2-23-2024

African swine fever virus early protein pI73R suppresses the type-I IFN promoter activities

Danh C. Lai

Jayeshbhai Chaudhari

Hiep L.X. Vu

Virus Research 343 (2024) 199342

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

African swine fever virus early protein pI73R suppresses the type-I IFN promoter activities

Danh Cong Lai [a], Jayeshbhai Chaudhari [b], Hiep L.X. Vu [a,b,*]

[a] Nebraska Center for Virology, University of Nebraska-Lincoln, United States
[b] Department of Animal Science, University of Nebraska-Lincoln, United States

ARTICLE INFO

Keywords:
cGAS-STING signaling
Interferon suppression
Viral virulence gene
Z-DNA binding protein

ABSTRACT

African swine fever virus is known to suppress type-I interferon (IFN) responses. The main objective of this study was to screen early-expressed viral genes for their ability to suppress IFN production. Out of 16 early genes examined, I73R exhibited robust suppression of cGAS-STING-induced IFN-β promoter activities, impeding the function of both IRF3 and NF-κB transcription factors. As a result, I73R obstructed IRF3 nuclear translocation following the treatment of cells with poly(dA:dT), a strong inducer of the cGAS-STING signaling pathway. Although the I73R protein exhibits structural homology with the Zα domain binding to the left-handed helical form of DNA known as Z-DNA, its ability to suppress cGAS-STING induction of IFN-β was independent of Z-DNA binding activity. Instead, the α3 and β1 domains of I73R played a significant role in suppressing cGAS-STING induction of IFN-β. These findings offer insights into the protein's functions and support its role as a virulence factor.

1. Introduction

African swine fever virus (ASFV) is the causative agent of a devastating swine disease. Although disease outcomes vary based on ASFV strain virulence, infectious dose, infection route, and host genetics (Salguero, 2020), pigs infected with highly virulent ASFV strains causing the current pandemics often face mortality rates of 100 %. Primary measures to control ASF include early detection, culling infected animals, and enhancing biosecurity measures (Gallardo et al., 2019). Recently, Vietnam has approved the use of two live attenuated ASF vaccines, yet their utilization remains limited.

ASFV is the singular member of the *Asfivirus* genus within the *Asfaviridae* family (Alonso et al., 2018). The virus has a double-stranded DNA genome between 170 and 190 kbp which encodes for over 150 proteins (Dixon et al., 2013). Despite possessing a DNA genome, ASFV replicates within perinuclear viral factories in the cytoplasm of infected cells (Jouvenet et al., 2004). Monocytes and macrophages are the primary target cells for ASFV replication (Gómez-Villamandos et al., 2013). Additionally, viral antigens are found in hepatocytes, endothelial cells, renal tubular epithelial cells, and neutrophils of infected pigs, indicating that these cells are also permissive for ASFV (Meloni et al., 2022).

ASFV is highly sensitive to the antiviral effects of type-I interferons (IFNs) (Fan et al., 2020). However, the virus has evolved diverse mechanisms to suppress IFN induction. Early investigations have identified the multi-gene families MGF360 and MGF505 as critical suppressors of type-I IFNs (Afonso et al., 2004). Deletion of the MGF360 and MGF505 genes from highly virulent ASFV strains completely attenuates the virus (Afonso et al., 1998; O'Donnell et al., 2015). Remarkably, ASFV strains lacking the MGF360 and MGF505 genes provide full protection against a lethal challenge with the virulent parental virus (O'Donnell et al., 2015). The initial findings underscore the significance of viral genes that suppress IFN production as major viral virulence factors and essential targets for the rationale design of live-attenuated vaccine candidates.

Cyclic GMP-AMP synthase (cGAS) is an essential cytosolic DNA sensor (Motwani et al., 2019). Upon recognizing dsDNA, cGAS activates the production of 2′3′ cyclic GMP–AMP (cGAMP), which triggers the activation of Stimulator of Interferon Genes (STING) (Decout et al., 2021). Activated STING traffics from the endoplasmic reticulum (ER) to the Golgi, where it recruits and serves as a docking site for TANK-binding kinase 1 (TBK1) auto-phosphorylation. (Chen et al., 2016; Decout et al., 2021). Subsequently, phosphorylated TBK1 phosphorylates and activates interferon regulatory factor 3 (IRF3) and nuclear factor kappa-light-chain-enhancer of activated B (NF-κB), which

* Corresponding author.
E-mail address: hiepvu@unl.edu (H.L.X. Vu).

https://doi.org/10.1016/j.virusres.2024.199342
Received 22 January 2024; Received in revised form 21 February 2024; Accepted 23 February 2024

translocate to the nucleus to induce type-I IFN production. (Liu et al., 2021; Ni et al., 2018).

Many proteins of ASFV were discovered to play vital roles in downregulating the host's innate immunity. For instance, EP364R and C129R (Dodantenna et al., 2022), MGF505-7R (Li et al., 2021), MGF505-11R (Yang et al., 2021), MGF360-13L (Luo et al., 2023), L83L (Cheng et al., 2023), E184L (Zhu et al., 2023), p17 (Zheng et al., 2022) and H240R (Ye et al., 2023) inhibit IFN-β production by degrading 2′, 3′-cGAMP and STING. Besides, A137R (Sun et al., 2022b), DP96R (Wang et al., 2018), I215L (Huang et al., 2021), MGF505-3R (Cheng et al., 2022) and MGF360-11L (Yang et al., 2022) antagonize the production IFN-β by promoting the degradation of TBK1 or inhibiting the phosphorylation of TBK1.

For DNA viruses, immediate early and early genes are the first set of viral genes transcribed after viral infection. These gene products regulate both host and virus gene expression replication (Stinski and Meier, 2007). Moreover, they also play pivotal roles in counteracting the host's innate immune responses. In this study, we employed an IFN-β promoter-based luciferase assay to screen a subset of ASFV early genes for their ability to suppress cGAS/STING-mediated activation of the IFN-β promoter. We identified I73R, a small viral protein, as a potent suppressor of the cGAS/STING signaling pathway. Additionally, our findings indicate that pI73R inhibits cGAS/STING activation of the IFN-β promoter by interfering with both the IRF3 and NF-κB transcription factors.

2. Materials and method

2.1. Cells, transfection, antibodies and reagent

HEK-293T cell line (ATCC CRL-3216) and Hela cell line (ATCC CCL-2) were grown in Dulbecco's modified Eagle's medium (Gibco, Rockford, IL, USA) supplemented with 10 % fetal bovine serum (Gibco, Rockford, IL, USA) at 37 °C in a 5 % CO_2 atmosphere. Polyethylenimine (PEI) and Lipofectamine 3000 (Invitrogen, Waltham, MA, USA) were used for DNA transfection. Poly(dA:dT) was purchased from InvivoGen (San Diego, CA, USA).

The mouse monoclonal antibodies specific to Flag-tag were obtained from GenScript Biotech (Piscataway, NJ, USA). The mouse monoclonal GFP and β-actin antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The horseradish peroxidase (HRP) and Alexa Fluor 594 labeled anti-mouse IgG secondary antibodies were from Invitrogen (Waltham, MA, USA).

