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Short communication

Caveolin-1-mediated endocytic pathway is involved in classical swine fever virus Shimen infection of porcine alveolar macrophages

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ABSTRACT

Macrophages are at the frontline of defense against pathogenic microorganisms. However, very little is known about the cell invasion mechanism of classical swine fever virus (CSFV) Shimen strain. To elucidate the infective mechanism of this important pathogen, we screened deep-sequencing data derived from macrophages infected with CSFV Shimen and uninfected macrophages, and identified a role of caveolin-1 (*CAV1*). Digital gene expression profiling showed that *CAV1* was differentially expressed in CSFV Shimen-infected macrophages. Quantitative polymerase chain reaction and western blot analyses showed that the transcription and translation of *CAV1* were significantly up-regulated in CSFV Shimen-infected macrophages. In addition, immunofluorescent confocal microscopy analysis suggested that CAV1 was temporally colocalized with CSFV E2 throughout the course of the infection. Through the overexpression of recombinant *CAV1* or the silencing of *CAV1* expression using small interfering RNA in macrophages, we demonstrated that *CAV1* expression is beneficial for the replication of CSFV Shimen. However, RNA silencing of *CAV1* did not prevent viral replication, which may indicate that CSFV can also enter macrophages by other mechanisms. Our findings suggest that CAV1-mediated endocytosis is advantageous for productive CSFV Shimen infection in macrophages, providing a new insight into the mechanisms of evasion of host immunity for successful viral infection.

1. Introduction

Classical swine fever virus (CSFV) (genus *Pestivirus*, family *Flaviviridae*) is a single-stranded, positive-sense RNA virus surrounded by a viral envelope (Paton and Greiser-Wilke, 2003). As a highly epidemic and fatal illness, classical swine fever (CSF) is listed as a notifiable disease by the World Organization for Animal Health. The virulent strain of CSFV causes acute CSF, with typical symptoms of hemorrhagic lymphadenitis and diffuse hemorrhage in the skin, kidney, and other organs. The CSF epidemic has thus far been effectively controlled by the C-strain vaccine and culling (Moennig et al., 2003). However, since the 1990s, atypical mild swine fever has been the main clinical form of the disease reported in most countries (Edwards et al., 2000), which has led to new difficulties in the prevention and control of the disease, especially because it lacks typical pathologic characteristics that can be readily distinguished from other pig diseases.

To achieve a persistent infection, CSFV must first compromise the host immune defense. Previous studies have shown that CSFV infects macrophages, dendritic cells, and other immune cells, and it replicates in these cells rather than causing a cytopathic effect (Bauhofer et al., 2005; Borca et al., 2008). Macrophages protect the host from infection and act at the frontline of immune defense (Mercer and Greber, 2013). The function of macrophages appears to be subverted by CSFV; however, the mechanism by which CSFV invades macrophages remains unknown. Here, using digital gene expression (DGE) profiling, we identified caveolin-1 (CAV1), a component expressed in the endocytic pathways, as a factor potentially involved in the infection of macrophages by CSFV. Further experiments in CSFV-infected macrophages were

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conducted to prove that CAV1 is the critical factor that confers susceptibility to CSFV.

2. Materials and methods

2.1. Study design, cells, and virus strain

DGE analysis (Audic and Claverie, 1997) was performed to screen for differentially expressed genes based on high-throughput sequencing data obtained from complementary DNA libraries derived from control porcine macrophages and those infected with CSFV Shimen, according to previously described methods (Ning et al., 2016). This analysis identified *CAV1* as a differentially expressed gene, and further testing was conducted to confirm whether CAV1 is a critical factor for the susceptibility of a macrophage cell line to infection by CSFV Shimen. The porcine alveolar macrophage cell line 3D4/21 (CRL 2843) was obtained from the American Type Culture Collection (Manassas, VA, USA). The CSFV Shimen strain was obtained from the Control Institute of Veterinary Bioproducts and Pharmaceuticals (Beijing, China). Cell culture and virus inoculation were conducted for 0, 12, 24, and 48 h (Ning et al., 2014). Macrophages were cultured in 25 cm^2 tissue culture flasks, at a density of 2×10^7 cells per flask, before each experiment in a 37 °C incubator under a 5% CO_2 atmosphere. After a 1 h incubation with CSFV at a multiplicity of infection (MOI) of 5, the medium was aspirated and fresh medium containing 2% (v/v) fetal calf serum was added.

2.2. Quantification of CAV1 expression using quantitative polymerase chain reaction (qPCR)

The transcription and translation of *CAV1* were analyzed in CSFV Shimen-infected porcine macrophages at different time points post-infection. Quantitative polymerase chain reaction (qPCR) assays were conducted to detect changes in the mRNA expression of *CAV1* in macrophages during the first 48 h of CSFV Shimen infection using the following specific oligonucleotide primers: 5′-CAGGGCAACATCTACAAGC-3′ and 5′-AAGAGGGCAGACAGCAAAC-3′. The thermal cycler program was 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 68 °C for 20 s. Each qPCR analysis was performed in triplicate.

