The A137R Protein of African Swine Fever Virus Inhibits Type I Interferon Production via the Autophagy-Mediated Lysosomal Degradation of TBK1

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ABSTRACT African swine fever is a lethal hemorrhagic disease of pigs caused by African swine fever virus (ASFV), which greatly threatens the pig industry in many countries. Deletion of virulence-associated genes to develop live attenuated ASF vaccines is considered to be a promising strategy. A recent study has revealed that the A137R gene deletion results in ASFV attenuation, but the underlying mechanism remains unknown. To elucidate the mechanism of the A137R gene regulating ASFV virulence, an ASFV mutant with the A137R gene deleted (ASFV-DΔA137R) was generated based on the wild-type ASFV HLJ/2018 strain (ASFV-WT). Using transcriptome sequencing analysis, we found that ASFV-DΔA137R induced higher type I interferon (IFN) production in primary porcine alveolar macrophages (PAMs) than did ASFV-WT. Overexpression of the A137R protein (pA137R) inhibited the activation of IFN-β or IFN-stimulated response element. Mechanistically, pA137R interacts with TANK-binding kinase 1 (TBK1) and promotes the autophagy-mediated lysosomal degradation of TBK1, which blocks the nuclear translocation of interferon regulator factor 3, leading to decreased type I IFN production. Taken together, our findings clarify that pA137R negatively regulates the cGAS-STING-mediated IFN-β signaling pathway via the autophagy-mediated lysosomal degradation of TBK1, which highlights the involvement of pA137R regulating ASFV virulence.

IMPORTANCE African swine fever (ASF) is a lethal viral disease of pigs caused by African swine fever virus (ASFV). No commercial vaccines and antiviral treatments are available for the prevention and control of the disease. Several virulence-associated genes of ASFV have been identified, but the underlying attenuation mechanisms are not clear. Compared with the virulent parental ASFV, the A137R gene-deleted ASFV mutant promoted the expression of type I interferon (IFN) in primary porcine alveolar macrophages. Further analysis indicated that the A137R protein negatively regulated the cGAS-STING-mediated IFN-β signaling pathway through targeting TANK-binding kinase 1 (TBK1) for autophagy-mediated lysosomal degradation. This study not only facilitates the understanding of ASFV immunoevasion strategies, but also provides new clues to the development of live attenuated ASF vaccines.

KEYWORDS African swine fever virus, A137R protein, IFN-β, TBK1, autophagy-mediated lysosomal pathway

African swine fever (ASF) is a highly contagious and hemorrhagic disease of pigs in many countries. From the first outbreak in Kenya in 1921, ASF has been prevalent in Europe, South America, and the Caribbean. The disease has caused significant
economic losses due to its high mortality and wide spread since its emergence in China in 2018 (1–3). African swine fever virus (ASFV), the causative agent of ASF, belongs to the *Asfarviridae* family. As a nucleocytoplasmic large DNA virus, ASFV encodes more than 160 proteins, some of which are involved in viral DNA replication and repair, virion morphogenesis, and evasion of innate immune responses (4, 5).

Pathogen-associated molecular patterns (PAMPs) are sensed by the pathogen pattern recognition receptors (PRRs) to trigger the innate immune response. Upon binding to cytosolic double-stranded DNA (dsDNA), cyclic GMP-AMP (cGAMP) synthase (cGAS) recruits ATP and GTP to generate cGAMP. As a second messenger, cGAMP activates the cGAS-stimulator of interferon (IFN) genes (STING). Activated STING recruits TANK-binding kinase 1 (TBK1) to phosphorylate interferon regulator 3 (IRF3), leading to IFN-β production. Compared with the attenuated ASFV NH/P68 strain, the virulent Armenia/07 strain suppresses IFN-β production via the cGAS-STING pathway (6). It has been shown that the ability of ASFV to downregulate IFN production is associated with its virulence (7–11). So far, several ASFV proteins regulating IFN production have been identified. The ASFV A276R protein inhibits IFN-β production through targeting IRF3 (12); the I239L protein inhibits double-stranded RNA (dsRNA)-stimulated activation of IFN-β and CCL5, whereas the underlying mechanism remains to be elucidated (13); the DP96R protein negatively regulates type I IFN production by inhibiting the phosphorylation of TBK1 (14). The members of multigene families 360 (MGF360) and MGF505 are considered to be IFN antagonists (8–11, 15–17). The MGF360-12L protein disrupts the interaction between p65 and importin alpha to suppress the nuclear translocation of NF-κB (15), and the MGF505-11R protein degrades STING to antagonize type I IFN production via the lysosomal, ubiquitin-proteasome, and autophagy pathways (16). In addition, the MGF505-7R protein is involved in the pathogenicity of ASFV through targeting multiple adaptors of innate immune signaling pathways (9–11).

