Interferon-Inducible Oligoadenylate Synthetase-Like Protein Acts as an Antiviral Effector against Classical Swine Fever Virus via the MDA5-Mediated Type I Interferon Signaling Pathway

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Running title: Porcine OASL is an anti-CSFV ISG.

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ABSTRACT
Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), which poses a serious threat to the global pig industry. Interferons (IFNs) and IFN-stimulated genes (ISGs) play a key role in host antiviral defense. We have previously screened the porcine 2'-5'-oligoadenylate synthase-like protein (pOASL) as a potential anti-CSFV ISG using a reporter CSFV. This study aimed to clarify the underlying antiviral mechanism of pOASL against CSFV. We confirmed that CSFV replication was significantly suppressed in lentivirus-delivered, pOASL-overexpressing PK-15 cells, whereas silencing the expression of endogenous pOASL by small interfering RNAs markedly enhanced CSFV growth. In addition, the transcriptional level of pOASL was upregulated both in vitro and in vivo upon CSFV infection. Interestingly, the anti-CSFV effects of pOASL are independent of canonical RNase L pathway but depend on the activation of the type I IFN response. Glutathione S-transferase pulldown and coimmunoprecipitation assays revealed that pOASL interacts with MDA5, a double-stranded RNA sensor, and further enhances the MDA5-mediated type I IFN signaling. Moreover, we showed that pOASL exerts anti-CSFV effects in an MDA5-dependent manner. In conclusion, pOASL suppresses CSFV replication via the MDA5-mediated type I IFN signaling pathway.

Keywords: Classical swine fever virus; Interferon-stimulated genes; 2'-5'-Oligoadenylate synthase-like protein; Antiviral activity; Porcine MDA5; Type I interferon
The host innate immune response plays an important role in mounting the initial resistance to viral infection. Here, we identify the porcine 2'-5'-oligoadenylate synthase-like protein (pOASL) as an interferon (IFN)-stimulated gene (ISG) against classical swine fever virus (CSFV). We demonstrate that the anti-CSFV effects of pOASL depend on the activation of type I IFN response. In addition, we show that pOASL, as an MDA5-interacting protein, is a coactivator of MDA5-mediated IFN induction to exert anti-CSFV actions. This work will be beneficial to the development of novel anti-CSFV strategies by targeting pOASL.

Classical swine fever virus (CSFV) is the etiological agent of classical swine fever (CSF), which is an economically important viral disease of domestic pigs and wild boar worldwide. CSFV is a member of the Pestivirus genus belonging to the Flaviviridae family (1, 2). The virus contains a single-stranded, positive-sense RNA genome of approximately 12.3 kilobases (3, 4). The viral genome comprises a single, large open reading frame which encodes a polyprotein precursor of approximately 3,898 amino acids (aa) that is further proteolytically cleaved into 12 polypeptides comprising of four structural (C, E\textsuperscript{enn}, E1, and E2) and eight nonstructural proteins (N\textsuperscript{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (5, 6).

Initiation of innate immunity in response to viral infections relies on sensing viral nucleic acids by retinoic acid-inducible gene I (RIG-I)-like receptors (RLR) in the cytoplasm (7). Upon binding to viral RNAs, RIG-I or melanoma differentiation-associated gene 5 (MDA5) initiates a signaling cascade to induce type I interferons (IFNs) (8, 9). IFNs exhibit pleiotropic effects through inducing transcription of hundreds of IFN-stimulated genes (ISGs) (9–12).

The 2'-5'-oligoadenylate synthetases (OASs) are IFN-induced antiviral enzymes. The human OAS family consists of four members, i.e. OAS1–3 and...
Among them, OAS1–3 contain one, two, and three basic OAS units, respectively. The porcine OAS family consists of OAS1, OAS2, and OASL (13).

Members of the OAS family exert antiviral activity via the canonical OAS/RNase L-dependent or -independent antiviral pathways. The OAS/RNase L pathway has been studied extensively (10, 14). Activation of RNase L by the OAS family requires the OAS oligomerization unit and the processivity of synthesizing di-, tri-, and tetrameric 2′-5′-oligoadenylates (2-5A), which in turn bind and activate the RNase L. Upon binding to 2-5A, RNase L dimerizes and degrades cellular and viral RNAs, resulting in the reduction of protein synthesis (15, 16). Moreover, some studies indicate that some members of the OAS family exhibit antiviral activity via several RNase L-independent pathways (12, 17, 18). For instance, human OASL (hOASL) exerts antiviral effects through enhancing the RIG-I-mediated signaling by mimicking polyubiquitin (12).

