Classical swine fever virus and p7 protein induce secretion of IL-1β in macrophages

Zhi Lin, Wulong Liang, Kai Kang, Helin Li, Zhi Cao and Yanming Zhang

Correspondence
Yanming Zhang
ylzhangym@sohu.com

INTRODUCTION

Classical swine fever virus (CSFV) has a tropism for vascular endothelial cells and immune system cells. The process and release of pro-inflammatory cytokines, including IL-1β and IL-18, is one of the fundamental reactions of the innate immune response to viral infection. In this study, we investigated the production of IL-1β from macrophages following CSFV infection. Our results showed that IL-1β was upregulated after CSFV infection through activating caspase-1. Subsequent studies demonstrated that reactive oxygen species may not be involved in CSFV-mediated IL-1β release. Recently, research has indicated a novel mechanism by which inflammasomes are triggered through detection of activity of viroporin. We further demonstrated that CSFV viroporin p7 protein induced IL-1β secretion which could be inhibited by the ion channel blocker amantadine and also discovered that p7 protein was a short-lived protein degraded by the proteasome. Together, our observations provided an insight into the mechanism of CSFV-induced inflammatory responses.

RESULTS

CSFV infection induces IL-1β secretion from macrophages

To assess the effect of CSFV on IL-1β transcription and secretion, 3D4/2 macrophages that supported CSFV replication in vitro (Weingartl et al., 2002) were infected with CSFV. At specified time points, total cellular RNA was extracted from mock-infected or CSFV-infected cells and the expression of IL-1β mRNA was quantified by real-time reverse transcription (RT)-PCR. At the same time, cell culture supernatants were collected and the release of mature IL-1β...
was measured using an IL-1β-specific ELISA kit. The results showed a 10-fold increase in the level of IL-1β mRNA in infected cells at 24 h post-infection (p.i.) (Fig. 1a) and a 16-fold increase at 48 h p.i. (Fig. 1a). We also found increased secretion of IL-1β at both 24 and 48 h p.i. (163 and 216 pg ml⁻¹, respectively) compared with the control group (11 pg ml⁻¹) (Fig. 1b). We also confirmed that the IL-1β-stimulatory activity of CSFV required viral replication, as UV-inactivated virus did not induce IL-1β production from treated 3D4/2 macrophages (Fig. 1b).

Reactive oxygen species (ROS) production has been shown to be involved in the induction of IL-1β (Allen et al., 2009). In a previous study, CSFV protein NS5A was shown to stimulate ROS accumulation in swine umbilical vascular endothelial cells (SUVECs) (He et al., 2012). Using dihydroethidium (DHE), a ROS fluorescent probe, we discovered that CSFV infection also stimulated ROS accumulation in 3D4/2 macrophages (Fig. 1c). To determine whether ROS was responsible for IL-1β secretion by CSFV infection, cells were incubated with the ROS inhibitor pyrrolidine dithiocarbamate (PDTC; an antioxidant) (100 µM). However, we observed 20% inhibition in IL-1β secretion from CSFV-infected cells incubated with PDTC (Fig. 1b).

These results demonstrated that CSFV infection could induce the transcriptional expression and secretion of mature IL-1β, whilst ROS was not involved in this process.

**Activation of caspase-1 by CSFV**

Post-translational processing and secretion of IL-1β are processed by caspase-1. Caspase-1, consisting of two subunits of p20 and p10, is activated through autocleavage. After activation, caspase-1 cleaves the pro-inflammatory cytokine pro-IL-1β to mature IL-1β. To determine whether CSFV infection activated caspase-1, mock-infected and CSFV-infected cellular lysates were analysed by Western blotting at specified time points. The results showed higher levels of mature caspase-1 (p10) at 24 and 48 h p.i. (Fig. 2a).

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**Fig. 1.** CSFV infection induces IL-1β secretion from macrophages. (a) RNA from mock-infected or CSFV-infected macrophages was isolated at 24 and 48 h p.i., and IL-1β mRNA expression was measured by quantitative real-time RT-PCR. (b) Macrophages were incubated with CSFV for 24 and 48 h. CSFV-infected macrophages were incubated with PDTC (100 µM) for 24 h. Macrophages were incubated with UV-inactivated virus for 24 h. Cell-free supernatants were collected and analysed for IL-1β by ELISA. The results are shown as mean ± SD from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared with mock-infected control cells. (c) Fluorescence detection of ROS levels in CSFV-infected macrophages. Nuclei were stained for 15 min at room temperature with Hoechst 33342 (blue). DHE was used for detecting ROS (red).
Benzylxycarbonyl-Tyr-Val-Ala-Asp(OMe)-fluoromethylketone (z-VAD-FMK), a caspase-1 inhibitor, significantly inhibited IL-1β release from CSFV-infected macrophages (Fig. 2b). These data suggested that CSFV induced secretion of IL-1β through activation of caspase-1.