Most studies focus on IFN production antagonized by viral early-expressed proteins. However, the late-expressed viral proteins also block IFN production. The ASFV E120R protein directly interacts with IRF3 and disrupts the recruitment of IRF3 to TBK1, suppressing the activation of IRF3 (18). Interestingly, a series of late-expressed genes are also associated with ASFV virulence, such as E184L (19), A137R (20), EP402R (21, 22), and I177L (23). More specifically, an ASFV mutant with the A137R gene deleted is attenuated and able to confer protection against the challenge of the parental ASFV Georgia2010 (ASFV-G) (20). However, the mechanism of the A137R protein (pA137R or p11.5) regulation of ASFV virulence remains unclear. Therefore, we analyzed the differentially expressed genes (DEGs) of the primary porcine alveolar macrophages (PAMs) infected with the wild-type ASFV HLJ/2018 strain (ASFV-WT) sharing a high identity with ASFV-G or the A137R gene-deleted ASFV mutant (ASFV-ΔA137R) by transcriptome sequencing (RNA-seq) analysis. Compared with ASFV-WT, ASFV-ΔA137R infection significantly increased type I IFN production in PAMs. Mechanistically, pA137R inhibits the cGAS-STING-mediated IFN-β signaling pathway by interacting with and degrading TBK1 in a dose-dependent manner via the autophagy-mediated lysosomal pathway, leading to the inhibition of IRF3 nuclear translocation. Altogether, our results dissect for the first time the underlying mechanism of pA137R suppressing type I IFN production, which is associated with ASFV virulence.

**RESULTS**

**ASFV-ΔA137R induces higher type I IFN production in PAMs than does ASFV-WT.** To explore the underlying molecular mechanism of pA137R regulation of ASFV virulence, we generated ASFV-ΔA137R based on the virulent ASFV HLJ/2018 strain by homologous recombination. The A137R gene was replaced by a cassette containing the enhanced green fluorescent protein (EGFP) gene under the control of the ASFV p72 promoter (p72EGFP). ASFV-ΔA137R was purified after 10 rounds of limiting dilution based on the EGFP expression (Fig. 1A), and the purified mutant virus was identified by Western blotting (Fig. 1B). As expected, pA137R was detectable in the PAMs infected with ASFV-WT, but not with ASFV-ΔA137R, while p72 could be detected in the PAMs...
FIG 1 Differential expression pattern in A137R gene-deleted ASFV mutant (ASFV-ΔA137R)-infected primary porcine alveolar macrophages (PAMs) by transcriptome sequencing (RNA-seq) analysis. (A and B) Identification of ASFV-ΔA137R by fluorescence microscopy (A) and Western blotting (B). (C) Replication characteristics of ASFV-ΔA137R and the wild type ASFV HLJ/2018 strain (ASFV-WT) in PAMs. PAMs were infected with ASFV-ΔA137R or ASFV-WT at a multiplicity of infection (MOI) of 0.01 and collected at the indicated time points to determine the viral titers by 50% hemadsorption doses (HAD50). (D) Venn diagram of the differentially expressed genes (DEGs) in the ASFV-ΔA137R- versus ASFV-WT-infected PAMs. PAMs were infected with ASFV-ΔA137R or ASFV-WT (MOI = 1) and collected at 4, 12, and 20 hours post-infection (hpi), and then the total mRNAs of PAMs were used to generate RNA-seq libraries. A total of 384 DEGs (log2 fold change of >1) were expressed in the three groups: the numbers of special genes from ASFV-ΔA137R versus ASFV-WT at 4, 12, and 20 hpi are 1,161, 1,951, and 543, respectively. (E) Heat map of the 34 DEGs involved in IFN response induced by ASFV-ΔA137R versus ASFV-WT at 4, 12, and 20 hpi. (F) Kyoto Encyclopedia of Genes and Genomes enrichment analysis in the ASFV-ΔA137R- or ASFV-WT-infected PAMs. Error bars denote standard errors of the means. A red asterisk indicates the signaling pathway of interest.
infected with either ASFV-ΔA137R or ASFV-WT. To determine the expected deletion of the A137R gene, the genome of ASFV-ΔA137R was analyzed by next-generation sequencing (NGS). Compared with the parental ASFV-WT, no undesirable variations were found in the genome of ASFV-ΔA137R, except a 391-bp deletion of the A137R gene replaced by the p72EGFP expression cassette. We then analyzed the effect of the A137R gene deletion on viral growth in vitro. PAMs were infected with ASFV-ΔA137R or ASFV-WT at a multiplicity of infection (MOI) of 0.01, and the viral titers were assayed by 50% hemadsorption doses (HAD50) at the indicated time points. The growth profile of ASFV-ΔA137R was similar to that of ASFV-WT in PAMs (Fig. 1C).

The virulence of ASFV depends on the viral replication efficiency in target cells or the blockage of the immune responses by viral proteins. Since the A137R gene deletion does not influence the replication of ASFV-WT in vitro, we thus systemically analyzed the DEGs of the PAMs infected with ASFV-ΔA137R or ASFV-WT (MOI = 1) by RNA-seq analysis. Compared with the ASFV-WT-infected PAMs, the transcription levels of 2,458, 3,676, and 1,671 genes were altered upon ASFV-ΔA137R infection at 4, 12, and 20 hours post-infection (hpi), respectively (Fig. 1D). Notably, ASFV-ΔA137R, than did ASFV-WT (Fig. 1E). The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis indicated that the upregulated genes upon ASFV-ΔA137R infection were involved in the IFN signaling pathway, such as cytosolic DNA-sensing, JAK-STAT, and Toll-like receptor (TLR) signaling pathways (Fig. 1F).

To verify the roles of pA137R regulation of the IFN signaling pathway, the transcription level of IFN-β was examined by reverse transcription-quantitative PCR (RT-qPCR) in the ASFV-ΔA137R- or ASFV-WT-infected PAMs. As shown in Fig. 2A, ASFV-ΔA137R, but not ASFV-WT, significantly enhanced IFN-β transcription in the PAMs at 12 to 20 hpi but not at 4 hpi, which was consistent with the RNA-seq analysis. Sendai virus (SeV), an IFN agonist, activates the retinoic acid inducible gene (RIG-I)-mediated IFN signaling pathway (24). ASFV-ΔA137R also induced higher transcription levels of IFN-α, IFN-β, and downstream IFN-stimulated genes (ISGs), including ISG54 and ISG56, in the PAMs upon SeV stimulation than did ASFV-WT (Fig. 2B to E). Taken together, these data indicate that pA137R inhibits the IFN signaling pathway and plays an important role in evading innate immune response.

