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RPS3-induced antiviral cytokines inhibit the proliferation of classical swine fever virus

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Summary. – Classical swine fever virus (CSFV) infection results in serious economic losses to the pig industry. This positive-sense RNA virus hijacks cell host proteins for its own replication. Although previous studies have shown that RPS3, a 40S ribosomal subunit protein, is mainly required for DNA repair, apoptosis and inflammation, the effect of RPS3 on CSFV replication remains uncertain. Thus, we investigated the potential role of RPS3 in CSFV infection in RPS3-knockdown and -overexpressing cell lines using real-time fluorescence quantitative PCR (RT-qPCR) and indirect immunofluorescence assays. Results showed that knockdown of RPS3 by lentiviruses enhanced CSFV replication, whereas overexpression of RPS3 by lentiviruses inhibited CSFV replication. These findings indicated the antiviral role of RPS3 in CSFV infection. Subsequent experiments revealed that CSFV replication was inhibited in cells cultured with the supernatants of RPS3-overexpressing cell, suggesting that the RPS3-mediated inflammatory response was involved in CSFV infection. Furthermore, enzyme-linked immunosorbent assay (ELISA) revealed that the secretion of antiviral cytokines (IL-8 and INF-β) was increased in cells with sufficient RPS3 expression but decreased in cells lacking RPS3 expression. RT-qPCR and immunofluorescence assays revealed that CSFV infection inhibited RPS3-mediated antiviral cytokine secretion. Taken together, these findings reveal that RPS3 is a novel antiviral factor that inhibits CSFV proliferation by increasing antiviral cytokine secretion.

Keywords: classical swine fever virus; ribosome protein S3; IL-8; INF-β

Introduction

Classical swine fever (CSF) is a viral infectious disease affecting pigs and is caused by classical swine fever virus (CSFV). CSF has high infectivity and lethality, causing serious economic losses and hindering the healthy and sustainable development of the pork industry (Dreier *et al.,* 2007; Luo *et al*., 2014). CSFV belongs to the family *Flaviviridae* and the genus *Pestivirus* (Ji *et al*., 2015). It is an enveloped virus with a 12.3 kb single-stranded RNA genome, including a single open reading frame flanked by 5' and 3' untranslated regions. The open reading frame of CSFV can be translated into a polyprotein precursor composed of 3898 amino acid residues. This precursor is gradually cleaved by cellular and virus-specific proteases into four structural proteins and eight nonstructural proteins (Luo *et al*., 2017; Yu *et al*., 2019).

The ribosome is a complex ribonucleoprotein apparatus consisting of a large and a small subunit, the latter of which mediates the interactions between mRNA and tRNAs (Wilson and Doudna Cate, 2012). Certain ribosomal proteins are involved in other extraribosomal functions (Naora 1999), such as apoptosis (Naora *et al*., 1998), transcriptional regulation (Wan *et al*., 2007; Wier *et al*., 2012; Pham *et al*., 2013) and DNA repair (Kim and Linn, 1989; Grabowski *et al*., 1991; Wilson *et al*., 1994; Kim *et al*., 1995; Yoon *et al*., 2002; Jung *et al*., 2003). RPS3, a 40S ribosomal subunit protein, is mainly required for DNA repair (Jang *et al*., 2004; Lee *et al*., 2010; Kim *et al*., 2013), apoptosis (Lee *et al*., 2010) and transcriptional regulation (Wan *et al*., 2007; Wier *et al*., 2012; Pham *et al*., 2013). Previous studies have shown that RPS3 has two independent domains located in its C-terminal and N-terminal regions (Kuo *et al*.,

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Abbreviations: CSFV = classical swine fever virus; ELISA = enzyme-linked immunosorbent assay; NCshRNA = negative control shRNA; RT-qPCR = real-time fluorescence quantitative PCR; SD = standard deviation; shRNA = short hairpin RNA

1992; Thayer *et al*., 1995; Yacoub *et al*., 1996; Jang *et al*., 2004). The conserved 15–26 amino acid sequence of the RPS3 N-terminus is critical for apoptosis (Jang *et al*., 2004). Studies have also shown that the overexpression of RPS3 can enhance caspase-3 and caspase-8 activation, suggesting that RPS3 acts upstream of the caspase pathway (Lee *et al*., 2010). Additionally, the conserved 96–178 amino acid sequence in the C-terminal region of RPS3 is required for DNA repair (Kuo *et al*., 1992; Thayer *et al*., 1995; Yacoub *et al*., 1996). Furthermore, the interaction between RPS3 and p65 is essential for the activation of nuclear transcription factor kappa B (NF-κB) signaling, as it enhances IL-2 and IL-8 production (Naora *et al*., 1998). RPS3 is hijacked by certain viruses and bacteria to inhibit the immune response and suppress host cell proliferation to promote its own replication. For example, human immunodeficiency virus type 1 (HIV-1) Tat protein interacts with RPS3 to disrupt spindle formation, thereby preventing cell proliferation (Kim and Kim, 2018). Metalloprotease NleC suppresses host NF-κB signaling-mediated immune responses by interfering with the interaction between p65 and RPS3 in enteropathogenic *Escherichia coli* infection (Andrea *et al*., 2015). Moreover, our previous study has shown that the NS4B protein of CSFV binds to RPS3 (Lv *et al*., 2018). However, the precise role of RPS3 in CSFV infection remains unclear.