In a previous study, we screened the porcine OASL (pOASL) as a candidate anti-CSFV ISG (19). Some studies have demonstrated that hOASL has an N-terminal OAS-like domain without 2′-5′ OAS activity (12, 20, 21). Moreover, pOASL also contains only one OAS-like domain, lacking two C-terminal tandem ubiquitin-like domains (13). However, whether pOASL exhibits 2′-5′ OAS activity and how it exerts anti-CSFV actions remain unclear.

This study was attempted to elucidate the role of pOASL in enhancing the MDA5-mediated signaling and clarify the mechanism by which pOASL exerts anti-CSFV activity. We have revealed a model whereby pOASL, induced upon CSFV infection, binds to MDA5 and functions as an anti-CSFV ISG via the MDA5-mediated type I IFN signaling pathway.

MATERIALS AND METHODS

Cells and viruses. PK-15 (a porcine kidney cell line) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) containing 5% fetal calf
sera (FCS) (Sigma-Aldrich) in 5% CO₂ at 37°C. BHK-21 (a Syrian baby hamster kidney cell line) and HEK293T (a human embryonic kidney cell line) cells were cultured similarly in DMEM plus 10% FCS.

rCSFV-Fluc, a reporter CSFV harboring the firefly luciferase (Fluc) gene (22), was used in the antiviral assays of pOASL. rCSFV-Fluc and the CSFV Shimen strain (Shimen), the parent virus of rCSFV-Fluc, were propagated in PK-15 cells. Sendai virus (SeV) was propagated in 9-day-old specific-pathogen-free chicken embryos.

Plasmid construction and cell transfection. The pOASL gene (GenBank accession no. NM_001031790) was cloned into the vectors pCMV-HA (Clontech), p3xFlag-CMV-10 (Sigma-Aldrich), or pFUGW (Addgene), creating pHA-pOASL, pFlag-pOASL, or pFUGW-pOASL. The primers for amplification of the pOASL gene are listed in Table 1.

HEK293T cells seeded in six-well plates (Corning) were transfected with 2 μg of pHA-pOASL and 2 μl of X-tremeGENE HP DNA transfection reagent (catalog no. 06366236001; Roche) as per the manufacturer’s instructions. At 6 h posttransfection (hpt), the medium was changed as fresh DMEM containing 10% FCS and incubated for another 48 h for further assays.

Establishment and identification of a cell line overexpressing pOASL. To construct a stable cell line overexpressing pOASL, HEK293T cells grown in 10-cm cell dishes were cotransfected with 10.5 μg of pFUGW-pOASL or pFUGW-EGFP harboring the egfp gene (serving as a control) in combination with 7 μg of psPAX2 and 3.5 μg of pMD2.G, and the resulting recombinant lentiviruses Lenti-pOASL and Lenti-EGFP were harvested at 48 hpt from the supernatants of the transfected cells. Subsequently, to generate stable cell lines PK-pOASL and PK-EGFP, PK-15 cells were transduced with the above lentiviruses at a multiplicity of infection (MOI) of 1. The expression of EGFP-pOASL and EGFP in the cells was immunoblotted with a mouse anti-EGFP monoclonal antibody (MAb) (1:1,000) (catalog no. A00185; GeneScript).
**Cell viability assay.** To evaluate the effects of the ectopic expression on the growth of the cell lines, cell viability assay was carried out using cell counting kit-8 (CCK-8) (catalog no. CK04; Dojindo) according to the manufacturer’s instructions.

**Indirect immunofluorescence assay (IFA) and virus titration.** CSFV was titrated based on IFA as described previously (19). Viral titers were calculated as described previously (23) and expressed as median tissue culture infective doses (TCID₅₀)/ml.

**Real-time RT-PCR.** Total RNA was isolated from the CSFV-infected PK-15 cells or swine organs using TRIzol reagent (catalog no. 15596026; Invitrogen) and reverse transcribed into the cDNA using reverse transcriptase XL (AMV) (catalog no. 2621; TaKaRa) following the manufacturer’s instructions. A real-time reverse transcription-PCR (qRT-PCR) assay was performed to quantify the genomic RNA copy numbers of CSFV (24).

**RNA interference assay.** Three specific small interfering RNAs (siRNAs) targeting different regions of the pOASL, pRNaseL, or pMDA5 gene and a non-targeting control siRNA (siNC) were commercially synthesized by GenePharma, and the siRNAs are listed in Table 2. The siRNA transfection assay was performed as described previously (19). PK-15 cells (5 × 10⁴ cells/well) were plated into 24-well plates and transfected with 0.2 μM sipOASLs or siNC using X-tremeGENE siRNA transfection reagent (catalog no. 4476093001; Roche) as per manufacturer’s instructions. At 36 hpt, the transfected cells were inoculated with rCSFV-Fluc or Shimen at an MOI of 0.1. After 2 h, the cells were replaced with fresh DMEM containing 2% FCS and incubated at 37°C. At 48 h postinfection (hpi), the supernatants and lysates of the cells were collected for further analysis.