**pA137R inhibits the cGAS-STING-mediated IFN-β signaling pathway.** The cGAS-STING-mediated IFN-β signaling pathway is significantly suppressed upon the virulent ASFV Armenia/07 infection, but the viral proteins involved remain elusive (5). The RNA-seq analysis revealed that the cytosolic DNA-sensing signaling pathway was activated in the ASFV-ΔA137R-infected PAMs, and thus we further investigated the effects of pA137R on the cGAS-STING pathway. Human embryonic kidney (HEK293T) cells were cotransfected with p3×Flag-cGAS and -STING and reporter plasmids, along with pMyc-A137R or pCMV-Myc, for 24 h. The results indicated that overexpression of pA137R significantly inhibited the activation of the IFN-β or IFN-stimulated response element (ISRE) promoter in a dose-dependent manner (Fig. 3A and B). Similarly, pA137R also markedly suppressed the transcription levels of IFN-β, ISG54, and ISG56 (Fig. 3C). These data suggest that pA137R negatively regulates the cGAS-STING-mediated IFN-β signaling pathway.

**pA137R inhibits the IFN-β signaling pathway through targeting TBK1.** To identify which adapters in the cGAS-STING pathway were antagonized by pA137R, HEK293T cells were cotransfected with pMyc-A137R and reporter plasmids along with p3×Flag-cGAS, -STING, -TBK1, or -IRF3-5D (the active form of IRF3), respectively. We showed that pA137R markedly suppressed the activation of the IFN-β promoter induced by cGAS, STING, or TBK1, but not IRF3-5D (Fig. 4A to D). Therefore, we speculated that pA137R may inhibit the activation of the IFN-β promoter through targeting TBK1. Glutathione S-transferase (GST) pulldown assay indicated that pA137R specifically interacted with TBK1 but not with other adapters, including cGAS, STING, or IRF3, of the cGAS-STING pathway (Fig. 5A). The colocalization of pA137R with TBK1 was observed in the cytoplasm, with a colocalization coefficient of 0.74 (Fig. 5B). PAMs were infected with ASFV-WT for 48 h, and the endogenous TBK1 was subjected to...
coimmunoprecipitation (co-IP) assay using anti-pA137R polyclonal antibodies (PAbs). As shown in Fig. 5C, TBK1 was precipitated by pA137R in the ASFV-infected PAMs. The colocalization of pA137R and TBK1 (a colocalization coefficient of 0.75) was further demonstrated by laser confocal microscopy (Fig. 5D). Collectively, these results indicate that pA137R blocks the IFN-β signaling pathway by interacting with TBK1.

**pA137R promotes the autophagy-mediated lysosomal degradation of TBK1.** Previous studies have revealed that the IFN signaling pathway is antagonized by several viruses, including human cytomegalovirus (HCMV) (25), Zika virus (ZIKV) (26), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (27), through targeting or degrading the host proteins. Since pA137R interacts with TBK1 and inhibits the activation of IFN-β (Fig. 3 to 5), we investigated the effects of pA137R on the transcription and expression of TBK1. p3×Flag-TBK1 and pMyc-A137R were cotransfected into HEK293T cells. The results showed that TBK1 protein expression was inhibited by overexpression of pA137R in a dose-dependent manner, while the mRNA level of TBK1 remained unchanged (Fig. 6A and B), indicating that pA137R may degrade the TBK1 protein. We further analyzed the TBK1 expression in the PAMs infected with ASFV-ΔA137R or ASFV-WT at 24 hpi. We found that ASFV-WT downregulated the expression...
The A137R protein (pA137R) inhibits the cGAS-STING-mediated beta interferon (IFN-β) signaling pathway. (A and B) pA137R inhibits the activation of the IFN-β (A) or IFN-stimulated response element (ISRE) promoter (B) in a dose-dependent manner. HEK293T cells were cotransfected with pIFN-β/pISRE-Fluc expressing firefly luciferase (0.05 μg), pTK-Rluc expressing Renilla luciferase (0.01 μg), and p3×Flag-cGAS (0.01 μg) and -STING (0.05 μg), along with pMyc-A137R (0.1, 0.25, or 0.5 μg) for 24 h followed by luciferase reporter assay. The expression of cGAS, STING, or pA137R was analyzed by Western blotting using mouse anti-Flag or -Myc monoclonal antibody. GAPDH was used as a loading control. (C) pA137R inhibits the mRNA levels of IFN-β and IFN-stimulated genes (ISGs). HEK293T cells were cotransfected with p3×Flag-cGAS and -STING along with pMyc-A137R (0.5 μg) or pCMV-Myc (0.5 μg) for 24 h, and the mRNA levels of IFN-β, ISG54, and ISG56 were quantified by reverse transcription-quantitative PCR. Error bars denote standard errors of the means. All the data were analyzed using Student’s t test: **, P < 0.01; ***, P < 0.001.
of TBK1, while the TBK1 expression was significantly increased upon ASFV-DΔA137R infection in PAMs, although it was lower than that in the uninfected PAMs (Fig. 6C). Degradation of cellular proteins mainly relies on the ubiquitin-proteasome, autophagosome, or lysosome pathway. Therefore, HEK293T cells were cotransfected with p3×Flag-cGAS (A), -STING (B), -TBK1 (C), or -IRF3-5D (the activated form of IRF3) (D), along with pMyc-A137R or pCMV-Myc, and the luciferase activities were measured at 24 hours post-infection. The expression of cGAS, STING, TBK1, IRF3-5D, or pA137R was analyzed by Western blotting using mouse anti-Flag or -Myc monoclonal antibody. GAPDH was used as a loading control. Error bars denote standard errors of the means. All the data were analyzed using Student’s t test: ***, P < 0.001; ns, not significant.