Therefore, in this study, we aimed to explore the antiviral role of RPS3 in CSFV infection using both RPS3-knockdown and -overexpressing cell lines. Our results might be valuable in establishing RPS3 as a potential target for anti-CSFV treatment.

Materials and Methods

Cells and virus. Porcine alveolar macrophages (PAMs; ATCC; CRL-2845), and human embryonic kidney (HEK293T) cells were maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) or high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco), both supplemented with 10% fetal bovine serum (Cat # 10099; Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Cat # V900929; Sigma-Aldrich, St. Louis, MI, USA), in a 5% CO_2 incubator at 37°C. The CSFV Shimen strain was obtained from the China Institute of Veterinary Drug Control (Beijing, China) and propagated in PAM cells. Experiments involving CSFV were standardized according to the Laboratory Biosafety Manual and strictly performed in a P3 biosafety laboratory.

Construction and transfection of plasmids. RPS3 amplification primers were designed based on the published porcine RPS3 gene sequence (NM_001044601.1). The RPS3 gene was amplified from the cDNA by PCR and cloned into pCDH-CMV-MCSEF1-GFP-Puro to obtain the CMV-RPS3 plasmid. Three pairs of short hairpin RNA (shRNA) sequences targeting the porcine RPS3 gene and one negative control sequence were designed (http://rnaidesigner.thermofisher.com/rnaiexpress) and then synthesized by Beijing Aoke Dingsheng Biotechnology Co., Ltd (Beijing, China). After annealing, interference sequences were inserted into the pCDH-U6-GreenPuro interference vector to generate three different shRNA sequences against RPS3 (shRPS3-1, shRPS3-2, shRPS3-3) and a negative control shRNA (NCshRNA).

Cell line production. HEK293T cells in the logarithmic phase were seeded into 6-well plates prior to lentivirus packaging and cultured in an incubator at 37°C and 5% CO_2 for 12 h. Subsequently, 0.67 μg each of pGag/Pol, pRev, and pVSV-G as well as 2 μg of the overexpression or knockdown lentivectors plasmids (CMV, CMV-RPS3; shRPS3-1, shRPS3-2, shRPS3-3, and NCshRNA) were co-transfected into HEK293T cells using Turbofect Transfection Reagent (Cat # R0531; Thermo Fisher Scientific, Waltham, MA, USA) when the cell density reached approximately 85%. Following 16 h of incubation, the culture medium was discarded and replaced with advanced DMEM containing 20 ml/l fetal bovine serum, 0.01 mmol/l cholesterol (Cat # C8667; Sigma-Aldrich), 0.01 mmol/l L-α-phosphatidylcholine (Cat # P443; Sigma-Aldrich), chemically defined lipids (1:1000 dilution, Cat # 11905031; Invitrogen, Carlsbad, CA, USA), and 4 mmol/l L-glutamine (Cat # G7513; Sigma-Aldrich). After further incubation for 48 h, the cell supernatants were collected and centrifuged at 1,500 rpm for 10 min to remove cell debris. The supernatants were considered as lentiviral cultures. Lentivirus titers were tested via a median tissue culture infectious dose ($TCID_{50}$) assay in HEK-293 T cells (Li *et al*., 2019). Lentiviral cultures (multiplicity of infection (MOI), 1) and 6 μg/ml polybrene were used to infect PAM cells. Following a 12-h infection period, PAM cells were cultured in fresh medium supplemented with 5 mg/ml puromycin (Cat # P8833; Sigma-Aldrich) to screen for stable cell lines. The negative control vectors (CMV and NCshRNA) were used as controls and processed similarly.

Western blotting analysis. Cells were harvested and lysed using RIPA buffer containing phenylmethanesulfonyl fluoride (PMSF) (Cat # P0013B; Beyotime, Haimen, China). Protein concentration was measured using a bicinchoninic acid (BCA) Protein Assay Kit (Cat # ab102536; Abcam, Cambridge, UK). Protein samples were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Cat # ISEQ00010; Merck Millipore, Burlington, MA, USA). After blocking with 5% milk for 2 h at room temperature, the membranes were incubated with the primary antibodies: rabbit anti-RPS3 monoclonal antibody (1:2,000 dilution, Cat # AF1045; Beyotime) or mouse anti-β-actin monoclonal antibody (1:2,000, Cat # AF5001; Beyotime) at 4°C overnight. The membranes were then incubated with horseradish peroxidase goat anti-mouse IgG (Cat # SA00001-1; Proteintech, Rosemont, IL, USA) or goat anti-rabbit IgG (1:4,000, Cat # 10285-1-AP; Proteintech) second-