2.2. Plasmids

Sequences of ASFV early genes based on the ASFV Georgia 2007/1 genome (GenBank: FR682468.2) were codon-optimized for optimal expression in human cells. A flag-tag sequence (DYKDDDDK) was fused to the carboxyl terminal of the genes to facilitate protein detection. The genes were then chemically synthesized using a commercial DNA synthesis service (Integrated DNA Technologies, San Diego, CA, USA) and subcloned into the pCI vector (Promega, Madison, WI, USA). The I73R deletion mutants were constructed using site-directed mutagenesis. The sequence authenticity of the ASFV genes and the I73R deletion mutants was confirmed through sequencing.

The IFN-β promoter-Firefly luciferase (pIFN-β-FLuc) plasmid was obtained from Addgene (plasmid ID:102597). The control plasmid pRL-TK (encoding Renilla luciferase) was obtained from Promega (Madison, WI, USA). The human cGAS and STING genes were PCR-amplified from the plasmids obtained from Addgene (plasmid ID: 86675 for cGAS and ID: 102598 for STING). The HA tag (YPYDVPDYA) was fused to the amino-terminal of the cGAS and STING to facilitate protein detection. IRF3-GFP was PCR amplified from a plasmid obtained from Addgene (plasmid ID: 127663) and cloned into the pCI vector. The IRF3-5D-GFP plasmid was generated using site-directed mutagenesis, according to a previous report (Irie et al., 2012). The NF-κB p65 was constructed by PCR amplifying from the cDNA of HEK-293T cells and fused in-frame into the GFP gene and cloning into the pCI vector. The 5X-NF- κB response element (RE) and 4X-IRF3-RE reporter plasmids were constructed by inserting five and four tandem repeats of NF- κB-RE and IRF3-RE into the pGL4.3 vector.

2.3. Luciferase reporter assays

To identify ASFV genes capable of suppressing cGAS/STING-induced IFN-β promoter activation, HEK-293T cells were cultured in 96-well plates and transfected with 30 ng of either a control plasmid (pCI) or individual ASFV early gene-expressing plasmids. Additionally, cells were co-transfected with 10 ng of the pIFN-β-Fluc plasmid (encoding firefly luciferase), 2 ng of pRL-TK plasmid (Renilla luciferase for normalization), 30 ng of pCI-cGAS, and 30 ng of pCI-STING plasmids. At 24 h post-transfection (hpt), cells were lysed using lysis buffer, and luciferase activities were measured utilizing a dual luciferase kit (Promega, Madison, WI, USA), following the manufacturer's instructions.

To confirm the I73R ability to suppress IFN-β promoter, Hela cells in 24-well plates were transfected with varying amounts of either a control plasmid (pCI) or pCI-I73R plasmid. Simultaneously, the cells were co-transfected with 50 ng of the pIFN-β-Fluc and 10 ng of pRL-TK plasmid. Following 24 hpt, the cells were stimulated by transfection with 500 ng of poly(dA:dT). After 12 hours, luciferase activities were quantified using a dual luciferase kit.

To investigate the inhibitory effects of I73R on the IRF3 or NF-κB transcription factors, HEK-293T cells were seeded in 24-well plates and transfected with various amounts of either the control plasmid (pCI) or the pCI-I73R plasmid, together with 50 ng of 4X-IRF3-RE or the 5X-NF-κB-RE plasmids, along with 150 ng of pCI-cGAS, 150 ng of pCI-STING plasmids and 10 ng of pRL-TK plasmid. Another set of experiments involved HEK-293T cells seeded in 24-well plates and transfected with different amounts of the control plasmid (pCI) or the pCI-I73R plasmid co-transfected with either 150 ng of pCI-IRF3-5D-GFP or pCI-p65-GFP along with 50 ng of the pIFN-β-Fluc and 10 ng of pRL-TK plasmid. At 24 hpt, luciferase activities were measured using a dual luciferase kit (Promega, Madison, WI, USA).

In each instance, the Firefly luciferase value for every treatment was normalized by the Renilla luciferase value. The promoter activity was expressed as the fold change in the normalized Firefly luciferase value of the stimulated cells relative to that of unstimulated cells.

2.4. Quantitative reverse transcriptase PCR (qRT-PCR) assay

The total RNA was extracted using the Direct-zol™ RNA MicroPrep kit (Zymo research, Orange, CA, USA) and cDNA was prepared using the LunaScript® RT SuperMix Kit (New England Biolabs, Beverly, MA, USA). qPCR reaction was conducted using TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific, USA) with 120 ng of cDNA and the primers and probe set. The primers and probes detecting human β-Actin (Hs01077958_s1) and human IFN-β (Hs01060665_g1) were obtained from Invitrogen (Waltham, MA, USA). β-Actin was used as an internal reference. The relative IFN-β mRNA levels were normalized to β-Actin mRNA levels and calculated using $2^{-\Delta\Delta CT}$ method.

2.5. Immunofluorescence and confocal microscopy

Hela cells cultured in 4-well culture chambers (BD Biosciences, San Jose, CA, USA) were transfected with 500 ng of pCI-I73R-Flag and 200 ng pCI-IRF3-GFP for 24 h. The cells were then stimulated by transfection with 500 ng poly(dA:dT). Twelve hours later, the cells were fixed in 4 % paraformaldehyde for 10 min at room temperature, followed by permeabilization in 0.5 % Triton X-100 at room temperature for 15 min. The cells were incubated with the anti-Flag antibodies at 4 °C overnight,

followed by the Goat Anti-Mouse IgG H+L (Alexa Fluor® 488) for 1 h at room temperature. Subsequently, the cells were counterstained with DAPI (Thermo Scientific, Waltham, MA, USA) for 5 min. The cells were visualized with a Nikon A1R-Ti2 confocal system (Nikon, Melville, NY, USA).

2.6. Western blot analysis

Cells were lysed with RIPA buffer (ThermoFisher Scientific, Waltham, MA, USA) supplemented with phosphatase and protease inhibitors for 30 min, followed by centrifuged clarification at 15,000 × g