**CSFV p7 protein stimulates IL-1β release**

Recently, intracellular accumulation of ROS, influenza A virus M2 ion channel protein, encephalomyocarditis virus viroporin 2B protein and hepatitis C virus (HCV) p7 protein were shown to induce IL-1β secretion through the activation of the inflammasome complex (Ichinohe et al., 2010; Ito et al., 2012; Shrivastava et al., 2013). CSFV p7 protein is an ion channel protein, which induces Ca2+ flux in cells (Guo et al., 2013a) and may be involved in CSFV-mediated IL-1β production. To prove this hypothesis, 3D4/2 macrophages were transfected with plasmid pEGFP-p7 or pEGFP-C3. After 24 h, the level of IL-1β mRNA was quantified by real-time RT-PCR. Cell culture supernatants were collected and the release of mature IL-1β was measured using an IL-1β-specific ELISA kit. We observed increased expression of IL-1β mRNA (3.5-fold) (Fig. 3a) and secretion of IL-1β (75 pg ml⁻¹) in p7-expressing cells (Fig. 3b).

**GFP-tagged p7 is a short-lived protein degraded by the proteasome in different cells**

Many virus proteins are transiently produced but then decay following uptake in cells. As we had no efficient anti-p7 antibody to perform immunofluorescence studies, a GFP tag was used for fluorescence microscopy analysis. For this, 3D4/2 macrophages were transfected with plasmids expressing either GFP or GFP-p7. After 24 h, GFPs were observed by direct fluorescence and the numbers of GFP-positive cells were evaluated after Hoechst 33342 nuclear staining. As expected, fluorescence analyses showed that the GFP control protein (Fig. 4a, b) could be detected in cells untreated or exposed to proteasome inhibitor MG132 (10 μM). In contrast, GFP-p7 was nearly undetectable (Fig. 4c) and could mainly be detected after MG132 treatment (Fig. 4d).

Furthermore, we tested if p7 was unstable in SUVECs in order to verify whether p7 protein was unstable in different cells. Once again, Western blot analysis showed that p7 was accumulated in cells in the presence of proteasome inhibitor MG132 (Fig. 5). The above results suggested that p7 protein was a short-lived protein degraded by the proteasome in different mammalian cells.

Our observations showed that p7 protein was accumulated in cells in the presence of proteasome inhibitor MG132 and it may have influenced IL-1β expression. To examine this possibility, we measured IL-1β production following MG132 treatment. The results showed that IL-1β production was slightly increased following MG132 treatment (Fig. 6). To determine whether p7-induced inflammasome activation was associated with its ion channel characteristics, cells were treated with the ion channel blocker amantadine and transfected with p7 or pEGFP-C3. As expected, cells treated with amantadine showed reduced IL-1β secretion (Fig. 6). Thus, CSFV p7 protein appeared to be associated with CSFV-mediated IL-1β production, although the exact mechanism(s) requires further study.

**DISCUSSION**

In this study, our findings demonstrate that CSFV induces the expression and maturation of IL-1β in macrophages, mediated by activation of caspase-1. Although ROS production has been involved in the induction of IL-1β (Allen et al., 2009), 20% inhibition in IL-1β secretion is observed from CSFV-infected cells incubated with PDTC, suggesting that
ROS may not be involved in CSFV-induced IL-1β expression. Viroporin has been implicated in the induction of IL-1β (Wang, 2013). Our results show that CSFV viroporin p7 protein induces IL-1β production that can be inhibited by the ion channel blocker amantadine and we also discovered that p7 protein is a short-lived protein degraded by the proteasome.