Table 1. Primers used in this study

Primers	Sequence (5'–3')	Application
CMV-RPS3-F CMV-RPS3-R	CGGAATTCGCCACCATGGATTACAAGGATGACGACGATAAGGCGGTGCAGATTTCCA CGGGATCCTCATTACTTATCGTCGTCATCCTTGTAATCTGCTGTGGGTACTGGC	Cloning
shRPS3-1-F shRPS3-1-R	GATCCGCAGAGTCTCTGCGATACAAACAAGAGTTTGTATCGCAGAGACTCTGCTTTTTG AATTCAAAAAGCAGAGTCTCTGCGATACAAACTCTTGTTTGTATCGCAGAGACTCTGCG	RNAi
shRPS3-2-F shRPS3-2-R	GATCCGACAGAGAGCTAAATCCATGACAAGAGTCATGGATTTAGCTCTCTGTCTTTTTG AATTCAAAAAGACAGAGAGCTAAATCCATGACTCTTGTCATGGATTTAGCTCTCTGTCG	RNAi
shRPS3-3-F shRPS3-3-R	GATCCGGATCAAGGTAAAGATCATGCCAAGAGGCATGATCTTTACCTTGATCCTTTTTG AATTCAAAAAGGATCAAGGTAAAGATCATGCCTCTTGGCATGATCTTTACCTTGATCCG	RNAi
NCshRNA-F NCshRNA-R	GATCCGCTTAAACGCATAGTAGGACTTCAAGAGAGTCCTACTATGCGTTTAAGCTTTTTG AATTCAAAAAGCTTAAACGCATAGTAGGACTCTCTTGAAGTCCTACTATGCGTTTAAGCG	RNAi
CSFV-F CSFV-R	GAGAAGGACAGCAGAACTAAGC TTACCGCCCATGCCAATAGG	RT-qPCR
β-actin-F β-actin-R	CAAGGACCTCTACGCCAACAC TGGAGGCGCGATGATCTT	RT-qPCR
IL-8-F IL-8-R	TTCGATGCCAGTGCATAAATA CTGTACAACCTTCTGCACCCA	RT-qPCR
INF-β-F INF-β-R	CGCTCTCCTGATGTGTTTCTCC CAAATTGCTGCTCCTTTGTTGG	RT-qPCR

ary antibody at room temperature for 2 h. Finally, the signal was determined using a horseradish peroxidase enhanced chemiluminescence analysis method. The relative expression levels of RPS3 were estimated using density readings of the gel bands, and the ratios were calculated relative to a β-actin control.

Virus titration by indirect immunofluorescence assay. PAM cells were seeded in 96-well plates and inoculated with infectious progeny viruses collected from culture supernatants. After 48 h, the cells were fixed with 1:1 stationary liquid (methanol: acetone) at 4°C for 20 min. Following three washes with phosphate-buffered saline (PBS), the fixed cells were permeabilized with 1% Triton X-100 for 10 min, washed three times with PBS, and treated with 5% skim milk for 2 h. Subsequently, the cells were incubated with mouse CSFV-positive serum (1:100) at 4°C for 12 h. Following three washes with PBS, the cells were incubated with rabbit anti-pig IgG (whole molecule)-FITC antibody (1:500, Cat # F1638; Sigma-Aldrich) for 1 h at room temperature. The fluorescence-positive wells were observed under a fluorescence inversion microscope (Nikon, Tokyo, Japan). The viral titers were determined as $TCID_{50}$/ml using the Reed and Muench method (Zhang *et al*., 2019).

Real-time quantitative PCR. RT-qPCR was conducted to detect the expression of IL-8 mRNA, INF-β mRNA and CSFV RNA using specific primers. The primer sequences used in this study are shown in Table 1. Total cellular RNA was extracted using a TRIzol Reagent (Cat # 15596018; Invitrogen) and quantified using a NanoDrop 10000 spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized using the Evo M-MLV RT for PCR Kit (Cat # AG11604; Accurate Biotechnology). The mRNA expression was estimated using the TB Green™ Premix Ex Taq™ II (Cat # RR820A; Takara Bio, Shiga, Japan) according to the manufacturer's protocol on a CFX Connect Real-Time PCR system (Bio-Rad, Hercules, CA, USA). The data were analyzed using the comparative threshold (Ct) method. Relative fold changes in gene expression were normalized against β-actin- expression.

Enzyme-linked immunosorbent assay (ELISA). IL-8 and INF-β protein levels were measured using an ELISA kit (SEM366Hu, Cat # SEA080Po; Wuhan USCN Business, Wuhan, China) according to the manufacturer's instructions. Briefly, cell supernatants were collected at the indicated time points, and centrifuged at 1000 rpm for 5 min at 4°C to remove cell debris. Next, 100 µl of detection antibody labeled with horseradish peroxidase was added to standard wells and sample wells, which were subsequently incubated for 1 h at 37°C. Subsequently, 50 µl substrate A and 50 µl substrate B were added to each well, followed by incubation at 37°C for 15 min. Finally, stop solution was added, and the optical density value was measured at 450 nm using the SpectraMax M5 Microplate Reader (Molecular Devices, San Francisco, CA, USA).

Statistical analysis. Data were analyzed by t-test using the GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). All experiments were performed at least three times, and the results were expressed as the mean ± standard deviation (SD). P-values <0.05 were considered significant.

Results

Knockdown of RPS3 enhances CSFV production

To investigate the role of endogenous RPS3 in CSFV replication, three dshRNAs against RPS3 (shRPS3-1, shRPS3-2,