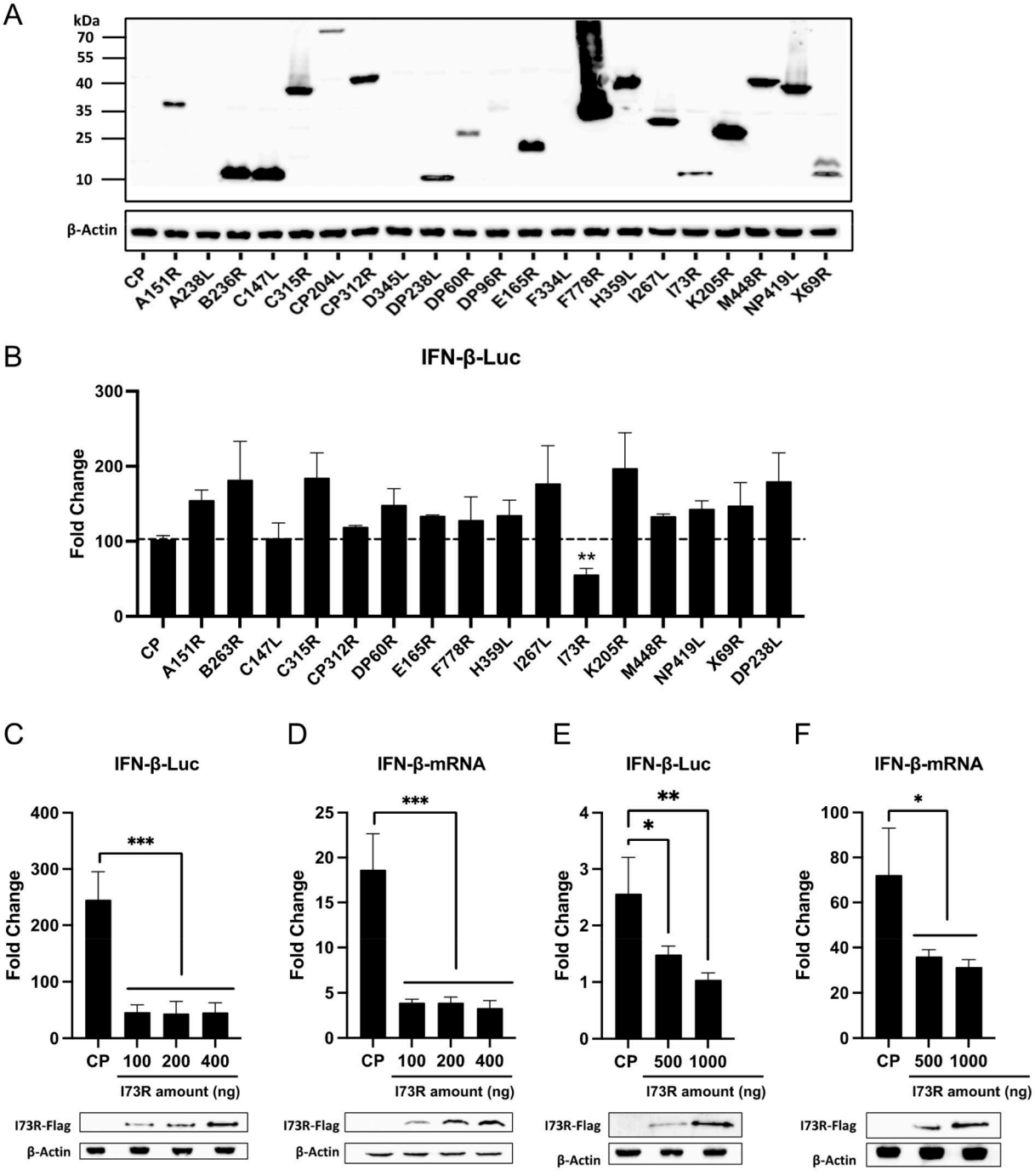

Fig. 1. Screening of ASFV early genes for inhibition of IFN-β luciferase expression. (A) HEK-293T cells were transfected with indicated plasmids. At 24 hpt, whole cell lysates were collected and resolved in an SDS-PAGE. Flag-tagged ASFV proteins were detected by immunoblotting with an anti-flag antibody. (B) HEK-293T cells in 96-well plates were co-transfected with the pIFN-β-Fluc, pRL-TK, pcGAS-HA, pSTING-HA, and either the control plasmid or one of the ASFV early genes. At 24 hpt, luciferase activities were determined using a dual luciferase assay kit. (C-D) HEK-293T cells in 24-well plates were co-transfected with the pIFN-β-Fluc, pRL-TK, pcGAS-HA, pSTING-HA, and increasing amounts of the pCI-I73R-Flag plasmid. The control plasmid was added to the transfection mixture to keep the total DNA amount constant. At 24 hpt, luciferase activities (C) and endogenous IFN-β mRNA (D) were quantified. (E-F) Hela cells in 24-well plates were cotransfected with the pIFN-β-Fluc, pRL-TK, and increasing amounts of the pCI-I73R-Flag plasmid. At 24 hpt, the cells were stimulated with poly(dA:dT) for 12 h, and luciferase activities (E) and endogenous IFN-β mRNA (F) were quantified. For panels B-F, data are expressed as the fold changes in Fluc activities or endogenous IFN-β mRNA in stimulated cells relative to unstimulated cells. The bottom panels show the expression of the I73R-Flag protein and β-actin, which was used as a loading control. Asterisks indicate statistically significant differences compared to the control plasmid. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. CP: control plasmid

for 15 min at 4 °C. The cell lysates were mixed with 4x Laemmli Sample Buffer (Bio-Rad, Hercules, CA, USA), and boiled for 5 min. The samples were separated by SDS-PAGE and transferred onto the Immobilon®-P transfer Membrane (Millipore Sigma, Burlington, MA, USA). The membranes were blocked with 5 % nonfat dried milk in PBS (Bio-Rad, Hercules, CA, USA) for 2 h at room temperature. Subsequently, the membranes were incubated with primary antibodies at 4 °C overnight. After three washes in PBS-T20, the membranes were incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. After washing, the membranes were incubated with Clarity™ Western ECL Substrate (Bio-Rad, Hercules, CA, USA) for 5 min, and then protein bands were visualized and imaged by the ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA). The β-Actin was detected as the internal control.

2.7. Statistical analysis

All experiments were performed at least three independent times. All data were expressed as the mean ± standard deviations. Graphs and statistical analyses were performed using GraphPad Prism 9.0. Data were analyzed using a one-way Analysis of Variance (ANOVA), followed by Tukey's multiple comparison test.

3. Results

3.1. ASFV I73R gene exhibited strong suppression of IFN-β promoter activity

Twenty-one early ASFV genes highly expressed within 6 hours post-infection (Cackett et al., 2020; Cackett et al., 2022; Ju et al., 2021) were selected and cloned into a mammalian expression plasmid. Five genes, A238L, CP204L, D345L, DP96R, and F334L, did not express after 24 hours post-transfection in HEK-293T cells (Fig. 1A) and were excluded from subsequent studies.

A luciferase-based reporter assay was utilized to identify ASFV genes capable of suppressing IFN-β promoter activity. In this assay, HEK-293T cells were cotransfected with individual ASFV genes, the IFN-β promoter plasmid, and the cGAS and STING plasmids. Among the 16 genes assessed, only the I73R gene exhibited significant suppression of the INF-β promoter activity compared to the control plasmid (Fig. 1B). Increasing I73R amounts did not further enhance its ability to suppress the IFN-β promoter activity (Fig. 1C).

To confirm the IFN-β promoter suppression effects of the I73R gene, HEK-293T cells were stimulated by cotransfection with the cGAS and STING plasmid, and different amounts of the I73R plasmid and the endogenous IFN-β mRNA levels were quantified. Cells transfected with the I73R plasmid exhibited significantly lower IFN-β mRNA abundances compared to those transfected with a control plasmid (Fig. 1D). The transfection of higher amounts of the I73R plasmid did not further reduce the endogenous IFN-β mRNA levels.