FIG 4 The A137R protein (pA137R) inhibits the beta interferon (IFN-β) signaling pathway through targeting TBK1. (A to D) HEK293T cells were cotransfected with pIFN-β-Fluc expressing firefly luciferase (0.05 μg), pTK-Rluc expressing Renilla luciferase (0.01 μg), and p3×Flag-cGAS (A), -STING (B), -TBK1 (C), or -IRF3-5D (the activated form of IRF3) (D), along with pMyc-A137R or pCMV-Myc, and the luciferase activities were measured at 24 hours post-infection. The expression of cGAS, STING, TBK1, IRF3-5D, or pA137R was analyzed by Western blotting using mouse anti-Flag or -Myc monoclonal antibody. GAPDH was used as a loading control. Error bars denote standard errors of the means. All the data were analyzed using Student’s t test: ***, P < 0.001; ns, not significant.
pA137R using the ATG5-knockout HeLa cells. Consistent with the inhibitor treatment assay, the degradation of TBK1 by pA137R was counteracted in the ATG5-knockout HeLa cells compared with the parental wild-type (WT) cells (Fig. 6F). Altogether, these results indicate that the autophagy-mediated lysosomal pathway is responsible for the degradation of TBK1 by pA137R.

pA137R blocks the nuclear translocation of IRF3. With the activation of the cGAS-STING pathway, the activated IRF3 is translocated into the nucleus and triggers IFN-β transcription. Since IRF3 is activated by TBK1, we speculated that TBK1 degradation may influence IRF3 translocation from the cytoplasm into the nucleus. HEK293T cells were transfected with pMyc-A137R or pCMV-Myc for 24 h and then stimulated with rabbit anti-Flag or mouse anti-Myc MAbs for 24 h, and the localization of pA137R and TBK1 was visualized by laser confocal microscopy using in-house mouse anti-pA137R or rabbit anti-TBK1 PAb and the indicated secondary antibody, respectively. Cell nuclei (blue) were stained with DAPI as described above. Bars, 10 μm.
FIG 6 The A137R protein (pA137R) promotes the autophagy-mediated lysosomal degradation of TBK1. (A and B) pA137R decreases TBK1 expression. (A) HEK293T cells were transfected with pMyc-A137R (0, 0.25, 0.5, or 1 μg), and subjected to isolation of cellular total mRNAs followed by quantification of TBK1 transcription level by reverse transcription-quantitative PCR. (B) HEK293T cells were cotransfected with p3′Flag-TBK1 (1 μg) together with pMyc-A137R (0, 0.25, 0.5, or 1 μg), and subjected to isolation of total cellular proteins followed by analysis by Western blotting. (C) The A137R gene-deleted ASFV mutant (ASFV-ΔA137R) resulted in higher TBK1 expression in primary porcine alveolar macrophages (PAMs) than did the wild-type ASFV HU18 strain (ASFV-WT). PAMs were infected with ASFV-ΔA137R or ASFV-WT for 24 hours post-infection (hpi) at a multiplicity of infection (MOI) of 1, and the expression of TBK1 or pA137R was analyzed by Western blotting using the indicated antibodies. (D) Viability of HEK293T cells treated with the indicated inhibitors. HEK293T cells were grown for 24 h and then treated with dimethyl sulfoxide (DMSO [negative control]) (0, 10, 100, or 200 μM), MG132 (0, 5, 10, or 20 μM), bafilomycin A1 (BafA1) (0, 50, 100, or 200 nM), or 3-methyladenine (3-MA) (0, 5, 10, or 20 mM) for 8 h. The cell viability was determined as described above. (E) HEK293T cells were cotransfected with p3′Flag-TBK1 and pMyc-A137R or pCMV-Myc for 24 h and then treated for 8 h with DMSO (negative control), MG132 (20 μM), BafA1 (100 nM), or 3-MA (10 mM), respectively. The expression of TBK1 or pA137R was analyzed as described above. GAPDH was used as a loading control. (F) pMyc-A137R and p3′Flag-TBK1 were cotransfected into autophagy-related protein 5 (ATG5)-knockout or wild-type (WT) HeLa cells for 36 h, and the expression of TBK1 or pA137R was examined with the indicated antibodies as described above. GAPDH was used as a loading control. Error bars denote standard errors of the means. All the data were analyzed using Student’s t test. ns, not significant.
with SeV or poly(dA-dT). The subcellular localization of IRF3 in the absence or presence of pA137R was determined by laser confocal microscopy (Fig. 7A and B). In the unstimulated group, the IRF3 was mainly located in the cytoplasm. Upon SeV or poly(dA-dT) stimulation, approximately 30 to 40% of IRF3 translocated into the nucleus in the pCMV-Myc-transfected cells. However, only 10% of IRF3 was detected in the nucleus when there was ectopic expression of pA137R. Thus, pA137R degrades TBK1, leading to blockage of the nuclear translocation of IRF3.