**Animal infection experiment.** To test the expression level of pOASL in CSFV-infected pigs, three pigs were left uninfected or infected with 10⁵ TCID₅₀ Shimen. Various organs (heart, liver, spleen, lung, kidney, tonsils, and lymph nodes) were collected at 3 d postinoculation (25). The mRNA expression level
of pOASL was evaluated by qRT-PCR as described previously (19).

**Dual luciferase reporter assay.** HEK293T cells in 24-well plates were transfected with pHA-pOASL or pCMV-HA (0.5 μg each) together with the promoter reporter plasmid pIFN-β-Fluc, pISRE-Fluc, or pNF-κB-Fluc (0.2 μg each) and the internal reference reporter plasmid expressing TK-Renilla luciferase (Rluc) (10 ng). At 24 hpt, the transfected cells were stimulated with SeV at 20 hemagglutinin units (HAUs)/ml or left untreated for 24 h, and assayed for reporter activities using Dual-Luci assay kit (Promega). The luciferase activities were presented as relative expression levels of Fluc/Rluc.

**Coimmunoprecipitation (Co-IP) and Western blotting.** HEK293T cells were cotransfected with pFlag-pOASL and pMyc-pMDA5 or pMyc-pRIG-I expressing the porcine MDA5 (pMDA5) or RIG-I (pRIG-I) (2 μg each). At 48 hpt, the cells were lysed with NP-40 lysis buffer (catalog no. P0013F; Beyotime) containing phenylmethanesulfonyl fluoride (PMSF) to a final concentration of 1 mM for 1 h at 4°C. To remove cellular debris, the lysates were centrifuged at 12,000 × g at 4°C for 25 min and protein G-agarose (catalog no. 11243233001; Roche) was used to preclear the supernatants for 4 h at 4°C, and then incubated with an anti-Flag MAb (catalog no. F1804; Sigma-Aldrich) or with an irrelevant isotype-matched MAb (catalog no. A001 85; GeneScript) acting as a negative control for 5 h at 4°C. The immunoprecipitates were washed five times with the NP-40 lysis buffer and dissolved in PBS for Western blotting as mentioned above.

**GST pulldown assay.** To produce GST-tagged pOASL protein, the pOASL gene was subcloned into the prokaryotic expression vector pGEX-6p-1 (GE Healthcare). The resulting recombinant plasmid pGST-pOASL was transformed to express the GST-tagged pOASL (GST-pOASL) in *Escherichia coli* BL21(DE3). GST-pOASL was purified as described previously (19). GST pulldown assay was performed by incubating the GST-pOASL bound-resin with the Myc-tagged pMDA5 produced in HEK293T cells for 5 h at 4°C. Immunoblotting was performed to probe the pulled down proteins using a
mouse anti-GST PAb (catalog no. AB101; Tiangen) and a mouse anti-Myc MAb (catalog no. SAB4700447; Sigma-Aldrich).

**Confocal imaging.** BHK-21 cells were cotransfected with pFlag-pOASL and pMyc-pMDA5 (2 μg each). At 36 hpt, the cells were immunostained with respective antibodies, and observed using a Leica SP2 confocal microscope (Leica Microsystems) as described previously (19).

**Statistical analysis.** All data were analyzed using SPSS 18.0 software. Differences were considered to be significant if an unadjusted $P$-value is less than 0.05.

**RESULTS**

**pOASL inhibits CSFV replication.** To explore the antiviral activity of pOASL against CSFV, we established PK-pOASL or PK-EGFP cells stably expressing pOASL or EGFP. The cell growth and viability of PK-pOASL and PK-EGFP cells were indistinguishable from those of the parent PK-15 cells (Fig. 1A). We have demonstrated that overexpression of pOASL inhibits rCSFV-Fluc replication (19). The anti-CSFV activity of pOASL was examined in PK-pOASL and PK-EGFP cells upon infection with the parental CSFV Shimen strain at MOI of 0.1 and 1. The extracellular titers of the progeny virus (Fig. 1B), the number of viral genomic copies (Fig. 1C), and the intracellular expression level of the N<sup>pro</sup> protein (Fig. 1D) were significantly reduced in PK-pOASL cells, compared to those in PK-EGFP cells at 24 or 48 hpi. However, the suppression of CSFV replication by OASL is different between MOI of 0.1 and 1. Considering that pOASL exerts antiviral effects against CSFV, we tried to investigate the antiviral kinetics of pOASL against CSFV replication. The intracellular viral genomic copy numbers of CSFV in the infected cells were quantified at different time points postinfection. The results showed that the inhibition of CSFV at an MOI of 1 by pOASL reached a peak at 12 hpi and then decreased thereafter (Fig. 1E).