To further confirm the suppression effects of I73R, we used the poly (dA:dT) as a stimulator of IFN-β promoter activities instead of cGAS and STING plasmids. Hela cells, which retain a functional cGAS-STING signaling pathway (Wu et al., 2013), were cotransfected with the I73R expression plasmid and IFN-β promoter plasmid, followed by stimulation with poly(dA:dT). Compared to the control plasmid, the presence of I73R resulted in a significant reduction in luciferase activity (Fig. 1E) and endogenous IFN-β mRNA (Fig. 1F).

Together, the results strongly indicate that the ASFV I73R gene can effectively suppress the production of IFN-β triggered by the cGAS-STING signaling pathway.

3.2. ASFV I73R inhibits both IRF3- and NF-κB-mediated IFN-β promoter actiation

The IFN-β promoter is activated by both NF-κB and IRF3 transcription factors. Therefore, luciferase-based reporter assays were employed to identify which transcription factors were suppressed by the I73R gene. Cotransfection of HEK-293T cells with the 4X-IRF3-RE luciferase reporter, together with cGAS and STING plasmids, resulted in an approximately 3000-fold increase in luciferase activity (Fig. 2A). In the presence of I73R, the luciferase activity was reduced to approximately 1000 folds. Similarly, when HEK-293T cells were cotransfected with 5X-NF-κB-RE luciferase, cGAS, and STING plasmids, an average of 200-fold luciferase activity was observed (Fig. 2B). However, in the presence of I73R, the luciferase activity was diminished, resembling levels seen in unstimulated control cells.

To confirm the inhibitory effects of I73R on IRF3 and NF-κB, we stimulated cells with the IRF3-5D, a constitutively active form of IRF3 (Grandvaux et al., 2002), and p65, a component of the NF-κB. The IRF3-5D plasmid, when cotransfected with IFN-β luciferase plasmid, induced over 300 folds of luciferase activity (Fig. 2C). When IRF3-5D and ASFV I73R were cotransfected to cells, the luciferase activity was dramatically reduced to approximately 50 folds. Similarly, the p65-GFP plasmid also strongly induced IFN-β-driven expression of luciferase activity. The presence of I73R significantly downregulated the IFN-β promoter activation induced by p65-GFP (Fig. 2D).

Collectively, the results indicate that the I73R gene effectively suppressed both IRF3- and NF-κB-mediated IFN-β promoter activity.

3.3. ASFV I73R inhibits IRF3 nucleus translocation

After activation, phosphorylated IRF3 relocates to the cell nucleus and binds to the IRF3-RE within the IFN-β promoter to induce IFN-β expression (Honda et al., 2006). Since I73R inhibits IRF3-mediated IFN-β promoter activation, we sought to determine if it could block IRF3 nuclear translocation. To explore this, Hela cells were co-transfected with the IFR3-GFP plasmid and either the I73R-Flag plasmid or a control plasmid, followed by poly(dA:dT) stimulation. As expected, unstimulated IRF3-GFP predominantly localized in the cell cytoplasm. Upon poly(dA:dT) stimulation, IRF3-GFP translocated into the cell nucleus (Fig. 3). However, in the presence of I73R, IRF3-GFP remained sequestered in the cytoplasm, indicating a potential hindrance to its nuclear translocation.

3.4. ASFV I73R inhibits the cGAS-STING signaling pathway independent of its Z-DNA binding activity

ASFV I73R is a high-affinity Z-DNA binding protein (Liu et al., 2023; Sun et al., 2022a). Three residues, Asn44, Tyr48, and Trp68, are critical for its Z-DNA binding activity (Sun et al., 2022a). To evaluate the importance of Z-DNA binding activity in suppressing the IFN-β promoter, we created an I73R mutant (I73R-mut) by simultaneously replacing these three active Z-DNA binding residues with alanine. In the IFN-β luciferase reporter assay, the I73R-mut construct showed similar suppression of IFN-β promoter activity induced by cGAS/STING compared to the wild-type I73R construct (Fig. 4A). Similarly, cells transfected with the I73R-mut construct exhibited similar levels of endogenous IFN-β mRNA as those transfected with the wild-type I73R upon stimulated with cGAS and STING (Fig. 4B). Thus, the Z-DNA binding activity of I73R is not essential for its suppression of IFN-β promoter activity induced by cGAS and STING.

3.5. ASFV I73R domains associated with its suppression of the cGAS-STING signaling pathway

The I73R protein comprises three α helices and three β pleated sheet domains (Sun et al., 2022a). To identify the domains associated with the IFN-β promoter suppression, we generated six deleted mutants by sequentially removing each domain from the I73R gene (Fig. 5A). Upon transfection into HEK-293T cells, these mutants displayed varying levels of protein expression (Fig. 5B). Notably, mutants Δα1 and Δα2 showed

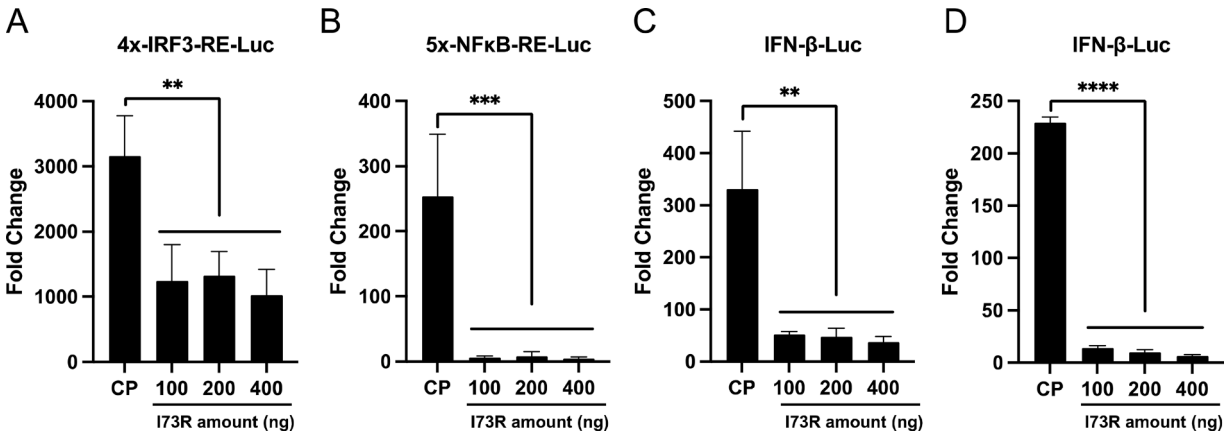

Fig. 2. ASFV I73R inhibits IRF3- and NF-κB-mediated IFN-β promoter activity. (A-B) HEK-293T cells in 24-well plates were co-transfected with either the p4X-IRF3-RE-Fluc (A) or the p5X-NFκB-RE-Fluc (B) plasmid, together with pRL-TK, pcGAS-HA, pSTING-HA, and increasing amounts of the pCI-I73R-Flag plasmid. (C-D) HEK-293T cells were cotransfected with either IRF3-5D-GFP (C) or p65-GFP (D), and the pIFN-β-Fluc, pRL-TK, pcGAS-HA, pSTING-HA, and increasing amounts of the pCI-I73R-Flag plasmid. At 24 hpt, luciferase activities were quantified using a Dual-Luciferase assay kit. Data are expressed as the fold changes in Fluc activities in stimulated cells relative to unstimulated cells. Asterisks indicate statistically significant differences compared to the control plasmid. $^{**}p \leq 0.01$, *** $p \leq 0.001$, $^{****}p < 0.0001$. CP: control plasmid.