**DISCUSSION**

Previous studies have shown that knocking out the immunoregulatory genes usually alters ASFV virulence (8–11, 17, 29–31). pA137R is a late-expressed viral structural protein and associated with ASFV virulence (20, 32), but the underlying attenuation mechanism is not clear. In this study, we found that ASFV-ΔA137R significantly increased type I IFN production in PAMs compared with ASFV-WT (Fig. 1 and 2). pA137R suppressed the cGAS-STING-mediated IFN-β signaling pathway through targeting TBK1 (Fig. 3 and 4). Furthermore, we demonstrated that pA137R interacts with TBK1, promotes its degradation via the autophagy-mediated lysosomal pathway, and affects IRF3 nuclear translocation to block type I IFN production (Fig. 8). To our knowledge, this is the first study to elaborate the mechanism of pA137R regulation of ASFV virulence.

TBK1 undergoes autophosphorylation and ubiquitination to facilitate the activation of downstream adaptors (e.g., IRF3 or NF-κB) during viral infections. However, viral proteins target TBK1 to downregulate IFN production by disrupting TBK1-associated complexes or degrading TBK1: e.g., the HCMV UL94 protein (25), the herpes simplex virus 1 (HSV-1) US11 (33) and γ34.5 (34) proteins, the foot-and-mouth disease virus Lpro protein (35), the swine acute diarrhea syndrome coronavirus nucleocapsid protein (36),...
the heartland virus nonstructural proteins (37), and the SARS-CoV-2 M protein (38). In this study, we found that type I IFN production was inhibited by pA137R in PAMs upon ASFV infection (Fig. 1 and 2), and the TBK1 expression was also downregulated in the HEK293T cells with pA137R-overexpressed or ASFV-WT-infected PAMs (Fig. 6). TBK1, a critical adaptor of the cGAS-, RIG-I-, and melanoma differentiation-associated antigen 5 (MDA-5)-mediated signaling pathways (39), is degraded by ubiquitin-specific protease 38 (USP38) (40), major vault protein (MVP) (41), and dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase 2 (DYRK2) (42) via the proteasomal or autophagy-mediated lysosomal pathway. It has been revealed that the ASFV DP96R protein degrades TBK1 (14), but the underlying mechanism remains unknown. We have demonstrated that pA137R interacts with and degrades TBK1 via the autophagy-mediated lysosomal pathway (Fig. 5 and 6). The expression of TBK1 in the ASFV-ΔA137R-infected PAMs was higher than that in the ASFV-WT-infected PAMs but lower than that in the uninfected PAMs, implying that other ASFV proteins besides pA137R might also degrade TBK1.

FIG 8 Schematic model of the A137R protein (pA137R) negatively regulating beta interferon (IFN-β) production upon ASFV infection. ASFV dsDNA can be sensed by cGAS, a cytosolic DNA sensor, and transcribed into dsRNA by type III RNA polymerase (RNA Pol-III) (47) to trigger the activation of the IFN signaling pathway. To evade innate immune responses, pA137R interacts with and degrades TBK1 via the autophagy-mediated lysosomal pathway, followed by the inhibition of IRF3 nuclear translocation to suppress the IFN-β production upon ASFV infection.
Vaccinia virus (VACV) replicates in the cytoplasm and generates dsRNA (43). The A52 (44), K7 (45), and E3 (46) proteins of VACV inhibit the TLR-mediated NF-\(\kappa\)B activation. Similarly, ASFV (13, 47, 48) and HSV-1 (49, 50) also regulate the dsDNA or dsRNA-mediated antiviral responses. In this study, we found that ASFV-\(\Delta\)A137R induced higher transcription of the IFN genes and ISGs in the PAMs upon SeV stimulation than did ASFV-WT, and pA137R negatively regulated the cGAS-STING-mediated IFN-\(\beta\) signaling pathway (Fig. 2 and 3), which indicated that pA137R is an IFN antagonist in the dsDNA (cGAS) or dsRNA (RIG-I or MDA-5)–mediated IFN-\(\beta\) signaling pathway. RNA-seq analysis demonstrated that the JAK-STAT and TLR signaling pathways were activated, and the expression of chemokines (e.g., CCL2, CCL8, and CXCL8) and proinflammatory cytokines (e.g., interleukin-1\(\alpha\) [IL-1\(\alpha\)] and IL-1\(\beta\)) was upregulated in the ASFV-\(\Delta\)A137R-infected PAMs at 12 to 20 hpi, implying that pA137R probably exerts multiple actions to evade the innate immune responses.

It is generally accepted that the early-expressed viral proteins play a key role in regulating IFN production, but several studies have shown that the late-expressed viral proteins are also involved in this process and associated with viral virulence. UL94, a late-expressed protein and structural component of HCMV, inhibits IFN-\(\beta\) production by disrupting the dimerization of MITA and recruitment of TBK1 to MITA (25, 51, 52). The HSV-1 US11 protein interacts with RIG-I and MDA-5 and antagonizes IFN-\(\beta\) production (50); the herpesviral \(g34.5\) protein prevents protein synthesis via the protein kinase R (PKR) signaling pathway or inhibits the production of IFN-\(\beta\) by promoting TBK1 degradation and disrupting the interaction between TBK1 and IRF3 (33, 53). IFN-\(\beta\) inhibits viral infections, and low-virulence viruses in turn increase IFN-\(\beta\) production, such as West Nile virus (54), Japanese encephalitis virus (55), bluetongue virus (56), and ASFV (6–11). Deletion of the IFN antagonist from the virulent ASFV results in attenuation (7–11). The A137R gene is associated with the virulence of ASFV (20), its deletion resulted in more production of IFN-\(\alpha\), IFN-\(\beta\), ISG54, and ISG56 in PAMs (Fig. 1 and 2).