To further examine the antiviral effects of pOASL on CSFV, three specific
siRNAs were used to silence the expression of endogenous pOASL in PK-15 cells, resulting in efficient reduction of pOASL expression (Fig. 2A and B). Subsequently, the replication efficiency of rCSFV-Fluc or Shimen was assessed upon silencing pOASL. Compared with the siNC-transfected control cells, the intracellular Fluc activities and the expression of the Npro protein were increased in the sipOASLs-transfected PK-15 cells (Fig. 2C–D). Similarly, the extracellular viral genomic copy numbers and viral titers were increased significantly in these cells (6.6- and 7.9-fold, respectively) (Fig. 2E–F). The results demonstrate that knockdown of pOASL promotes CSFV replication.

**pOASL is activated upon CSFV infection.** Now that pOASL is able to inhibit CSFV replication, we attempted to confirm the effects of CSFV infection on the expression of pOASL. PK-15 cells were infected with Shimen and the transcription of pOASL was determined by qRT-PCR. As a control, PK-15 cells were treated with different doses of IFN-β. In accord with predictions, IFN-β activated the expression of pOASL in a dose-dependent manner (Fig. 3A). Similarly, the transcription level of pOASL was increased in the PK-15 cells infected with Shimen at an MOI of 0.1 at 3, 6, 12, 24, 36 and 48 hpi (Fig. 3B), as well as at an MOI of 0.1 and 1 at 6 hpi (Fig. 3C). However, the expression of pOASL was lower at 48 hpi than at 36 hpi at an MOI of 0.1, and lower at an MOI of 0.1 than at an MOI of 1 at 6 hpi. We also showed that pOASL mRNA was expressed in all tested CSFV-infected organs, including heart, liver, spleen, lung, kidney, tonsils and lymph nodes. High-level expression of pOASL was detected in liver, spleen, tonsils, and lymph nodes (Fig. 3D).

**The anti-CSFV effects of pOASL are independent of RNase L.** OAS are able to synthesize 2-5A, which leads to RNA degradation through activating RNase L (26). Considering that pOASL has an N-terminal OAS-like domain, we speculated that its antiviral activity likely depends on RNase L. We knocked down the RNase L expression in PK-15 cells using siRNaseLs (Fig. 4A–B). In both PK-EGFP and PK-pOASL cells, knockdown of RNase L resulted in increased luciferase activities (Fig. 4C), extracellular viral titers (Fig.
pOASL enhances the type I IFN response. The results presented above reveal that the anti-CSFV activity of pOASL is independent of the classical RNase L pathway. Hence, whether pOASL exerts antiviral actions by an alternative mechanism needs to be investigated. It has been documented that several ISGs exert antiviral effects via various signaling pathways (12, 27, 28).

To define the signaling pathway affected by pOASL, we conducted the dual-luciferase reporter assay. The results indicated that pOASL increased the promoter activities of IFN-β (Fig. 5A), ISRE (Fig. 5B), and NF-κB (Fig. 5C), implying that pOASL can enhance the IFN-β, ISRE, and NF-κB pathways.

pOASL interacts with the RNA sensor MDA5. It has been reported that hOASL interacts with RIG-I and enhances the RIG-I-mediated type I IFN signaling (12). CSFV has been verified to trigger the RIG-I and MDA5-dependent signaling pathway (29). Indeed, our data also demonstrated that pOASL activates the type I IFN response (Fig. 5). Based on above results, we hypothesized that pOASL plays a role in the RIG-I or MDA5-mediated signaling. The interaction between pOASL and pRIG-I or pMDA5 was investigated using Co-IP assay. The results showed that the Flag-tagged pOASL interacted with the Myc-tagged pMDA5 but not with the Myc-tagged pRIG-I (Fig. 6A and B). Furthermore, the Myc-tagged pMDA5 was shown to coimmunoprecipitate with the Flag-tagged pOASL but not the Flag-tagged pOAS1 (Fig. 6C). GST pulldown assay further confirmed that GST-pOASL but not GST interacted with pMDA5 (Fig. 6D). Furthermore, the pOASL-pMDA5 interaction was confirmed by confocal assay (Fig. 6E). To exclude the involvement of RNA in the pOASL-pMDA5 interaction, the cell lysates were
treated with RNases A and T1 followed by Co-IP. The results indicated that the
interaction of pOASL and pMDA5 was independent of RNA (Fig. 6F).