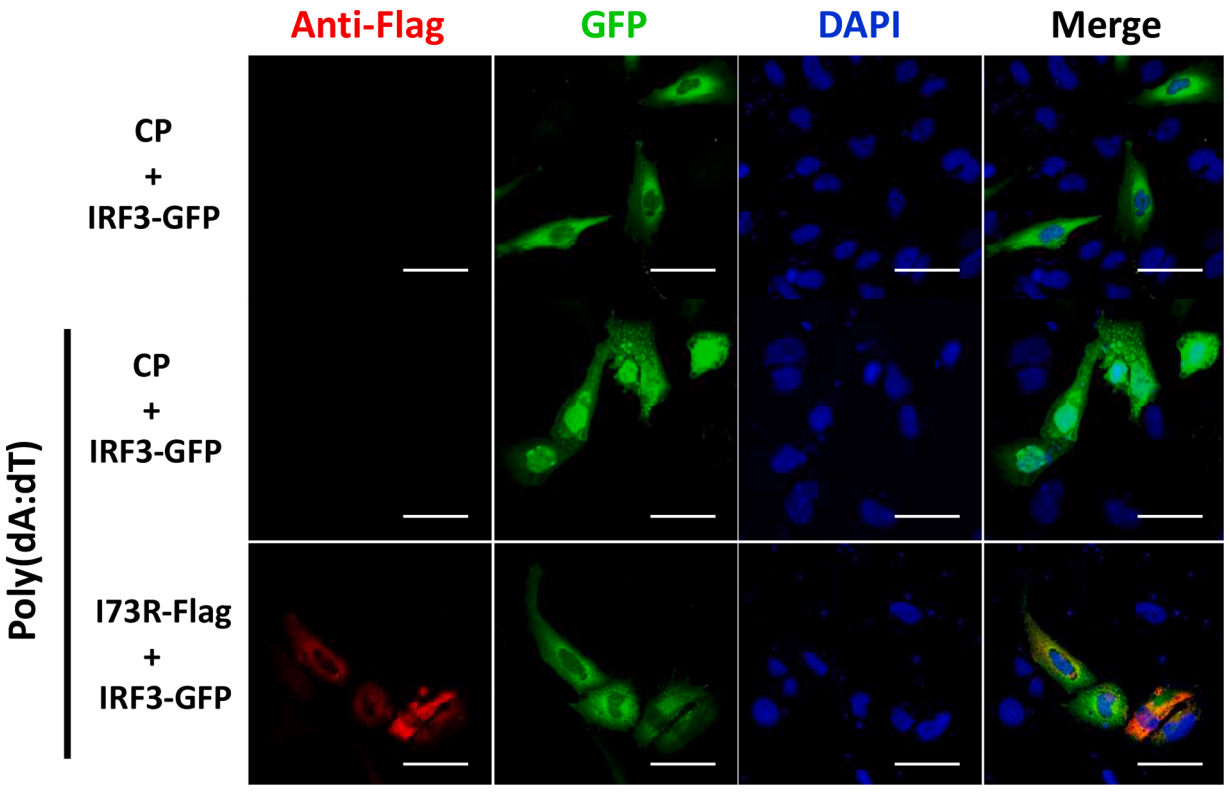

Fig. 3. ASFV I73R interferes with IRF3 nuclear translocation. Hela cells in 4-well culture chambers were transfected with either pI73R-Flag or the control plasmid (CP) along with pIRF3-GFP plasmid. At 24 hpt, the cells were stimulated with poly(dA:dT). At 12 h post-stimulation, the cells were fixed and subjected to an indirect immunofluorescence assay using an anti-Flag antibody (red). Cell nuclei were stained with DAPI (blue). Bars, 50 μm.

no detectable protein expression, highlighting the critical role of these domains in protein expression and stability. Additionally, mutants Δα3, Δβ1, and Δβ2 exhibited reduced protein expression compared to the wild-type I73R construct. When tested in the luciferase-based reporter assay, two mutants, Δα3 and Δβ1, completely lost the ability to suppress the IFN-β promoter activity (Fig. 5C and D). This was evident in the similar levels of luciferase activity and endogenous IFN-β mRNA in cells transfected with these mutants compared to those transfected with the control plasmid. These findings suggest the potential involvement of Δα3 and Δβ1 domains in suppressing IFN-β promoter activity. However, it is plausible that deleting these domains could have led to decreased protein expression and protein structural alterations, potentially affecting its ability to suppress the IFN-β promoter.

4. Discussion

ASFV contains a dsDNA genome which can be sensed by cGAS. ASFV infection of swine macrophages does not result in a substantial IFN production (Afonso et al., 2004). Therefore, we were interested in the identification of ASFV genes capable of suppressing the cGAS/STING

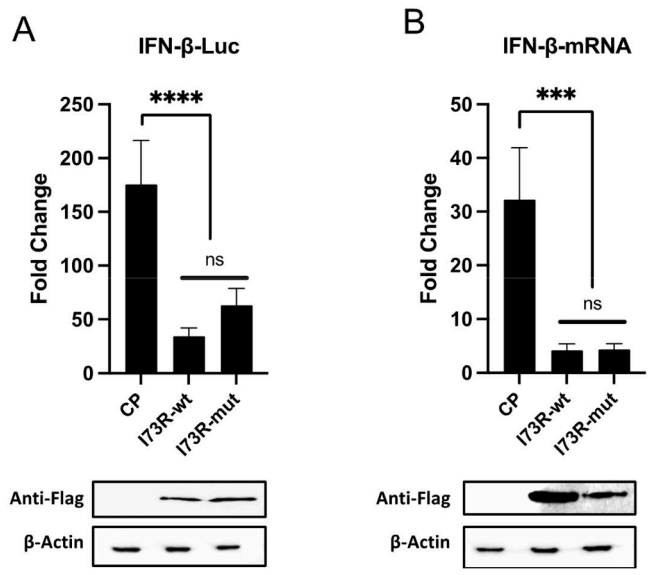

Fig. 4. ASFV I73R inhibits the cGAS-STING signaling pathway independent of its interaction with Z-DNA. HEK-293T cells in 24-well plates were cotransfected with pIFN-β-Fluc, pRL-TK, along with pcGAS-HA, pSTING-HA, and either the wild-type (wt) I73R-Flag or the mutant (mut) pI73R-Flag plasmid. At 24 hpt, luciferase activities (A) and endogenous IFN-β mRNA (B) were quantified. Data are expressed as the fold changes in Fluc activities or IFNβ mRNA abundance in stimulated cells relative to unstimulated cells. The bottom panels show the expression of the I73R-flag protein and β-actin. Asterisks indicate statistically significant differences compared to the control plasmid. **** $p<0.0001$. CP: control plasmid.

signaling pathway. Using the luciferase reporter assay, we screened 16 ASFV genes expressed early after infection and identified I73R as a potent suppressor of the cGAS/STING signaling axis. Our further studies revealed that I73R protein suppresses cGAS/STING-induced IFN-β promoter activity by impeding the action of both IRF3 and NF-κB transcription factors. The α3 and β1 domains of I73R play a crucial role in IFN-β promoter suppression.