IL-1β is one of the most potent pro-inflammatory cytokines, and has been shown to possess multiple and diverse properties in the response to infection (Piccioli & Rubartelli, 2013). Inflammasomes are known as platforms for caspase-1 activation and IL-1β maturation. The NLRP3 inflammasome and RIG-I inflammasome have been shown to be involved in RNA virus-induced caspase-1 activation (Ito et al., 2012; Poeck et al., 2010). A variety of viruses have been shown to induce IL-1β production through the NLRP3 inflammasome (Ito et al., 2012). In particular, flaviviruses related to CSFV, including West Nile virus, Japanese encephalitis virus and hepatitis C virus, promote IL-1β production through the NLRP3 inflammasome (Kaushik et al., 2012; Negash et al., 2013; Ramos et al., 2012). Prior works have revealed increased accumulation of IL-1β mRNA in swine macrophages and monocytes infected with CSFV by means of microarrays and quantitative real-time RT-PCR (Borca et al., 2008; Gladue et al., 2010; Knoetig et al., 1999; Zaffuto et al., 2007). Our report agrees with the findings that CSFV triggers IL-1β mRNA accumulation and also induces IL-1β secretion through activation of caspase-1. RIG-I plays a central role in the recognition of RNA viruses and activation of inflammasome signalling for IL-1β production (Kato et al., 2006; Loo et al., 2008; Poeck et al., 2010; Takeuchi & Akira, 2008). Recent studies have shown that CSFV can be recognized by RIG-I and MAD5 to induce NFκB activation (Dong et al., 2013; Hüsser et al., 2011). We consider that NFκB activation might be involved in the expression of pro-IL-1β, and CSFV might trigger RIG-I or NLRP3 to form an inflammasome and then activate caspase-1 for IL-1β maturation. However, this possibility needs further study.

Many viruses are known to encode viroporins that are involved in inflammasome activation. Influenza virus

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**Fig. 3.** CSFV p7 protein stimulates IL-1β release. (a) IL-1β mRNA expression in mock-treated, pEGFP-C3-transfected and pEGFP-C3-p7-transfected macrophages. (b) Supernatants from mock-treated, pEGFP-C3-transfected and pEGFP-C3-p7-transfected macrophages were collected and analysed for IL-1β by ELISA. The results are shown as mean±s.d from three independent experiments. *P<0.05 and ***P<0.001 compared with mock-infected control cells.

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**Fig. 4.** Protein stability of GFP-p7. Macrophages were transfected with (a, b) pEGFP-C3 or (c, d) pEGFP-C3-p7 expressing GFP-tagged p7. Cells were (b, d) treated with 10 μM proteasome inhibitor MG132 for 18 h or (a, c) left untreated. At 24 h, nuclei were stained for 15 min at room temperature with Hoechst 33342 and observed under a fluorescence microscope. Bar, 100 μm.

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**Fig. 5.** p7 is unstable in different cells. Macrophages and SUVECs were transfected with pEGFP-C3 or pEGFP-C3-p7 expressing GFP-tagged p7. After exposure to MG132 for 18 h, p7 was analysed by Western blot analysis.
viroplasm M2 protein (Ichinohe et al., 2010) and encephalomyocarditis virus viroporin 2B protein (Ito et al., 2012) induce IL-1β production by ionic perturbation. Recent studies also showed that porcine reproductive and respiratory syndrome virus ion channel-like protein E (Zhang et al., 2013) and HCV p7 RNA induce IL-1β secretion, which can be inhibited by KCl or the ion channel blocker amantadine (Shrivastava et al., 2013). Ca2+ signalling has been shown to be linked to NLRP3 inflammasome activation (Horng, 2014; Lee et al., 2012; Murakami et al., 2012). Previous work has demonstrated that CSFV p7 protein elevates Ca2+ levels in cells (Guo et al., 2013a). Our results indicate that CSFV p7 protein triggers IL-1β secretion and CSFV p7-mediated IL-1β secretion is inhibited by the ion channel blocker amantadine. Our observations, together with those of Guo et al. (2013a), suggest that CSFV p7 protein induces IL-1β secretion through elevated Ca2+ levels.

The proteasome system plays an important role in the degradation of short-lived proteins and some endogenously expressed proteins (Glickman & Ciechanover, 2002). Many virus proteins are produced transiently, but then decay in cells, including HCV NS2 protein, HCV p7 protein, CSFV NS2 protein and human immunodeficiency virus type 1 Vpu protein (Ciechanover, 1998; Franck et al., 2005; Guo et al., 2013b; Haqshenas, 2013). Recent studies have shown that HCV p7 is unaffected by proteasome inhibitors when expressed in a full-length virus context and only mutant p7 proteins that disrupt polyprotein processing are affected by MG132 (Bentham et al., 2013). It has been reported that pestivirus p7 protein is required for the generation of infectious virions, whereas mutations in bovine viral diarrhea virus p7 significantly affect the generation of infectious virions (Brohm et al., 2009; Harada et al., 2000).