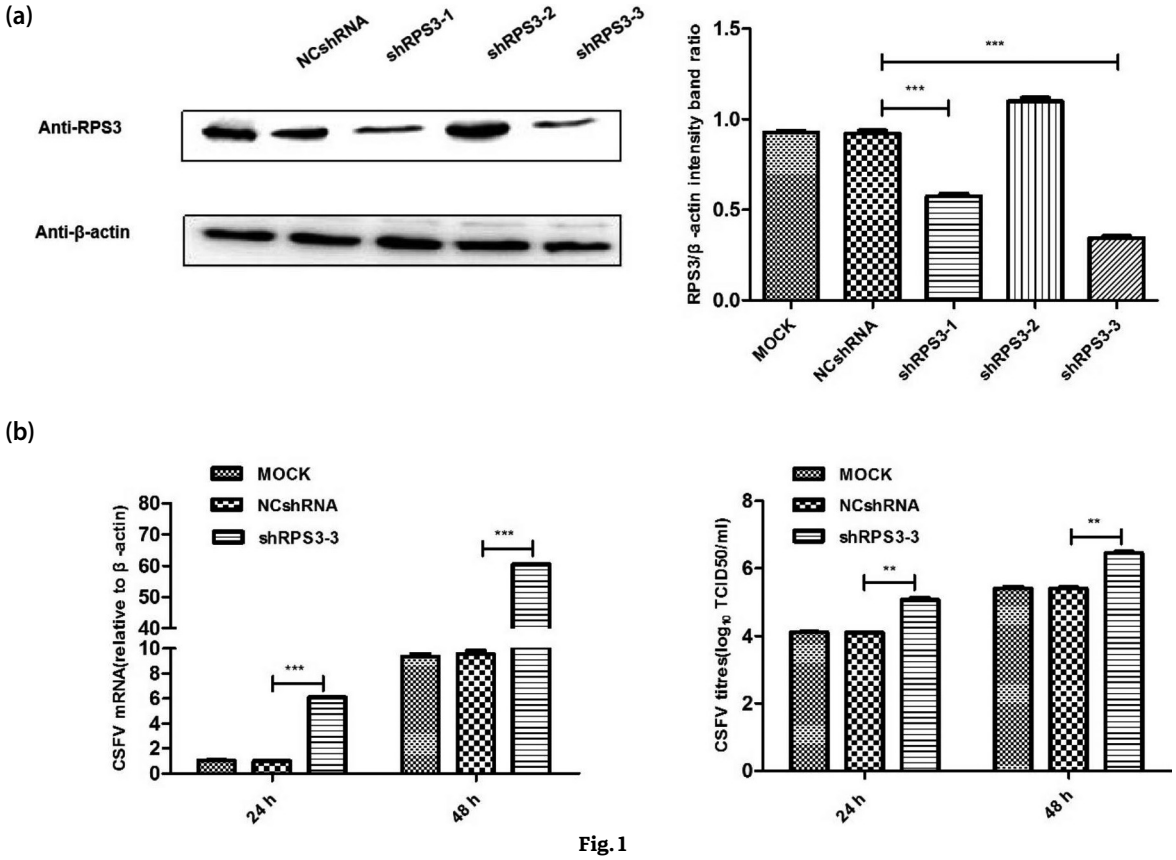

Fig. 1

Knockdown of RPS3 mediated by shRNA enhances CSFV replication in PAM cells

(a) Western blotting analysis of RPS3 protein levels in RPS3 knockdown cell lines using RPS3 antibody. β-actin served as an internal control. **(b)** qRT-PCR and $TCID_{50}$ assays of CSFV RNA levels and viral titers in mock cells, NTshRNA cells, and shRPS3-3 cells and cell supernatants. The cells were seeded in 6-well plates and infected with CSFV (MOI = 1). After infection for 24 and 48 h, the cells and cell supernatants were collected for viral RNA quantification and viral titration using qRT-PCR and $TCID_{50}$ assays, respectively.

shRPS3-3) and a negative control shRNA (NCshRNA) were designed and separately transduced into PAM cells via recombinant lentivirus to obtain stable RPS3-silenced cell lines. As shown in Fig. 1a, RPS3 protein expression levels were significantly reduced in shRPS3-1 and shRPS3-3 cell lines, with shRPS3-3 showing the highest silencing efficiency. Additionally, mock cells, NCshRNA cells and shRPS3-3 cells were infected with CSFV at the MOI of 1. After infection for 24 and 48 h, the cells and cell supernatants were separately collected for CSFV RNA quantification and viral titration. Compared with those in NCshRNA cells, CSFV RNA copy numbers and virus titers in shRPS3-3 cells were significantly increased (Fig. 1b). These findings indicated that CSFV replication was enhanced via knockdown of RPS3.

Overexpression of RPS3 inhibits CSFV proliferation

To further elucidate the effects of RPS3 on CSFV proliferation, a cell line that stably overexpressed RPS3 (CMV-RPS3 cells) and a negative control (CMV cells) were generated using recombinant lentiviruses. RPS3 protein expression levels were determined via western blotting analysis (Fig. 2a). Subsequently, mock cells, CMV cells and CMV- RPS3 cells were infected with CSFV (MOI = 1). Along with increased in RPS3 expression, CSFV RNA copy numbers were significantly decreased in CMV-RPS3 cells compared with those in CMV cells (Fig. 2b). Moreover, the number of infectious particles in the supernatants of CMV-RPS3 cells was significantly reduced (Fig. 2b). These results suggested that CSFV replication was restricted by the overexpression of RPS3.