I73R is a small protein composed of 72 amino acids with a molecular mass of 8.9 kDa (Sun et al., 2022a). During the early stages post-infection, I73R predominantly localizes within the cell nucleus (Sun et al., 2022a). As the infection progresses, the protein relocates to the cell cytoplasm. Previous studies demonstrated that I73R is not essential for viral replication in cell cultures (Liu et al., 2023). However, deletion of the I73R gene from the genome of a virulent ASFV strain completely abolished the virus virulence when tested in pigs. Notably, pigs infected with the recombinant ASFV lacking the I73R gene exhibited elevated levels of IL-6 and TNF-α, the inflammatory cytokines associated with the NF-κB signaling pathway, compared to those infected with the wild-type parental virus (Liu et al., 2023). This suggests a potential role of the I73R protein in inhibiting the NF-κB signaling pathway. In the present study, we demonstrate that I73R suppresses the cGAS/STING signaling pathway, thus providing further insights into the biological function of this protein. Given the pivotal role of interferons in suppressing virus infection, the ability of the I73R protein to hinder cGAS/STING-induced IFN-β promoter activity further substantiates its significance as an essential virulence factor.

Structural analysis revealed that the I73R protein might belong to the family of Zα-domain-containing proteins that bind left-handed helical conformation known as Z-DNA (Sun et al., 2022a). Several cellular and viral proteins are found to contain the Zα domain. Of them, the Vaccinia virus (VACV) E3L protein has been well characterized. E3L is an important viral virulence gene due to its ability to confer resistance to the antiviral effects of IFNs (White and Jacobs, 2012). Particularly, E3L is found to suppress RNase L and PKR, the two IFN-induced genes (White and Jacobs, 2012). In contrast to the IFN-resistant wild-type VACV, the mutant VACV lacking E3L (VACVΔE3L) is sensitive to IFN treatment and complete loss of pathogenicity (Kim et al., 2003). The E3L protein contains two domains: the Zα domain in the amino-terminus and the dsRNA binding domain in the carboxyl-terminus (Xiang et al., 2002), both of which are important for the suppression of IFNs. Different from VACV E3L, the ASFV I73R protein contains only the Zα domain and does not contain any additional domain. It binds all forms of nucleic acid, including sRNA, dsRNA, ssDNA, and dsDNA (Liu et al., 2023). Interestingly, it exhibited significantly higher binding affinity to ssRNA than to dsDNA. Three amino acid residues, Asn44, Tyr48, and Trp68, are essential for the protein to bind dsDNA (Sun et al., 2022a). However, amino acid residues critical for its binding to other forms of nucleic acids are not known, and their contribution to viral pathogenicity is not clear. For VACV E3L, the dsDNA binding affinity of the Zα domain plays an important role in the viral pathogenicity (Kim et al., 2003). Point mutations in the Zα domain that decrease its binding affinity to Z-DNA correlate with a decrease in neurovirulence in mice (Kim et al., 2003). On the contrary, for the ASFV I73R, mutating the DNA binding residues does not abolish its IFN antagonistic activities. Thus, the dsDNA binding affinity does not seem important for the ASFV I73R protein to suppress the cGAS/STING signaling pathway. In contrast, two domains, namely α3 and β1 are found to be necessary to suppress IFN-β promoter. Further studies are needed to understand how the α3 and β1 domains are associated with the suppression of IFN-β promoter.

We have attained conclusive results demonstrating the suppressive role of I73R in cGAS/STING induction of IFN-β production. However, ASFV harbors multiple genes capable of suppressing INF production. Therefore, it is essential to determine whether solely deleting the I73R gene from the ASFV genome is sufficient to abolish the virus's ability to suppress IFN induction. Previous studies revealed that pigs infected with a recombinant ASFV-ΔI73R virus displayed increased levels of TNF-α, a proinflammatory cytokine triggered by NF-κB signaling activation (Liu et al., 2023). Our current study revealed that I73R inhibits IFN-β promoter activity by suppressing the NF-κB transcription factor. Therefore, we anticipate that recombinant ASFV-ΔI73R viruses might indeed trigger robust IFN responses in cultured cells and infected pigs.

Funding

This study was funded by the Agriculture and Food Research Initiative competitive grant number 2022-67015-37264 of the USDA National Institute for Food and Agriculture.

Declaration

When writing this article, the authors utilized ChatGPT and Grammarly tools to enhance grammatical correctness and improve the clarity of the text. Following the use of these tools, the authors thoroughly reviewed and edited the content as necessary. The authors assume full responsibility for the publication's content.

CRediT authorship contribution statement

Danh Cong Lai: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jayeshbhai Chaudhari:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Hiep L.X. Vu:** Conceptualization, Methodology, Project administration, Funding acquisition, Resources, Formal analysis, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

