory responses (Li et al., 2017). In our present study, we found that infection with PRRSV strains of different virulence (JXwn06, HB-1/3.9, and CHsx1401) prior to CSFV-C infection could significantly upregulate the expression of pro-inflammatory cytokines including IL-6, IL-8, and TNF-α in PAM39 cells. This result consists with previous findings that both HP-PRRSV and low pathogenic PRRSV (LP-PRRSV) enhance the production of pro-inflammatory cytokines including IL-6 and TNF-α in serum and PAMs (Zhang et al., 2013). Among the pro-inflammatory cytokines, TNF-α is considered as one of the key pro-inflammatory cytokines involved in the innate immune response, and mediates the release of various cytokines, such as IL-6, IL-8, and IL-

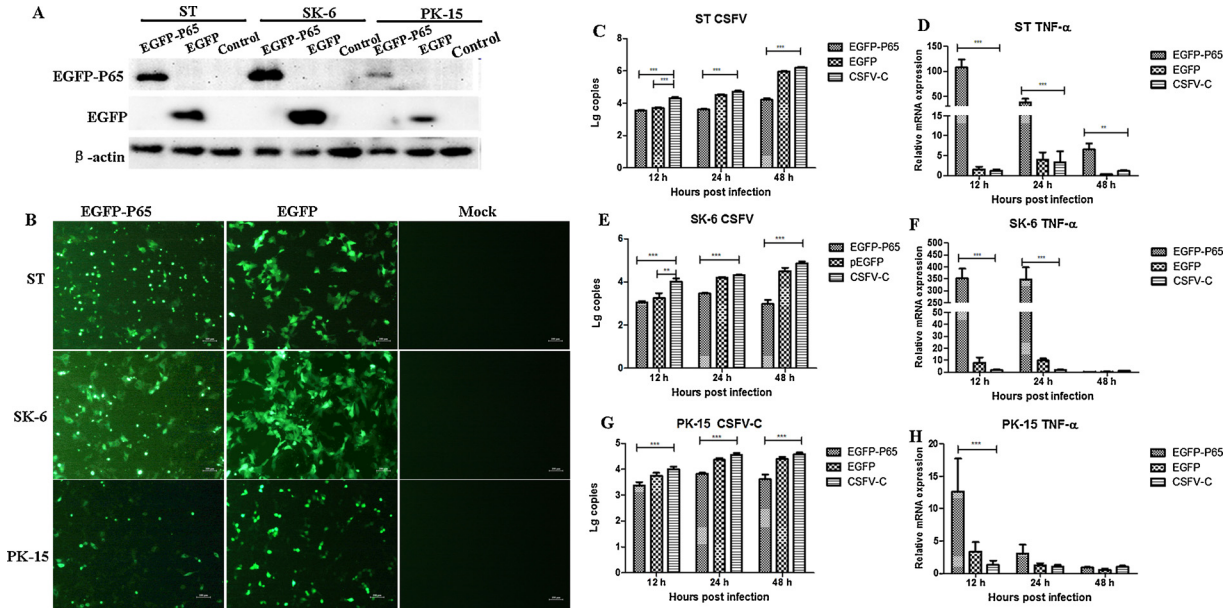

Fig. 6. Overexpression of P65 inhibited CSFV-C replication in various cells. ST, SK-6, and PK-15 cells were transfected with pEGFP-N1-P65 or pEGFP-N1 vector for 24 h. The expression levels of EGFP-P65 or EGFP were detected by **(A) Western blot and (B) IFA (40 × magnification).** Then, various cells were infected with CSFV-C, and CSFV-C RNA copies and TNF-α levels were detected by qRT-PCR in ST **(C and D)**, SK-6 **(E and F),** and PK-15 **(G and H)** cells.

1β by stimulated macrophages (Yamagishi et al., 2000). We also found that the addition of TNF-α could significantly reduce the replication of CSFV-C in various cell types.

NF-κB is a key player in regulating the expression of many pro-inflammatory molecules. Previous study showed that, in infected PK-15 cells, CSFV does not activate the NF-κB signaling pathway (Chen et al., 2012), and CSFV NS5A can inhibit the secretion of inflammatory cytokines induced by poly (I:C) through the suppression of the NF-κB signaling pathway (Dong and Tang, 2016). In contrast, PRRSV infection can induce the activation of NF-κB (Wang et al., 2013). Specifically, PRRSV N and Nsp2 proteins can activate the NF-κB signaling pathway and promote the expression of pro-inflammatory cytokines IL-6 and IL-8 (Fang et al., 2012). In this study, we found that the activation of the NF-κB signaling pathway could affect the replication of CSFV in various cells (PAM39, ST, SK-6, and PK-15). However, CSFV-C replication is limited to lymphoid tissues, and occasionally in other tissue such as kidney *in vivo* (Lorena et al., 2001), whereas PRRSV mainly replicate in PAMs and can persist in secondary lymphoid tissues (Hu et al., 2013). Whether CSFV-C replication *in vivo* can be influenced through the same manner we observed *in vitro* needs further investigation. Additionally, macrophages is only one of primary forms of the immune system *in vivo*, how does PRRSV infection affect the immune response and the replication of CSFV in the host also warrant further study.

Together, our results show that PRRSV infection-induced pro-inflammatory cytokine TNF-α can significantly inhibit the proliferation of CSFV, thus providing a possible scenario for CSFV vaccination failure caused by PRRSV infection in the field.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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