Given the positive role of RPS3 in the antiviral inflammatory cytokine secretion pathway, we incubated CSFV-infected PAM cells with supernatants of RPS3-overexpressing cells to examine the mechanism, by which RPS3 overexpression inhibits CSFV replication. After incubation for 24 and 48 h, the cells and cell supernatants were separately collected for CSFV RNA quantification and viral titration. The results showed that CSFV RNA

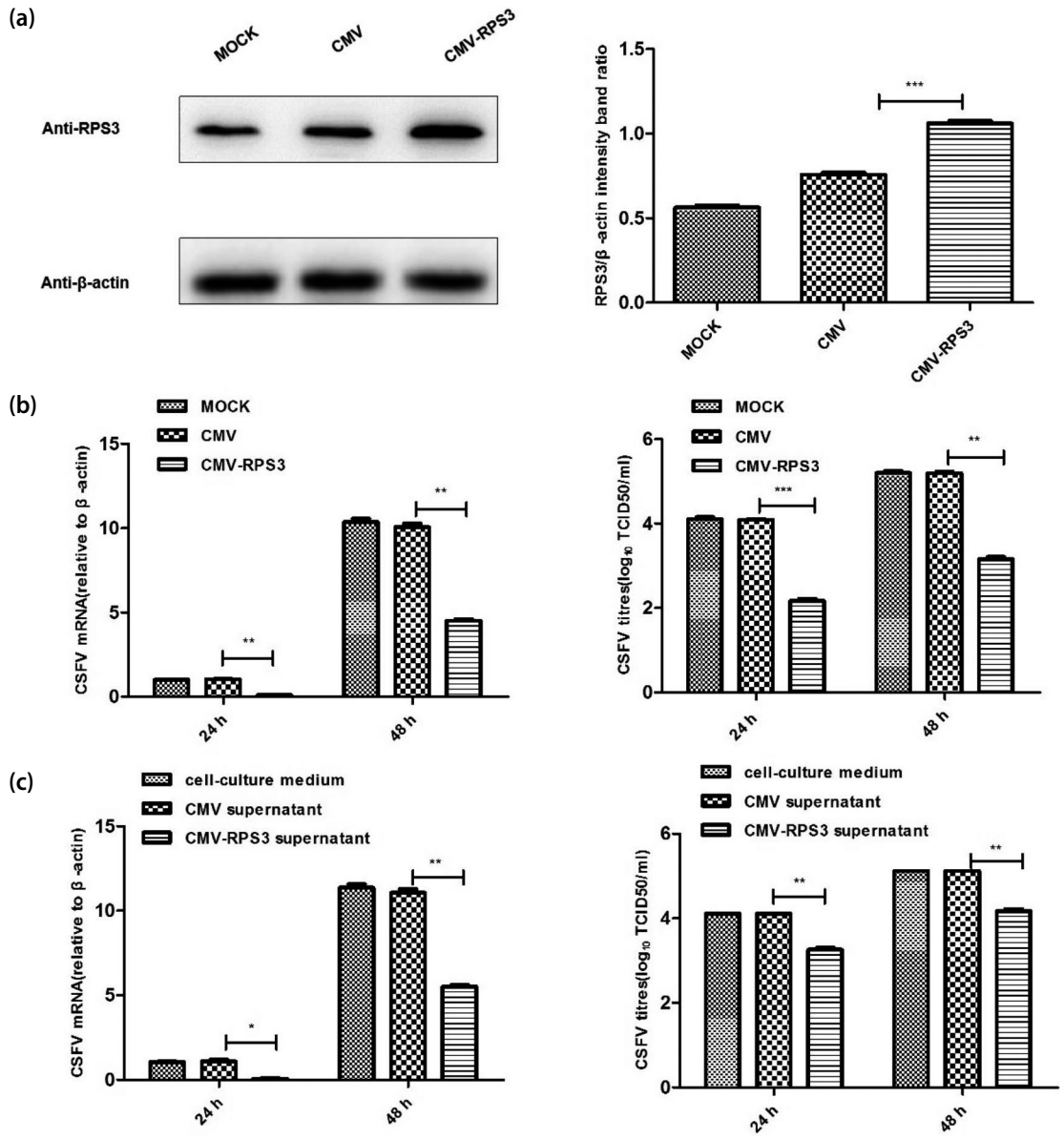

Fig. 2

Overexpression of RPS3 inhibits CSFV replication in PAM cells

(a) Western blotting analysis of RPS3 protein levels in RPS3 knockdown cell lines using RPS3 antibody. β-actin served as an internal control. **(b)** qRT-PCR and $TCID_{50}$ assays of CSFV RNA levels and viral titers in mock cells, CMV cells, and CMV-RPS3 cells and cell supernatants. The cells were seeded in 6-well plates, and infected with CSFV (MOI = 1). After infection for 24 and 48 h, the cells and cell supernatants were collected for viral RNA quantification and viral titration using qRT-PCR and $TCID_{50}$ assays. **(c)** qRT-PCR and TCID50 assays of CSFV RNA levels and viral titers in cells and cell supernatants. CSFV-infected PAM cells were separately cultured with the cell supernatants of CMV cells or CMV-RPS3 cells. After incubation for 24 and 48 h, the cells and cell supernatants were collected for viral RNA quantification and viral titration using qRT-PCR and $TCID_{50}$ assays, respectively.

copy numbers were significantly decreased in PAM cells cultured with CMV-RPS3 cell supernatants compared with those in PAM cells incubated with CMV cell supernatants. Moreover, the number of infectious particles in the supernatants of PAM cells cultured with CMV-RPS3 cell supernatants was significantly reduced (Fig. 2c). Taken together, these results revealed that the host factor RPS3 played an antiviral role in CSFV infection, indicating that the RPS3-mediated secretion of inflammatory cytokines might be involved in CSFV replication.