**pOASL enhances the MDA5-mediated type I IFN response.** Because
pOASL interacts with pMDA5 and activates the IFN-β signaling pathway, we
endeavored to investigate whether the action of pOASL is mediated via the
pMDA5 pathway. We tried to determine whether pOASL coexpressed with
pMDA5 affects the transcription of IFN-β and ISGs in PK-15 cells. The results
showed that the coexpression of pOASL with pMDA5 but not with pRIG-I
markedly upregulated the mRNA transcriptional levels of IFN-β, myxovirus
resistance protein 1 (Mx1) and guanylate-binding protein 1 (GBP1) (Fig. 7A–C).
Collectively, these studies support that pOASL positively regulates the
pMDA5-mediated type I IFN signaling.

**Antiviral activity of pOASL is abolished in MDA5-knockdown cells.**
MDA5 is one of the primary sensors of viral RNA for CSFV infection (29). In
light of the fact that pOASL displayed antiviral activity against CSFV depending
on the pMDA5-mediated type I IFN signaling (Fig. 7), we silenced the pMDA5
expression using siRNAs to further confirm the results. Marked reduction of
pMDA5 expression was observed in the cells silenced for pMDA5 (Fig. 8A). As
expected, significant reduction in CSFV replication was observed in the
siNC-transfected PK-pOASL cells. Upon silencing of pMDA5, however, pOASL
almost lost its ability to inhibit CSFV replication in PK-pOASL cells, as
assessed by the viral genomic copy numbers and the viral titers (Fig. 8B and
C). In contrast, in the cells with knockdown of pRIG-I (Fig. 8D), the anti-CSFV
activity of pOASL was not affected (Fig. 8E and F). These data indicate that
the anti-CSFV activity of pOASL is dependent on pMDA5.

**DISCUSSION**
In this study, we clarified the antiviral mechanism of pOASL by which pOASL
bound to pMDA5 and enhanced the pMDA5-mediated antiviral signaling. We
found that pOASL efficiently suppressed CSFV growth in PK-15 cells (Figs. 1
and 2). Interestingly, we found that pOASL expression was activated in vitro at earlier times after viral infection, and then declined (Fig. 3) and the anti-CSFV effects of pOASL depend on the enhancement of the type I IFN response but not on the canonical RNase L pathway (Figs. 4 and 5). Notably, we demonstrated that pOASL interacts with pMDA5 and enhances the pMDA5-mediated type I IFN antiviral signaling to antagonize the replication of CSFV (Figs. 6–8). Taken together, these findings suggest that pOASL is an anti-CSFV ISG via the pMDA5-mediated type I IFN signaling pathway.

The IFN-inducible OASs are important components for the antiviral activity of IFNs and involved in other cellular processes, including cell growth, apoptosis, gene regulation, cell differentiation, RNA splicing, and DNA replication (30, 31). In humans, there are three functional OAS genes (OAS1–3), including 8 to 10 OAS isoforms due to alternative mRNA splicing (32). In mice, in addition to OAS2 and OAS3, there are eight OAS1 genes (33, 34). The pig or cattle genome contains three OAS genes, OAS1, OAS2, and OASL. The human OASL gene has one OAS unit and two ubiquitin-like repeats on the C-terminus (35). While two tandemly duplicated OASL genes have been identified in the dog genome, only a single OASL ortholog has been found in the cattle or pig genome (30, 36). The porcine and bovine OASL genes contain a premature stop codon, resulting in truncated proteins that lack the typical C-terminal ubiquitin domains. Indeed, we demonstrated that the porcine OASL gene contains only a single OAS domain (37).

Viral infection usually upregulates the expression of ISGs, which inhibit viral replication. It has been reported that both OAS1 and OASL mRNA levels peaked around 10 h after influenza A infection, and then declined, and the decline observed in virus-infected cells was probably due to the synthesis of viral inhibitors of the innate immune system, such as the influenza A NS1 protein (38, 39). Similarly, our data also showed that the mRNA level of pOASL peaked around 12 h after infection, and then declined. Specifically, the mRNA level of pOASL was lower at 48 hpi than that at 24 hpi at an MOI of 0.1 (Fig.
3B), and lower at an MOI of 1 than at an MOI of 0.1 (Fig. 3C) at 6 hpi. A possible explanation is that CSFV has the ability to escape the antiviral action of pOASL. It has been shown that OASL is rapidly induced upon viral infection through the interferon regulatory factor 3 (IRF3) as well as IFN signaling (11, 40), and the CSFV Npro interacts with IRF3 and induces its proteasomal degradation to prevent type I IFN induction (41, 42). Thus, high doses of CSFV may increase the chance of IRF3 degradation, leading to the decreased expression of pOASL.