Thus, when expressed in a full-length virus context, the virus might prevent p7 from degradation. This may explain the conflict between Bentham et al. (2013) and Haqshenas (2013). Our results show that CSFV p7 protein is degraded via the proteasome in different cells, which also needs to be confirmed in a full-length virus context. Increased IL-1β expression was also observed within transfected cells following MG132 treatment.

Pro-inflammatory cytokines are important mediators in immune responses against viral infection. Further investigation is needed in order to examine the exact mechanism of CSFV-mediated inflammatory processes.

**METHODS**

**Virus and cell culture.** CSFV (Shimen strain) was purchased from the Control Institute of Veterinary Bio-products and Pharmaceuticals (China). The virus was propagated in swine alovular 3D4/2 macrophages (ATCC; CRL-2845); an m.o.i. of 10 TCID50 was used. Macrophages were cultured in RPMI 1640 medium (Gibco) containing 2 mM l-glutamine, 1.5 g sodium bicarbonate L-1, 4.5 g glucose L-1, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.1 mM non-essential amino acids and 10% FBS (Weingartl et al., 2002). SUVECs were cultured in high-glucose Dulbecco’s modified Eagle’s medium (Gibco) containing 10% heat-inactivated FBS (Hyclone) and 50 µg heparin ml-1 (Sigma-Aldrich).

**Constructs, transfection and reagents.** To facilitate cloning of the ampiclons, HindIII (F: CCGAAGCTTCTACCAATTGGGTCAAGG) and BamHI (R: TCGGGATCCGTTAAACCTTGGCAAC) sites (underlined) were engineered into the PCR oligonucleotides for amplification of the CSFV p7 gene according to the archived CSFV Shimen strain nucleotide sequence (GenBank accession number AF092448). The PCR product was subcloned into the mammalian expression vector pEGFP-C3. The recombinant plasmid was sequenced and named pEGFP-p7. Using Lipofectamine 2000 reagent (Invitrogen), the plasmids were transfected into overnight cultured SUVECs and macrophages in 12-well plates.

The specific proteasome inhibitor MG132 (Sigma) was used to inhibit the proteasomal degradation pathway. At 6 h post-transfection, fresh medium containing MG132 (10 µM) was added for another 18 h. For the GFP expression study, nuclei were stained for 15 min at room temperature with Hoechst 33342 (Beyotime). The ROS inhibitors PDTC and amantadine hydrochloride were purchased from sigma, z-VAD-FMK and DHE were purchased from Beyotime.

**Real-time RT-PCR.** The target IL-1β gene in mock-infected and CSFV-infected cells was quantified by real-time RT-PCR using the primers S: 5’-CAGCACCTCTCAAGCAGAACAA-3’ and A: 5’-GGCCAGCAACCATGTACACT-3’. Total cellular RNA was isolated using TRizol (Invitrogen) and the cDNA was reverse transcribed from 1 µg total RNA using a RT kit (Takara Bio). RNA expression was normalized to the housekeeping gene β-actin (P1: 5’-GTGCCACCG-GCAAATGCTTCC-3’; P2: 5’-ACCGACTGTGTCACCTTCC-3’). Quantitative real-time RT-PCR was carried out using a SYBR ExScript RT-PCR kit (Takara Bio). Reactions were performed under the following conditions: 10 min at 95 °C, and 40 cycles of 10 s at 95 °C and 30 s at 60 °C. Relative transcript levels were analysed using the ΔΔCt method as specified by the manufacturer (Livak & Schmittgen, 2001).

**Western blotting and ELISA.** Cells were incubated in radioimmuno-precipitation (RIPA) buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1%
NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 mM sodium orthovanadate, 1 mM sodium fluoride and 100 μg PMSF ml⁻¹ for 45 min on ice. Protein samples were separated by 15 % SDS-PAGE and transferred onto PVDF membranes (Millipore). Mouse anti-GFP mAb (Genescript) and rabbit anti-caspase-1 p10 (sc-514; Santa Cruz Biotechnology) were used as primary antibodies. Cellular glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected with mouse anti-porcine GAPDH antibody (Generscript) as an internal control protein. The secreted IL-1β protein in cell culture supernatant was determined by ELISA according to the manufacturer’s protocol (R & D Systems).

Statistical analysis. Differences in each groups were examined for statistical significance using Student’s t-test. A P value <0.05 was considered statistically significant.

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REFERENCE