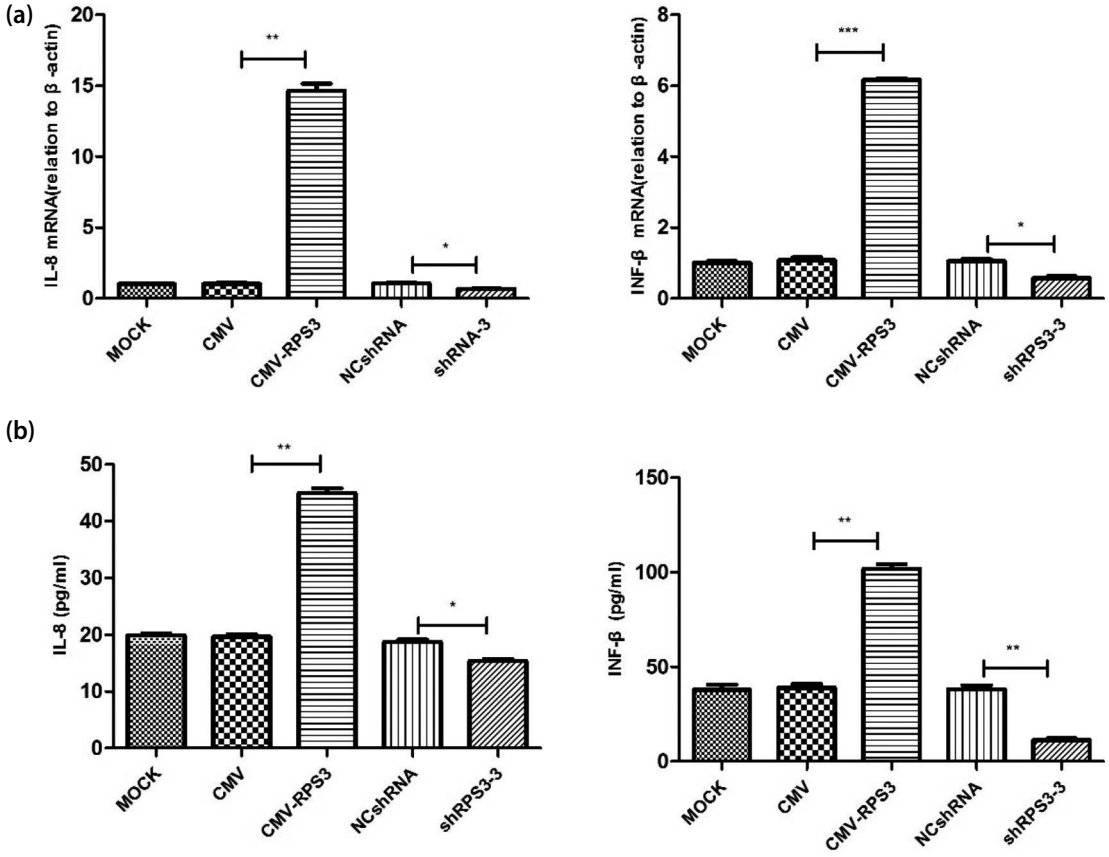

Fig. 3

RPS3 mediates IL-8 and INF-β secretion in PAM cells

(a, b) qRT-PCR and ELISA analyses of IL-8 and INF-β mRNA and protein levels in cells and cell supernatants. MOCK cells, CMV cells, CMV-RPS3 cells, NCshRNA cells and shRPS3-3 cells were seeded in 6-well plates. The cells and cell supernatants were collected at indicated time points for detection of mRNA and protein expression of IL-8 and INF-β using qRT-PCR and ELISA assays.

RPS3 enhances the expression of IL-8 and INF-β in PAM cells

Given the inhibitory effects of RPS3 and RPS3-overexpressing cell supernatants on CSFV proliferation, we examined the effect of RPS3 on expression of the inflammatory cytokines IL-8 and INF-β in PAM cells. PAM cells, CMV cells and CMV-RPS3 cells were seeded in 6-well plates and cultured for 24 h. Next, the cells and cell supernatants were collected, and the expression of IL-8 and INF-β was quantified using RT-qPCR and ELISA. RPS3 overexpression induced IL-8 and INF-β mRNA expression in the cells and promoted IL-8 and INF-β protein expression in the cell supernatants (Fig. 3a,b). These results suggested that RPS3 overexpression enhanced IL-8 and INF-β expression in PAM cells.

The effect of RPS3 silencing on IL-8 and INF-β expression was also detected in PAM cells. PAM cells, NCshRNA cells and shRPS3-3 cells were seeded in 6-well plates and cultured for 24 h. The cells and cell supernatants were then collected for examination of IL-8 and INF-β mRNA and protein expression. The results showed that knockdown of RPS3 inhibited IL-8 and INF-β mRNA expression in the cells, as well as IL-8 and INF-β protein expression in the cell supernatants (Fig. 3a,b). These findings suggested an RPS3-induced increase in IL-8 and INF-β expression in PAM cells.