There is an urgent need to define the determinants of ASFV virulence, which contributes to the development of treatments and vaccines. A low dose (10^2 HAD_{50}) of the A137R gene-deleted ASFV mutant (ASFV-G-\(\Delta\)A137R) results in ASFV attenuation and offers protection against challenge with the virulent parental ASFV-G, but the ASFV-G-\(\Delta\)A137R-inoculated pigs present high viremia titers (10^5 to 10^6) and shed the virus (20). It has been reported that pA137R is highly conserved among the genotype I and II strains (20). Because the attenuation of ASFV is relevant to the increased production of IFN (7–11) and ASFV-\(\Delta\)A137R promotes IFN-\(\beta\) production in PAMs, we speculate that pA137R promotes the autophagy-mediated lysosomal degradation of TBK1 to negatively regulate IFN-\(\beta\) production and attenuate ASFV virulence. However, we cannot rule out the differences in the genetic backgrounds of virus strains, which lead to changes in phenotypes. Deletion of EP402R from the virulent BA71 strain results in attenuation, whereas the phenotype is not observed in the ASFV Georgia 2007 and HLJ/2018 strains (20). However, the UK/CD2v-deleted ASFV strain also presents different attenuation in these strains (59, 61).

In conclusion, our research is the first to reveal that pA137R interacts with and degrades TBK1 via the autophagy-mediated lysosomal pathway to inhibit IFN-\(\beta\) production. These findings enrich the understanding of immunoevasion by ASFV and the mechanism of pA137R regulation of ASFV virulence.

MATERIALS AND METHODS

Cells and virus strain. PAMs were cultured in RPMI 1640 medium (catalog no. C11875500BT; Gibco) supplemented with 10% fetal bovine sera (FBS) (catalog no. 10091148; Gibco) and 2% antibiotics-antimycotics (catalog no. 15140122; Gibco) (62). HEK293T (62), ATG5-knockout HeLa (63), or HeLa (64) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (catalog no. D6429; Sigma-Aldrich) with 10% FBS at 37°C with 5% CO_2. The ASFV HLJ/2018 strain (GenBank no. MK333180.1) (58) and SeV (65) were propagated as described previously.
Construction of plasmids. The A137R gene was amplified from the genomic DNA (gDNA) of ASFV-WT and cloned into the EcoRI/XhoI sites of the pCMV-Myc (Clontech) or pGEX-6p-1 (GE Healthcare) vector, creating pMyc-A137R or pGST-A137R, respectively. The porcine WT and cloned into the EcoRI/XhoI sites of the pCMV-Myc (Clontech) or pGEX-6p-1 (GE Healthcare) vector, generating p3
amplifiers for amplification of plasmids are listed in Table 1.

Dual-luciferase reporter (DLR) assays. HEK293T cells were cotransfected with reporter plasmids (pIFN-β-Fluc or pISRE-Fluc) expressing the firefly luciferase (Fluc) and the internal reference renilla luciferase (Rluc) were described previously (65). The primers for amplification of plasmids in Table 1.