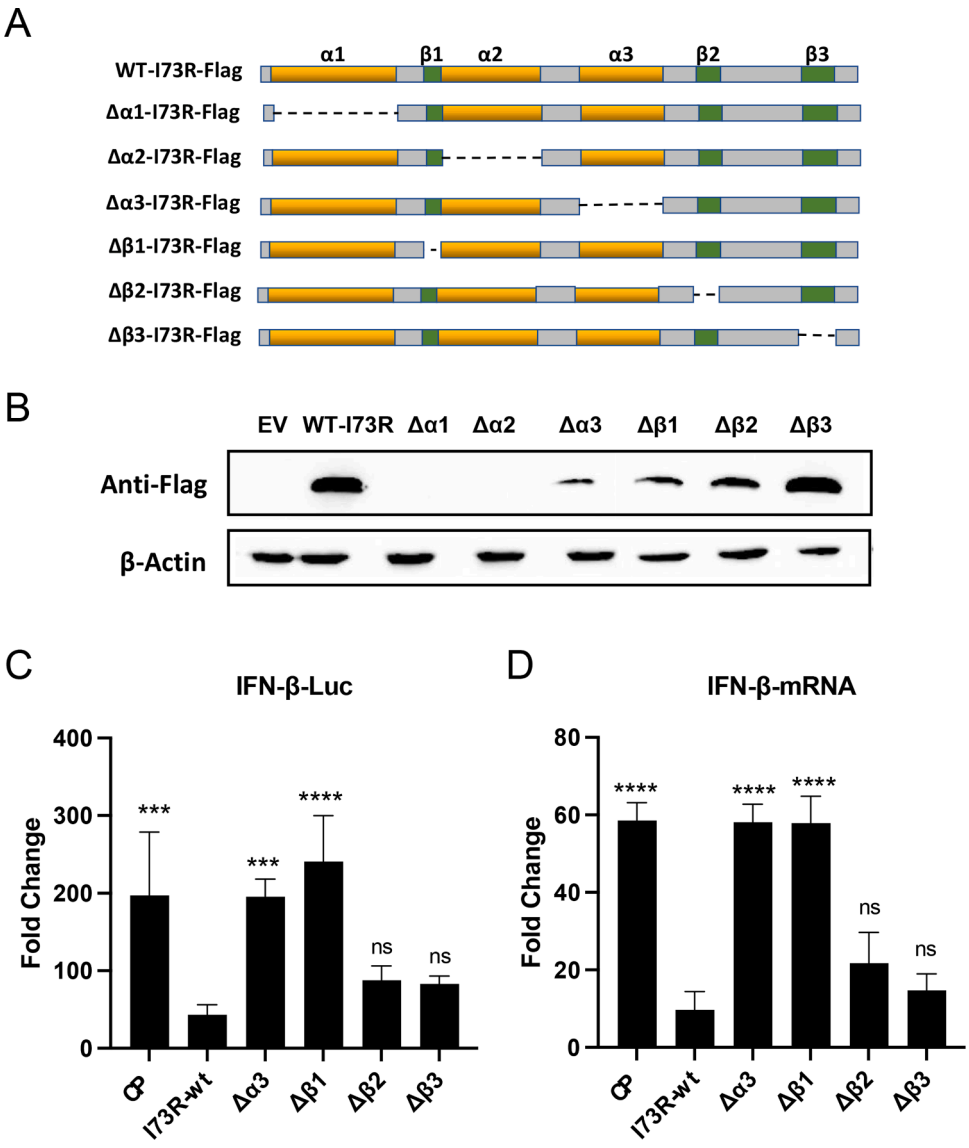

Fig. 5. I73R protein domains essential for its suppression of the IFN-β promoter. (A) Schematic representation of six domain-deleted constructs of the I73R proteins. (B) Protein expression of the I73R deletion mutants in HEK-293T cells detected by immunoblotting with an anti-flag antibody. (C-D) HEK-293T cells in 24-well plates were transfected with pIFN-β-Fluc, pRL-TK, pcGAS-HA, pSTING-HA, and either I73R-wild-type (wt) or individual domain-deletion constructs. At 24 hpt, luciferase activities (C) and endogenous IFNβ mRNA (D) were quantified. Data are expressed as the fold changes in Fluc activities or IFNβ mRNA abundance in stimulated cells relative to unstimulated cells. Asterisks indicate statistically significant differences compared to the control plasmid. *** $p \leq 0.001$, **** $p \leq 0.0001$. CP: control plasmid.

Data availability

Data will be made available on request.

References

Afonso, C., Piccone, M., Zaffuto, K., Neilan, J., Kutish, G., Lu, Z., Balinsky, C., Gibb, T., Bean, T., Zsak, L., 2004. African swine fever virus multigene family 360 and 530 genes affect host interferon response. J. Virol. 78, 1858–1864.

Afonso, C., Zsak, L., Carrillo, C., Borca, M., Rock, D., 1998. African swine fever virus NL gene is not required for virus virulence. J. Gen. Virol. 79, 2543–2547.

Alonso, C., Borca, M., Dixon, L., Revilla, Y., Rodriguez, F., Escribano, J.M., Consortium, I. R., 2018. ICTV virus taxonomy profile. Asfarviridae J. Gen. Virol. 99, 613–614.

Cackett, G., Matelska, D., Sýkora, M., Portugal, R., Malecki, M., Bähler, J., Dixon, L., Werner, F., 2020. The African swine fever virus transcriptome. J. Virol. 94, e00119–e00120.

Cackett, G., Portugal, R., Matelska, D., Dixon, L., Werner, F., 2022. African swine fever virus and host response: transcriptome profiling of the Georgia 2007/1 strain and porcine macrophages. J. Virol. 96 e01939-01921.

Chen, Q., Sun, L., Chen, Z.J., 2016. Regulation and function of the cGAS–STING pathway of cytosolic DNA sensing. Nat. Immunol. 17, 1142–1149.

Cheng, M., Kanyema, M.M., Sun, Y., Zhao, W., Lu, Y., Wang, J., Li, X., Shi, C., Wang, J., Wang, N., 2023. African swine fever virus L83L negatively regulates the cGAS-STING-mediated IFN-I pathway by recruiting tollip to promote STING autophagic degradation. J. Virol. 97 e01923-01922.

Cheng, M., Luo, J., Duan, Y., Yang, Y., Shi, C., Sun, Y., Lu, Y., Wang, J., Li, X., Wang, J., 2022. African swine fever virus MGF505-3R inhibits cGAS-STING-mediated IFN-β pathway activation by degrading TBK1. Anim. Dis. 2, 13.

Decout, A., Katz, J.D., Venkatraman, S., Ablasser, A., 2021. The cGAS–STING pathway as a therapeutic target in inflammatory diseases. Nat. Rev. Immunol. 21, 548–569.

Dixon, L.K., Chapman, D.A., Netherton, C.L., Upton, C., 2013. African swine fever virus replication and genomics. Virus Res. 173, 3–14.

Dodantenna, N., Ranathunga, L., Chathuranga, W.G., Weerawardhana, A., Cha, J.-W., Subasinghe, A., Gamage, N., Haluwana, D., Kim, Y., Jheong, W., 2022. African swine fever virus EP364R and C129R target cyclic GMP-AMP to inhibit the cGAS-STING signaling pathway. J. Virol. 96 e01022-01022.

Fan, W., Jiao, P., Zhang, H., Chen, T., Zhou, X., Qi, Y., Sun, L., Shang, Y., Zhu, H., Hu, R., 2020. Inhibition of African swine fever virus replication by porcine type I and type II interferons. Front. Microbiol. 11, 1203.

Gallardo, C., Fernández-Pinero, J., Arias, M., 2019. African swine fever (ASF) diagnosis, an essential tool in the epidemiological investigation. Virus Res. 271, 197676.