CSFV inhibits RPS3-mediated IL-8 and INF-β secretion

Given the role of RPS3 in promoting the secretion of the antiviral factors IL-8 and INF-β, we examined the effect of CSFV on the RPS3-mediated expression of IL-8 and INF-β in PAM cells. CMV-RPS3 cells were seeded in 6-well plates and cultured with CSFV for 24 and 48 h. Western blotting analysis showed reduced RPS3 protein levels in the CSFV-infected RPS3-overexpressing cells at 24 and 48 h (Fig. 4a). The cells and cell supernatants were subsequently collected for detection of the mRNA and protein expression of IL-8 and INF-β using RT-qPCR and ELISA. The results showed that IL-8 and INF-β mRNA lev-

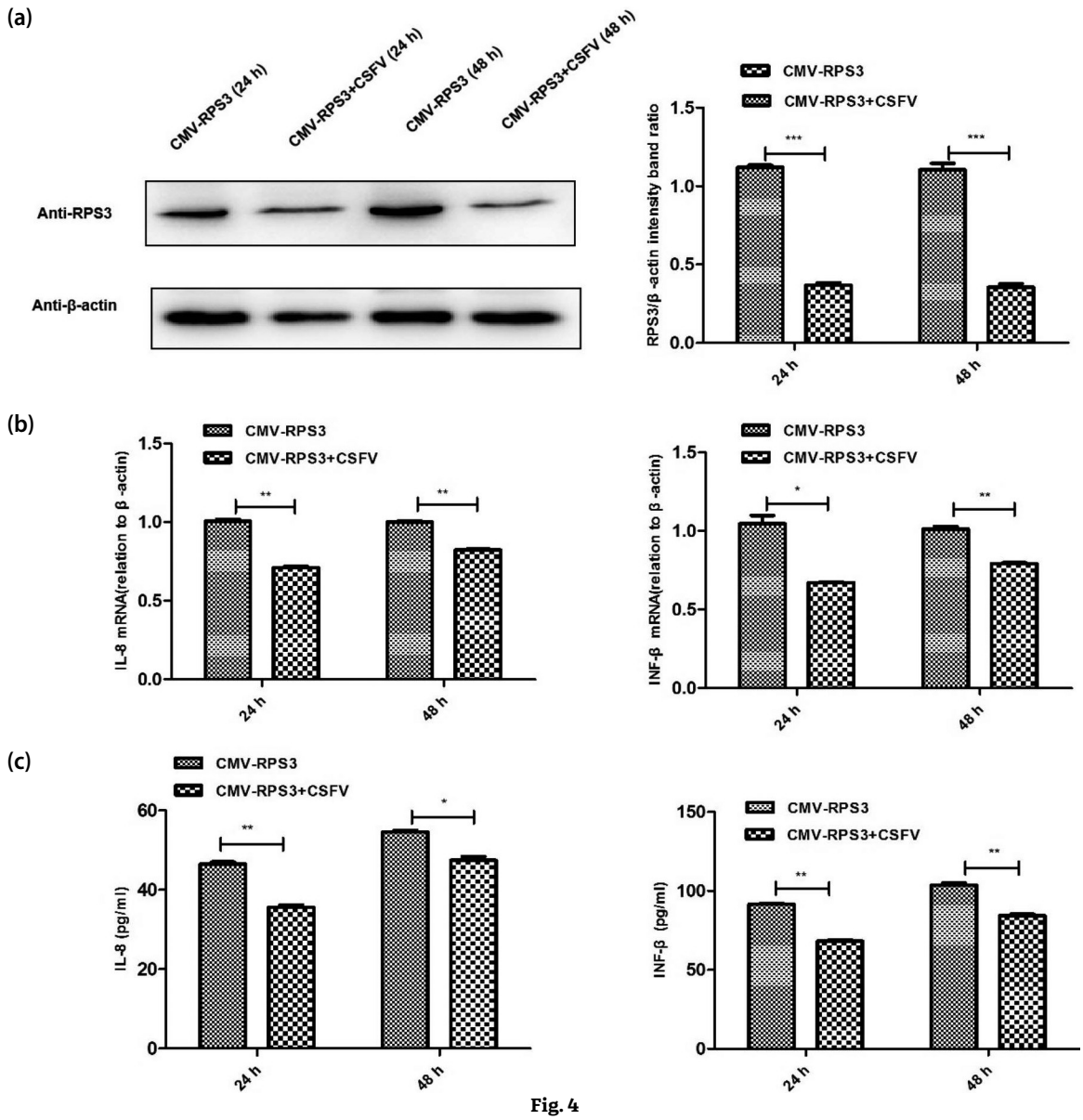

Fig. 4

CSFV inhibits RPS3-mediated IL-8 and INF-β protein expression

(a) Western blotting analysis of RPS3 protein levels in RPS3-overexpressing cells and CSFV-infected RPS3-overexpressing cells using RPS3 antibody. β-actin served as an internal control. **(b, c)** qRT-PCR and ELISA analyses of IL-8 and INF-β mRNA and protein levels in cells and cell supernatants. CMV-RPS3 cells were seeded in 6-well plates and infected without or with CSFV (MOI = 1). After infection for 24 and 48 h, the cells and cell supernatants were collected at indicated time points for detection of IL-8 and INF-β mRNA and protein expression using qRT-PCR and ELISA assays, respectively.

els in the CSFV-infected RPS3-overexpressing cells were markedly decreased compared with those in non-CSFV-infected RPS3-overexpressing cells (Fig. 4b). Similarly, we also observed reduced secretion of IL-8 and IFN-β in the supernatants of CSFV-infected RPS3-overexpressing cells at the indicated time points (Fig. 4c). These results suggest that CSFV inhibited the RPS3-induced expression of the inflammatory cytokines IL-8 and INF-β in PAM cells.