| Primers | Sequence (5'→3') | Description |
|---------|------------------|-------------|
| dA137R-RA-F | GGAGCTCGAGTCTGGAAGCTTTATCTGCAATTCAAATACATTAC | For left arm |
| dA137R-RA-R | CGGCCGCGGTACCTCGAGTTAGCCTTCTTTGATATT | For right arm |
| pIFN-β-R | TGGGTGGAATCATACTGGAACA | qPCR for human IFN-β |
| pIFN-β-F | GACACCCACTCCTCACTATT | qPCR for human GAPDH |
| hGAPDH-R | ACCACCCCTTTGCTGAGAG | qPCR for human ISG54 |
| hGAPDH-F | GGCTGGAATGAAACCGTCAT | qPCR for human ISG54 |
| hISG54-F | CTGCAACCATGAGTGAGAA | qPCR for human ISG54 |
| hISG54-R | CCTTTGAGGTGCTTTAGATAG | qPCR for human ISG54 |
| hISG56-F | TTAGAAAACAGGGTCTTGGAGGAG | qPCR for human ISG56 |
| hISG56-R | GATAGGAGCAGACAAGGAA | qPCR for human ISG56 |
| hIFN-β-F | CAATGGAATGGAAGCAGTTCTTACC | qPCR for human IFN-β |
| hIFN-β-R | GGGCCCCTGGGATCCCCGGAATTCATGGAAGCAGTTCTTACC | qPCR for human IFN-β |
| p3/C2 Flag-IRF3-5D-R | GCCTTGTACTGGTCGTCGTCGAGGTCGAGCGGGTGGTCGTTGTCAATGTGCAGGTCCAC | For right arm |
| p3/C2 Flag-IRF3-5D-F | GTGGACCTGCACATTGACAACGACCACCCGCTCGACCTCGACGACGACCAGTACAAGGC | For right arm |
| p3/C2 Flag-TBK1-R | CCTCTAGAGTCGACTGGTACCCTAGAAGATATCTGAGCGG | For right arm |
| p3/C2 Flag-TBK1-F | CAAGCTTGCGGCCGCGAATTCAATGCAGAGCACTTCTAATC | For right arm |
| p3/C2 Flag-STING-R | CCTCTAGAGTCGACTGGTACCTCAGAAGATATCTGAGCGG | For right arm |
| p3/C2 Flag-STING-F | CAAGCTTGCGGCCGCGAATTCAATGCCCTACTCCAGCCTGC | For right arm |
| p3/C2 Flag-cGAS-R | CCTCTAGAGTCGACTGGTACCTCACCAAAAAACTGGAAA | For right arm |
| p3/C2 Flag-cGAS-F | CAAGCTTGCGGCCGCGAATTCAATGGCGGCCCGGCGGGGAA | For right arm |
| GST-A137R-R | TCAGTCACGATGCGGCCGCTCGAGTTAGCCTTCTTTGATATT | For right arm |
| GST-A137R-F | GGGCCCCTGGGATCCCCGGAATTCATGGAAGCAGTTCTTACC | For right arm |
| Myc-A137R-F | ATGGAGGCCCGAATACACAAGAAG | For amplification of A137R |
| Myc-A137R-R | CGGCCGCGGTACCTCGAGTTAGCCTTCTTTGATATT | For amplification of A137R |
| hTBK1-R | GGTAGTCCATAGGCATTAGAAG | qPCR for human TBK1 |
| hTBK1-F | GAAGAGGAGACACAACAAGAAG | qPCR for human TBK1 |
| Flag-IRF3-5D-R | GCCTTGTACTGGTCGTCGTCGAGGTCGAGCGGGTGGTCGTTGTCAATGTGCAGGTCCAC | For right arm |
| Flag-IRF3-5D-F | GTGGACCTGCACATTGACAACGACCACCCGCTCGACCTCGACGACGACCAGTACAAGGC | For right arm |
| Flag-IRF3-R | CCTCTAGAGTCGACTGGTACCCTAGAAGATATCTGAGCGG | For right arm |
| Flag-IRF3-F | CAAGCTTGCGGCCGCGAATTCAATGCAGAGCACTTCTAATC | For right arm |
| Flag-TBK1-R | CCTCTAGAGTCGACTGGTACCCTAGAAGATATCTGAGCGG | For right arm |
| Flag-TBK1-F | CAAGCTTGCGGCCGCGAATTCAATGCAGAGCACTTCTAATC | For right arm |
| Flag-STING-R | CCTCTAGAGTCGACTGGTACCTCAGAAGATATCTGAGCGG | For right arm |
| Flag-STING-F | CAAGCTTGCGGCCGCGAATTCAATGCAGAGCACTTCTAATC | For right arm |
| Flag-cGAS-R | CCTCTAGAGTCGACTGGTACCTCACCAAAAAACTGGAAA | For right arm |
| Flag-cGAS-F | CAAGCTTGCGGCCGCGAATTCAATGGCGGCCCGGCGGGGAA | For right arm |
| Flag-IRF3-R | CCTCTAGAGTCGACTGGTACCCTAGAAGATATCTGAGCGG | For right arm |
| Flag-IRF3-F | CAAGCTTGCGGCCGCGAATTCAATGCAGAGCACTTCTAATC | For right arm |
| Flag-TBK1-R | CCTCTAGAGTCGACTGGTACCCTAGAAGATATCTGAGCGG | For right arm |
| Flag-TBK1-F | CAAGCTTGCGGCCGCGAATTCAATGCAGAGCACTTCTAATC | For right arm |
| Flag-STING-R | CCTCTAGAGTCGACTGGTACCTCAGAAGATATCTGAGCGG | For right arm |
| Flag-STING-F | CAAGCTTGCGGCCGCGAATTCAATGCAGAGCACTTCTAATC | For right arm |
| Flag-cGAS-R | CCTCTAGAGTCGACTGGTACCTCACCAAAAAACTGGAAA | For right arm |
| Flag-cGAS-F | CAAGCTTGCGGCCGCGAATTCAATGGCGGCCCGGCGGGGAA | For right arm |
| Flag-IRF3-R | CCTCTAGAGTCGACTGGTACCCTAGAAGATATCTGAGCGG | For right arm |
| Flag-IRF3-F | CAAGCTTGCGGCCGCGAATTCAATGCAGAGCACTTCTAATC | For right arm |

LA, left arm; RA, right arm; d, deletion; r, recombinant; F, forward; R, reverse; h, human; p, porcine.

TABLE 1 Primers used in this study

May 2022 Volume 96 Issue 9 10.1128/jvi.01957-21
the gDNA of ASFV-WT as the template, and then fused to the p72EGFP expression cassette by overlapping PCR using the primers listed in Table 1, and the resulting fusion fragment was cloned into the pOK-12 vector. The transfer vector was sequenced and named pOK-p72-ΔA137R-EGFP.

The ASFV-ΔA137R mutant was generated by homologous recombination according to the previously described methods (61). Briefly, PAMs were seeded in 6-well plates coated with poly-L-lysine and incubated for 24 h at 37°C. The PAMs were transfected with 2 μg of transfer vector pOK-p72-ΔA137R-EGFP using X-tremeGENE HP for 16 h and then infected with ASFV-WT (MOI = 3). The recombinant viruses in PAMs were harvested until the EGFP expression, followed by purification using limiting dilution.