With hundreds of ISGs induced by IFN, we assume that any step of the viral life cycle (entry, uncoating, transcription, translation, assembly, or egress) could be targeted for inhibition (43). The OAS family is a well-known molecule that regulates the early phase of viral infection (44), and the mouse Oas1b acts mainly on the replication stages of WNV (17). In this study, we investigated the antiviral kinetics of pOASL on CSFV replication. The data demonstrated that the viral genomic RNA copy numbers were decreased in PK-pOASL cells than those in PK-EGFP cells from 6 to 12 hpi (Fig. 1E). However, our experiments do not define the exact stage(s) of the CSFV life cycle that pOASL targets. Thus, we need to further investigate pOASL targets the exact stage of CSFV replication using different methods in the future.

hOASL interacts with RIG-I and enhances the RIG-I-mediated antiviral signaling (12). Similar to hOASL, the mouse Oasl2 exhibits antiviral activity and its loss results in the reduction of IFN signaling and enhances RNA virus replication (12). However, it has been demonstrated that the mouse Oasl1 binds to the 5′-UTR of IRF7 to inhibit IRF7 translation, thus reducing IFN induction and increasing viral replication (45). In this study, we showed that interaction of pOASL with pMDA5 enhances the pMDA5-mediated type I IFN signaling pathway which increases IFN induction and efficiently inhibits CSFV replication.

The OAS proteins bind to virus-derived dsRNA and result in an allosteric activation of OAS, giving rise to the synthesis of 2-5A from ATP, which
activates RNase L. Upon 2-5A binding, RNase L dimerizes and degrades cellular as well as viral RNA in the cell, causing the inhibition of protein synthesis (14, 15). The OAS/RNase L system is a component of the IFN-regulated innate antiviral immunity. Our study revealed that pOASL inhibits viral replication at the early stages of the CSFV life cycle. We also demonstrated that pOASL contained only a single N-terminal OAS-like domain and lacked C-terminal double ubiquitin domains. To investigate the sensitivity of CSFV to the pOASL antiviral pathway at molecular level, we supposed that pOASL exerting antiviral effect is associated with OAS/RNase L system based on sequence alignment. The results showed that RNase L knockdown by specific siRNAs did not result in the change of CSFV replication. Thus we draw a conclusion that pOASL exerts antiviral effects independent of the canonical RNase L pathway, but depending on the uncanonical pathway.

It has been indicated that hOASL plays a critical role in resistance to HCV infection in vivo (46). The hOASL protein displays antiviral activity and activates RIG-I by mimicking polyubiquitin. HCV and CSFV are both single-stranded, positive-sense RNA viruses belonging to the family Flaviviridae. Hence, we supposed that pOASL exerts antiviral actions also depending on activation of the type I IFN signal pathway. In this study, we revealed that pOASL could enhance the type I IFN signaling pathway. hOASL interacts and colocalizes with RIG-I, and enhances RIG-I signaling via its C-terminal ubiquitin-like domain (12). Our study indicated that pOASL interacts with pMDA5 but not pRIG-I, and enhanced pMDA5-mediated IFN induction. Our study reveals a mechanism by which pOASL exerts an antiviral action via the MDA5-mediated IFN signaling pathway.

In conclusion, we demonstrate that pOASL is an anti-CSFV ISG that depends on the MDA5-mediated type I IFN signaling pathway.

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FIGURE LEGENDS

FIG 1 pOASL inhibits CSFV growth. (A) Cell viability of the cell lines stably overexpressing pOASL. (B–D) The influence of overexpression of pOASL on CSFV replication. PK-pOASL and PK-EGFP cells were infected with the Shimen strain of CSFV at a multiplicity of infection (MOI) of 0.1 and 1 for 24 and 48 h. Viral titers in the harvested supernatants were titrated and determined by median tissue culture infective doses (TCID_{50}) per milliliter (B). The genomic RNA copy numbers of CSFV in the supernatants were quantified using a real-time reverse transcription-PCR (C). The expression of N^pro in the cell lysates was examined by Western blotting (WB) using a rabbit anti-N^pro polyclonal antibody (PAb) (1:500) (D). GAPDH was included as an internal reference control. The N^pro expression in the cell lysates was evaluated using the Odyssey application software version 3.0. (E) The anti-CSFV activity of pOASL at earlier times after viral infection. PK-pOASL or PK-EGFP cells were infected with CSFV at a multiplicity of infection of 1 and harvested at 0, 1, 3, 6, 9, 12, 15, 18, 24, 36, and 48 h postinfection. The intracellular genomic copy numbers of CSFV were quantified by a real-time RT-PCR. Each sample was run in triplicate. Bars represent standard deviations. *, P < 0.05; **, P < 0.01.