Gómez-Villamandos, J., Bautista, M., Sánchez-Cordón, P., Carrasco, L., 2013. Pathology of African swine fever: the role of monocyte-macrophage. Virus Res. 173, 140–149.
Grandvaux, N., Servant, M.J., tenOever, B., Sen, G.C., Balachandran, S., Barber, G.N., Lin, R., Hiscott, J., 2002. Transcriptional profiling of interferon regulatory factor 3 target genes: direct involvement in the regulation of interferon-stimulated genes. J. Virol. 76, 5532–5539.
Honda, K., Takaoka, A., Taniguchi, T., 2006. Type I inteferon gene induction by the interferon regulatory factor family of transcription factors. Immunity 25, 349–360.
Huang, L., Xu, W., Liu, H., Xue, M., Liu, X., Zhang, K., Hu, L., Li, J., Liu, X., Xiang, Z., 2021. African swine fever virus pI215L negatively regulates cGAS-STING signaling pathway through recruiting RNF138 to inhibit K63-linked ubiquitination of TBK1. J. Immunol. 207, 2754–2769.
Irie, T., Kiyotani, K., Igarashi, T., Yoshida, A., Sakaguchi, T., 2012. Inhibition of interferon regulatory factor 3 activation by paramyxovirus V protein. J. Virol. 86, 7136–7145.
Jouvenet, N., Monaghan, P., Way, M., Wileman, T., 2004. Transport of African swine fever virus from assembly sites to the plasma membrane is dependent on microtubules and conventional kinesin. J. Virol. 78, 7990–8001.
Ju, X., Li, F., Li, J., Wu, C., Xiang, G., Zhao, X., Nan, Y., Zhao, D., Ding, Q., 2021. Genome-wide transcriptomic analysis of highly virulent African swine fever virus infection reveals complex and unique virus host interaction. Vet. Microbiol. 261, 109211.
Kim, Y.-G., Muralinath, M., Brandt, T., Pearcy, M., Hauns, K., Lowenhaupt, K., Jacobs, B. L., Rich, A., 2003. A role for Z-DNA binding in vaccinia virus pathogenesis. Proc. Natl. Acad. Sci. U.S.A. 100, 6974–6979.
Li, D., Yang, W., Li, L., Li, P., Ma, Z., Zhang, J., Qi, X., Ren, J., Ru, Y., Niu, Q., 2021. African swine fever virus MGF-505-7R negatively regulates cGAS–STING-mediated signaling pathway. J. Immunol. 206, 1844–1857.
Liu, X., Ao, D., Jiang, S., Xia, N., Xu, Y., Shao, Q., Luo, J., Wang, H., Zheng, W., Chen, N., 2021. African swine fever virus A528R inhibits TLR8 mediated NF-κB activity by targeting p65 activation and nuclear translocation. Viruses 13, 2046.
Liu, Y., Shen, Z., Xie, Z., Song, Y., Li, Y., Liang, R., Gong, L., Di, D., Liu, J., Liu, J., 2023. African swine fever virus I73R is a critical virulence-related gene: a potential target for attenuation. Proc. Natl. Acad. Sci. U.S.A. 120, e2210808120.
Luo, J., Cheng, M., Duan, Y., Xing, X., Lu, M., Sun, Y., Shi, C., Wang, J., Lu, Y., Li, X., 2023. African swine fever virus encoded protein MGF360-13L inhibits cGAS-STING-mediated IFN-I signaling pathway. Gene 874, 147490.
Meloni, D., Franzoni, G., Oggiano, A., 2022. Cell lines for the development of African swine fever virus vaccine candidates: an update. Vaccines 10, 707.
Motwani, M., Pesiridis, S., Fitzgerald, K.A., 2019. DNA sensing by the cGAS–STING pathway in health and disease. Nat. Rev. Genet. 20, 657–674.
Ni, G., Ma, Z., Damania, B., 2018. cGAS and STING: at the intersection of DNA and RNA virus-sensing networks. PLoS Path. 14, e1007148.
O'Donnell, V., Holinka, L.G., Gladue, D.P., Sanford, B., Krug, P.W., Lu, X., Arzt, J., Reese, B., Carrillo, C., Risatti, G.R., 2015. African swine fever virus Georgia isolate harboring deletions of MGF360 and MGF505 genes is attenuated in swine and confers protection against challenge with virulent parental virus. J. Virol. 89, 6048–6056.
Salguero, F.J., 2020. Comparative pathology and pathogenesis of African swine fever infection in swine. Front. Vet. Sci. 7, 282.
Stinski, M.F., Meier, J.L., 2007. Immediate-early viral gene regulation and function. In: Arvin, A., Campadelli-Fiume, G., Mocarski, E., Moore, P.S., Roizman, B., Whitley, R., Yamanishi, K. (Eds.), Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge.
Sun, L., Miao, Y., Wang, Z., Chen, H., Dong, P., Zhang, H., Wu, L., Jiang, M., Chen, L., Yang, W., 2022a. Structural insight into African swine fever virus I73R protein reveals it as a Z-DNA binding protein. Transbound. Emerg. Dis. 69, e1923–e1935.
Sun, M., Yu, S., Ge, H., Wang, T., Li, Y., Zhou, P., Pan, L., Han, Y., Yang, Y., Sun, Y., 2022b. The A137R protein of African swine fever virus inhibits type I interferon production via the autophagy-mediated lysosomal degradation of TBK1. J. Virol. 96 e01957-01921.
Wang, X., Wu, J., Wu, Y., Chen, H., Zhang, S., Li, J., Xin, T., Jia, H., Hou, S., Jiang, Y., 2018. Inhibition of cGAS-STING-TBK1 signaling pathway by DP96R of ASFV China 2018/1. Biochem. Biophys. Res. Commun. 506, 437–443.
White, S.D., Jacobs, B.L., 2012. The amino terminus of the vaccinia virus E3 protein is necessary to inhibit the interferon response. J. Virol. 86, 5895–5904.
Wu, J., Sun, L., Chen, X., Du, F., Shi, H., Chen, C., Chen, Z.J., 2013. Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. Science 339, 826–830.
Xiang, Y., Condit, R.C., Vijaysri, S., Jacobs, B., Williams, B.R., Silverman, R.H., 2002. Blockade of interferon induction and action by the E3L double-stranded RNA binding proteins of vaccinia virus. J. Virol. 76, 5251–5259.
Yang, K., Huang, Q., Wang, R., Zeng, Y., Cheng, M., Xue, Y., Shi, C., Ye, L., Yang, W., Jiang, Y., 2021. African swine fever virus MGF505-11R inhibits type I interferon production by negatively regulating the cGAS-STING-mediated signaling pathway. Vet. Microbiol. 263, 109265.
Yang, K., Xue, Y., Niu, H., Shi, C., Cheng, M., Wang, J., Zou, B., Wang, J., Niu, T., Bao, M., 2022. African swine fever virus MGF360-11L negatively regulates cGAS-STING-mediated inhibition of type I interferon production. Vet. Res. 53, 1–12.
Ye, G., Liu, H., Liu, X., Chen, W., Li, J., Zhao, D., Wang, G., Feng, C., Zhang, Z., Zhou, Q., 2023. African Swine Fever Virus H240R Protein Inhibits the Production of Type I Interferon through Disrupting the Oligomerization of STING. J. Virol. e00577-00523.
Zheng, W., Xia, N., Zhang, J., Cao, Q., Jiang, S., Luo, J., Wang, H., Chen, N., Zhang, Q., Meurens, F., 2022. African swine fever virus structural protein p17 inhibits cGAS-STING signaling pathway through interacting with STING. Front. Immunol. 13, 941579.
Zhu, Z., Li, S., Ma, C., Yang, F., Cao, W., Liu, H., Chen, X., Feng, T., Shi, Z., Tian, H., 2023. African swine fever virus E184L protein interacts with innate immune adaptor STING to block IFN production for viral replication and pathogenesis. J. Immunol. 210, 442–458.