Discussion

The ribosome is a complex ribonucleoprotein apparatus consisting of a large and a small subunit. Ribosomal proteins, which typically serve as molecules that mediate the interactions between mRNAs and tRNAs, have been shown to be involved in other extraribosomal functions, including apoptosis, proliferation, inflammation, tumori-

genesis and transcriptional regulation, cell cycle, and DNA repair (Kim and Linn 1989; Grabowski *et al*., 1991; Wilson *et al*., 1994; Kim *et al*., 1995; Naora *et al*., 1998; Naora, 1999; Yoon *et al*., 2002; Jung *et al*., 2003; Wan *et al*., 2007; Wier *et al*., 2012; Wilson and Doudna Cate, 2012; Pham *et al*., 2013). In fact, the ribosomal proteins RPS25 and RPL19 are essential for the replication of certain viruses (Petrova *et al*., 2019). RPS3, which is mainly required for DNA repair, apoptosis, and transcriptional regulation (Kuo *et al*., 1992; Thayer *et al*., 1995; Yacoub *et al*., 1996; Jang *et al*., 2004; Wan *et al*., 2007; Lee *et al*., 2010; Wier *et al*., 2012; Kim *et al*., 2013; Pham *et al*., 2013) was recently found to enhance HIV replication (Kim and Kim, 2018). However, its functional involvement in CSFV infection has not been clearly defined. Hence, in this study, we investigated the potential role of RPS3 in CSFV infection in RPS3-knockdown and -overexpressing cell lines generated via recombinant lentivirus.

Our results showed that CSFV replication was inhibited in RPS3-overexpressing cells but was enhanced in RPS3-silencing cells, indicating the antiviral role of RPS3 in CSFV infection. Similarly, previous studies have confirmed that RPS3 is involved in the replication of West Nile virus and yellow fever virus, which are members of the *Flaviviridae* family (Petrova *et al*., 2019). Indeed, our previous studies, using a yeast two-hybrid test, showed that the NS4B protein of CSFV may interact with RPS3 (Lv *et al*., 2018). Therefore, the mechanism of action of RPS3 in CSFV infection is worth exploring.

RPS3, a key mediator of antiviral immunity, is a non-Rel subunit of NF-κB, which can regulate the secretion of immune factors, such as IL-8 and INF-β (Wan *et al*., 2007). Thus, we cultured CSFV-infected cells with the supernatants of RPS3-overexpressing cells. Results suggested that CSFV production was markedly decreased in the cells cultured with the supernatant of RPS3-overexpressing cells, indicating that the RPS3-mediated antiviral immunity may play a key role in the inhibitory activity of RPS3 on CSFV replication.

To further elucidate the underlying mechanism, we evaluated the expression of IL-8 and INF-β in RPS3-overexpressing or -silenced cells. Our findings revealed that the mRNA and protein expression of both antiviral factors was enhanced by RPS3 overexpression but reduced by RPS3 knockdown. This is consistent with a previous finding that the NF-κB-mediated host innate immune response plays an important role in controlling certain viral infections, including those caused by CSFV, hepatitis C virus, bovine viral diarrhea virus and other members of the *Flaviviridae* family (Fernanda *et al*., 2015; Li *et al*., 2018; Ling *et al*., 2018; Cao *et al*., 2019). Thus, our current results implied that RPS3 played a key role in the antiviral immunity of PAM cells.

To further determine whether RPS3-mediated IL-8 and INF-β secretion was involved in the immunity of PAM cells against CSFV replication, CSFV was detected in RPS3-overexpressing cells. We found that CSFV infection inhibits RPS3-induced IL-8 and INF-β secretion, indicating that RPS3-mediated secretion of antiviral cytokines was responsible for the inhibition of CSFV production. These results are consistent with previous studies showing that pathogenic *Escherichia coli* utilizes the RPS3-mediated inflammatory response to promote its own infection (Andrea *et al*., 2015).

A previous study confirmed that in PAM cells, CSFV Shimen strain infection led to the activation of MAPK signaling pathways but failed to activate NF-kB signaling pathways (Cao *et al*., 2015). Infection with this strain is also known to increase of IL-8 expression, not INF-β expression in PAM cells through MAPK signaling pathways (Cao *et al*., 2015). Moreover, CSFV infection has been shown to increase the serum levels of IL-8 in 6-month-old pigs (von Rosen *et al*., 2013). Furthermore, NS2, a nonstructural protein of CSFV, can activate NF-κB, which is responsible for the up-regulation of IL-8 expression (Tang *et al*., 2011). In another study, CSFV and its nonstructural protein, NS4A were shown to induce IL-8 production through the mitochondrial antiviral signaling pathway in swine umbilical vein endothelial cells (Dong *et al*., 2017). CSFV NS4B was found to inhibit poly (I:C) stimulation-mediated activation of the TLR3 signaling pathway in PAM cells, thereby suppressing TRIF mRNA transcription, IRF3 protein translation and NF-κB p65 phosphorylation, ultimately affecting the secretion of IFN-β (Cao *et al*., 2019). Taken together, these findings suggest that CSFV and its nonstructural proteins can modulate the secretion of antiviral cytokines (IL-8 and INF-β) through various signaling pathways. Hence, it is possible that CSFV inhibits RPS3 via NF-kB signaling pathways, but this possibility needs to be explored further.

Conclusion

In conclusion, the current study revealed that RPS3-mediated antiviral immunity plays a key role against CSFV infection in PAM cells. The findings of this study advance our understanding of the mutual relationship between hosts and viruses and elucidate the mechanism underlying the immune evasion and persistent infection of CSFV.

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