2.3. Western blot analysis

To determine the abundance of CAV1 protein in the macrophages, western blotting was performed as described by Ning et al. (2014) using an anti-CAV1 antibody (#3238, Cell Signaling Technology, Danvers, MA, USA) and a monoclonal antibody against CSFV E2 protein (Ab-mart, Shanghai, China). Signals were visualized by enhanced chemiluminescence solution (Advansta, Menlo Park, CA, USA), using a GeneGnome XRQ Chemiluminescence Detector (Syngene, Cambridge, UK) to obtain images. The intensity of the signals for CAV1 and CSFV E2 were determined using the GeneSys software program (Vilber Lourmat, France) and normalized to that for β-actin.

2.4. Confocal microscopy

To confirm that CSFV uses the caveolin-dependent endocytic pathway, analysis of the temporal colocalization of CAV1 and CSFV E2 protein was conducted using laser-scanning confocal microscopy (Model LSM510 META, Zeiss, Oberkochen, Germany), according to previously reported protocols (Shi et al., 2003). CSFV-infected cells were fixed by methanol and stained with CAV1 and CSFV E2 monoclonal antibodies at 0, 1, and 2 d. Image analysis was performed using the standard system operating software provided with the microscope.

2.5. Gene overexpression and RNA interference

To evaluate the role of CAV1 in the invasion of CSFV into macrophages, a recombinant plasmid overexpressing *CAV1* was constructed. *Xba*I (F: GCTCTAGAATGTCGGGGGGCAAATAC) and *Bam*HI (R: CGGGATCCTTATATTTCTTTCTGCATGTTGATG) restriction sites were engineered into the PCR oligonucleotides for amplification of the *CAV1* gene according to the archived nucleotide sequences. The PCR product was subcloned into the mammalian expression vector pCDH-CMV-MCS-EF1-copGFP. Using Turbo-Fect™ Transfection Reagent (Thermo Scientific, Waltham, MA, USA), the plasmids were transfected into overnight-cultured macrophages at 37 °C in 12-well plates. At 24 h post-transfection, when the transfection efficiency reached 70%, the cells were treated with CSFV Shimen and harvested at 0, 12, 24, and 48 h after incubation, and qPCR was used to detect the expression level of CSFV genome equivalents (Ning et al., 2013).

In addition, a set of *CAV1*-targeted siRNA constructs, which were kindly provided by Professor Jingyu Wang, Northwest A&F University, China, were integrated in the pGFP-V-RS vector plasmid according to a previously described protocol (Huang et al., 2011). Macrophages at 75% confluence were transfected with the pGFP-V-RS vector expressing *CAV1*-specific shRNA (*CAV1* siRNA constructs in the pGFP-V-RS plasmid) and control siRNA (29-mer non-targeting scrambled pGFP-V-RS vector). CSFV-infected cells were harvested at 0, 12, 24, and 48 h post-infection, and the effects of *CAV1* silencing on the protein abundance of *CAV1* were determined by western blot analysis and the detection of CSFV RNA by qPCR (Ning et al., 2013), respectively.

2.6. Statistical analysis

Statistical analyses were conducted by one-way or two-way analysis of variance using SPSS 16.0 (SPSS Inc., Chicago, IL, USA), and a p value less than 0.05 was considered to be significant. Data were shown as the mean values ± standard error of 3 independent experiments.

3. Results

3.1. CAV1 mRNA expression is positively correlated with the infection of CSFV Shimen

The DGE analysis showed that the expression of *CAV1* was significantly up-regulated in macrophages infected with CSFV Shimen compared with its expression in the control cells (Fig. 1A). We compared the obtained *CAV1* expression profile with another DGE profile from CSFV-infected swine umbilical vein endothelial cells (Ning et al., 2016), which showed a similar expression pattern to that of CSFV Shimen-infected macrophages (Fig. 1A). Next, qPCR was carried out to measure the mRNA expression of *CAV1* throughout the 48 h course of CSFV infection. As shown in Fig. 1B, cells infected with CSFV Shimen showed a significant increase in *CAV1* mRNA expression compared with that of the uninfected control cells.

3.2. Upregulation of the abundance of CAV1 protein by CSFV Shimen infection in macrophages

To confirm that the upregulated expression of *CAV1* led to an increased abundance of CAV1 in macrophages after CSFV Shimen infection, we detected CAV1 and CSFV Shimen E2 protein by western blotting. E2 is a structural protein of CSFV, and its