FIG 2 Knockdown of pOASL enhances CSFV growth. (A and B) Knockdown efficiency of the pOASL by siRNAs. PK-15 cells transfected with a mixture of siRNAs (sipOASLs) targeting three regions of pOASL or siNC were collected at 36 h posttransfection (hpt). The knockdown efficiency of pOASL was assessed by a real-time reverse transcription-PCR (qRT-PCR) (A). For Western blotting (WB) analysis (B), PK-15 cells were initially pretreated with 0.1 μg of IFN-β (catalog no. RP0011S-005; Kingfisher) for 12 h and transfected with sipOASLs or siNC. At 36 hpt, pOASL and GAPDH were probed using a rabbit anti-OASL polyclonal antibody (catalog no. SAB2101671; Sigma) (1:500) and a mouse anti-GAPDH monoclonal antibody (1:1,000), respectively. (C) The influence of knockdown of pOASL on rCSFV-Fluc
replication. Pretreated PK-15 cells with sipOASLs or siNC (0.2 μM) for 36 h were infected with rCSFV-Fluc at an MOI of 0.1 for 48 h, and luciferase activities were measured using a luciferase reporter assay (Promega) and expressed in relative light units (RLU). (D–F) The effects of knockdown of pOASL on CSFV Shimen replication. PK-15 cells pretreated with sipOASLs or siNC (0.2 μM) for 36 h were infected with the CSFV Shimen at an MOI of 0.1 for 24 and 48 h. The expression levels of Npro and GAPDH were analyzed as described above (D). The supernatants were collected at 48 h postinfection and the CSFV genomic copy numbers assessed using qRT-PCR (E). The extracellular viral titers were determined and expressed as TCID_{50}/ml (F). Each sample was run in triplicate. Bars represent standard deviations. *, P < 0.05; **, P < 0.01.

FIG 3 pOASL is upregulated upon CSFV infection. (A) The expression of pOASL in PK-15 cells treated with IFN-β (catalog no. RP0011S-005; Kingfisher). The expression of pOASL was examined by a real-time RT-PCR (qRT-PCR) in IFN-β-treated PK-15 cells. (B and C) The expression of pOASL in CSFV-infected PK-15 cells. PK-15 cells were infected with the Shimen strain of CSFV at a multiplicity of infection (MOI) of 0.1 and tested for the expression of pOASL at 0, 3, 6, 12, 24, and 48 h postinfection (hpi) (B), or infected at an MOI of 0, 0.01, 0.1, and 1 and tested at 6 hpi (C) by the qRT-PCR. (D) The expression of pOASL in CSFV-infected pigs. Three healthy pigs negative for CSFV were inoculated with 10^{5} median tissue culture infective doses (TCID_{50}) CSFV Shimen. Various organs (heart, liver, spleen, lung, kidney, tonsils, and lymph nodes) were collected at 3 d postinoculation. The expression of pOASL in the different organs of the infected pigs was assessed by the qRT-PCR. Each sample was run in triplicate. Bars represent standard deviations. *, P < 0.05; **, P < 0.01.

FIG 4 The anti-CSFV effects of pOASL are independent of the RNase L
(A and B) The knockdown efficiency of the RNase L expression by siRNAs. PK-15 cells transfected with a mixture of three siRNAs (sipRNaseL-400, -599, or -750) targeting different regions of RNase L or siNC were harvested at 36 h posttransfection (hpt). The knockdown efficiency of RNase L was checked by a real-time reverse transcription-PCR (qRT-PCR) (A). For Western blotting analysis (B), PK-15 cells were pretreated with 0.1 μg of IFN-β (catalog no. RP0011S-005; Kingfisher) for 12 h and transfected with sipRNaseLs or siNC. At 36 hpt, RNaseL and GAPDH were probed using a rabbit anti-RNaseL polyclonal antibody (catalog no. ab191392; Abcam) (1:500) and a mouse anti-GAPDH monoclonal antibody (1:1,000), respectively. (C) The influence of knockdown of RNase L on the antiviral activity of pOASL against rCSFV-Fluc infection. PK-pOASL or PK-EGFP pretreated with siRNaseLs or siNC (0.2 μM) for 36 h were inoculated with rCSFV-Fluc at a multiplicity of infection (MOI) of 0.1 for 48 h, and the luciferase activities were assayed using the luciferase reporter assay system (Promega) and expressed as relative light units (RLU). (D and E) The effect of silencing RNase L on the antiviral activity of pOASL against the CSFV Shimen strain. PK-pOASL or PK-EGFP cells were infected with the Shimen strain at an MOI of 0.1 for 48 h after pretreated with siRNaseLs or siNC (0.2 μM) for 36 h. The viral titers were determined in the harvested supernatants at 48 h postinfection and presented as 50% tissue culture infectious doses (TCID₅₀) per milliliter (D) and The CSFV genomic RNA copy numbers were determined by using the qRT-PCR (E). Each sample was run in triplicate. Bars represent standard deviations. *, P < 0.05; **, P < 0.01.