NGS. To confirm the accurate deletion of the A137R gene in the ASFV genome, the gDNA of ASFV-ΔA137R was extracted from the infected PAMs using the QiAamp blood mini kit (catalog no. 51104; Qiagen), and the full-length sequence of the ASFV-ΔA137R genome was determined by NGS as described previously (66).

RNA-seq analysis. PAMs were infected with ASFV-ΔA137R or ASFV-WT at an MOI of 1. At 4, 12, and 20 hpi, the cells were used to extract total RNAs by using TRIzol reagent (catalog no. 15596026; Invitrogen) according to the manufacturer’s instructions. RNA quantification and qualification were assessed using the RNA Nano 6000 assay kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The mRNAs with poly(A) tails were enriched and purified from the total mRNAs by poly(T) oligonucleotide-coupled magnetic beads. RNA-seq libraries were prepared and assessed by AMPure XP system kit (Beckman Coulter, Beverly, USA) in the Agilent Bioanalyzer 2100 system. Subsequently, the RNA-seq libraries were sequenced on an Illumina Novaseq platform (Nova gene, China). KEGG enrichment analysis of DEGs was performed using Cluster Profiler (3.4.4).

RNA extraction and RT-qPCR. The total RNAs of the cells with indicated treatment or virus infection were extracted using the SimplyPure total RNA extraction kit (catalog no. BSC52M1; BioFlux) and transcribed into cDNA by FastKing gDNA Dispelling RT SuperMix (catalog no. KR0116; Tiangen). The CDNs from HEK293T cells or PAMs were used as templates for RT-qPCR using SYBR Premix Ex Taq (catalog no. RR8208; TaKaRa) to examine the mRNA level of human ISG56 and β-actin (catalog no. R0330; TaKaRa) by using the 2^-ΔΔCt method (9). The primers are listed in Table 1.

NGS analysis. The total RNAs of the cells with indicated treatment or virus infection were extracted with TRIzol reagent (catalog no. 15596026; Invitrogen) according to the manufacturer’s instructions. RNA quantification and qualification were assessed using the RNA Nano 6000 assay kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The mRNAs with poly(A) tails were enriched and purified from the total mRNAs by poly(T) oligonucleotide-coupled magnetic beads. RNA-seq libraries were prepared and assessed by AMPure XP system kit (Beckman Coulter, Beverly, USA) in the Agilent Bioanalyzer 2100 system. Subsequently, the RNA-seq libraries were sequenced on an Illumina Novaseq platform (Nova gene, China). KEGG enrichment analysis of DEGs was performed using Cluster Profiler (3.4.4).

Co-IP assay. PAMs were infected with ASFV-WT at an MOI of 1, and the lysates were collected at 48 hpi for the co-IP assay. Protein G agarose (catalog no. 11243233001; Roche) was incubated with the lysates and rabbit anti-pA137R PABS or a rabbit immunoglobulin G (catalog no. ab190492; Abcam) (isotype control antibody). The bound proteins were subjected to Western blotting using rabbit anti-TBK1 (catalog no. 35045; Cell Signaling Technology) and in-house mouse anti-pA137R PABS.

Laser confocal microscopy. HEK293T cells were cotransfected with p3×Flag-cGSTA, -STING, -TBK1, or -IRF3 (2 μg each) for 36 h and fixed in 4% paraformaldehyde (catalog no. BLS39A; Biosharp) for 30 min at room temperature. After being washed twice with PBS, the cells were permeabilized in 0.15% Triton X-100 (catalog no. 1139100; BioFroxx) for 15 min at room temperature. The cells were incubated with mouse anti-pA137R MAb (1:500) (catalog no. M4439-100UL; Sigma-Aldrich) or rabbit anti-Flag PABs (1:500) for 1.5 h at 37°C. Following incubation with Alexa Fluor 633-conjugated goat anti-mouse IgG (H + L) antibody (1:500) (catalog no. A21052; Invitrogen) or Alexa Fluor 488-conjugated donkey anti-rabbit IgG (H + L) antibody (1:500) (catalog no. A21206; Invitrogen) for 45 min, the cells were washed with PBS. Subsequently, the cells’ nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) (1:1,000) (catalog no. D8417; Sigma-Aldrich) and observed by laser confocal microscopy (LSM880, Zeiss).

Cell viability assay. Cell viability was determined by Cell Counting Kit-8 (catalog no. K1018; APEXBio) according to the manufacturer’s instructions.

Inhibitor treatment assay. HEK293T cells were cotransfected p3×Flag-TBK1 with pMyc-A137R or pCMV-Myc for 24 h. Then the cells were treated with 20 μM proteasome inhibitor MG132 (catalog no. M6999-1MG; Sigma-Aldrich), 10 mM autophagosome inhibitor 3-MA (catalog no. HY-19312; Medchemexpress), or 100 nM BafA1 (catalog no. HY-100538; Medchemexpress) for 8 h, and the lysates were examined by Western blotting analysis.

Nuclear translocation assay. HEK293T cells were transfected with pMyc-A137R or pCMV-Myc (0.5 μg each) for 24 h and then stimulated with SEV (MOI = 1) or poly(dA:dT) (2 μg/mL) for 8 h. The localization of IRF3 and pA137R was examined by laser confocal microscopy using rabbit anti-IRF3 PABs (1:100) (catalog no. 703154; Invitrogen), respectively, as described above. The colocalization coefficients were determined using the ImageJ software.

Statistical analysis. All data were determined in triplicates and analyzed with SPSS 22.0 software (SPSS Software, Inc.). Student’s t test was used to assess statistical significance. A P value of <0.05 was considered as significant.
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