FIG 5 pOASL enhances the type I IFN response. HEK293T cells were cotransfected with pHA-pOASL or pCMV-HA (Vector) plus pIFN-β-Fluc and pTK-Rluc (A), pISRE-Fluc and pTK-Rluc (B), or pNF-κB-Fluc and pTK-Rluc (C) for 24 h, treated with 20 hemagglutinin units (HAUs)/ml Sendai virus (SeV) for 24 h or left untreated, and assayed for the luciferase activities using a
dual-luciferase reporter assay (Promega). pTK-Rluc was used as an internal reference. The protein levels of HA-pOASL in HEK293T cells were measured by Western blotting (WB). GAPDH served as a loading control. Each sample was run in triplicate. Bars represent standard deviations. *, P < 0.05; **, P < 0.01.

**FIG 6** pOASL interacts with the MDA5 RNA sensor. (A–C) Coimmunoprecipitation (Co-IP) assay. HEK293T cells were cotransfected with pFlag-pOASL and either pMyc-pRIG-I, pMyc-pMDA5, or pCMV-Myc. The cell were harvested and subjected to Co-IP assay using anti-Flag (A) and anti-Myc monoclonal antibodies (MAb) (B). HEK293T cells were cotransfected with pMyc-pMDA5 and either pFlag-pOASL, pFlag-pOAS1, or p3xFlag-CMV-10. The cell lysates were subjected to Co-IP assay using anti-Flag MAb (C). The precipitated proteins were tested using the anti-Flag and anti-Myc antibodies by Western blotting. (D) GST pulldown assay. The GST and GST-pOASL fused proteins expressed in *Escherichia coli* BL21 were affinity-purified with glutathione beads and incubated with the Myc-pMDA5 protein. The proteins bound to beads were analyzed by immunoblotting using a mouse anti-GST polyclonal antibody (1:2,000) and a mouse anti-Myc MAb (1:1,000). (E) Confocal assay. BHK-21 cells were cotransfected with pFlag-pOASL and pMyc-pMDA5 and subjected to confocal assay. (F) The pMDA5-pOASL interaction is independent of RNA. HEK293T cells were cotransfected with pFlag-pOASL and pMyc-pMDA5. The cell lysates were harvested and treated with RNases A/T1. Co-IP was performed using an anti-Flag MAb (1:1,000). Lane*, protein molecular weight marker.

**FIG 7** pOASL enhances the MDA5-mediated type I IFN signaling pathway. (A–C) pOASL upregulated the transcriptional levels of IFN-β and ISGs in PK-15 cells. PK-15 cells were cotransfected with pHA-OASL (0, 0.1, 0.2, or 0.5 μg) plus pMyc-pMDA5 (0.5 μg), pMyc-pRIG-I (0.5 μg), or pCMV-Myc (0.5 μg).
for 24 h, the transcriptional levels of IFN-β (A), Mx1 (B), and GBP1 (C) in the
cells were determined by qRT-PCR assay. Each sample was run in triplicate.
Bars represent standard deviations. *, P < 0.05; **, P < 0.01.

FIG 8 The antiviral activity of pOASL is dependent on MDA5. (A) Knockdown efficiency of pMDA5 by siRNAs. PK-15 cells were transfected with
the mixture of three siRNAs (sipMDA5s) to silence pMDA5 by targeting
different regions or control siRNA (siNC) and collected at 36 h posttransfection
(hpt). The efficiency of pMDA5 knockdown was examined by a real-time
reverse transcription-PCR (qRT-PCR). (B and C) Silencing pMDA5 expression
reduces the antiviral activity of pOASL against CSFV. PK-pOASL or PK-EGFP
cells were transfected with sipMDA5s or siNC (0.2 μM) for 24 h and infected
with CSFV at an MOI of 0.1 for 48 h. Total RNA was extracted from the
harvested cells and quantified to evaluate CSFV replication by qRT-PCR (B).
The viral titers in the harvested supernatants were titrated and determined by
median tissue culture infective doses (TCID₅₀) per milliliter (C). (D) Knockdown
efficiency of pRIG-I expression by siRNAs. (E and F) Silencing pRIG-I
expression does not affect the anti-CSFV activity of pOASL as assessed by
the viral genomic copy numbers (E) and the viral titers (F). Each sample was
run in triplicate. Bars represent standard deviations. NS, not significant; *, P <
0.05; **, P < 0.01.
FIG. 2
FIG. 3
FIG. 6
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