*Original article*

# MGF360-12L of ASFV-SY18 is an immune-evasion protein that inhibits host type I IFN, NF-κB, and JAK/STAT pathways

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## Abstract

African swine fever virus (ASFV) causes feverous and hemorrhagic disease of domestic pigs and European wild boars with high mortality, yet no commercial vaccine is currently available. Several ASFV strains with natural deletion or gene-targeted knockout of multiple MGF360 and MGF505 genes are attenuated *in vitro* and *in vivo*, and can offer full protection against homologous challenge. However, the mechanisms underlying the protection are not fully understood. This study aims to investigate the effects of MGF360-12L of ASFV-SY18 on the cGAS-STING signaling pathway and explore the potential mechanisms. We identified that ASFV-SY18 MGF360-12L could inhibit cGAS-STING, TBK1, or IRF3-5D-stimulated IFN-β expression and ISRE activation. Specifically, MGF360-12L inhibits both the activation of PRD(III-I) in a dose-dependent manner, and suppresses the exogenous expression of TBK1 and IRF3-5D. MGF360-12L could block NF-κB activation induced by overexpression of cGAS-STING, TBK1, IKKβ. Downstream of the IFN-β signaling, MGF360-12L blocks the ISRE promoter activation by reducing total protein level of IRF9. Moreover, MGF360-12L protein can inhibit IFN-β-mediated antiviral effects. In conclusion, our findings suggest that MGF360-12L is a multifunctional immune-evasion protein that inhibits both the expression and effect of IFN-β, which could partially explain the attenuation of relevant gene-deleted ASFV strains, and shed light on the development of efficient ASFV live attenuated vaccines in the future.

**Key words:** African swine fever virus, MGF360-12L, type I IFN, NF-κB, JAK/STAT

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## Introduction

The host innate immune system can recognize invading microbial pathogens through germline-encoded pattern recognition receptors (PRRs) and exert antiviral effects primarily by up-regulating the expression of type I interferons (IFNs) and interferon-stimulated genes (ISGs) (Kumar et al. 2011). The cyclic GMP-AMP synthase (cGAS) is an essential DNA sensor that recognizes cytosolic double-stranded DNA and catalyzes the formation of the second messenger, cGAMP cyclic GMP-AMP (cGAMP) production (Xia et al. 2016). cGAMP binds to stimulator of interferon gene (STING), and the latter undergoes conformational changes, transfers from the endoplasmic reticulum to the Golgi complex, and serves as a platform for recruiting and phosphorylation of TBK1 and IRF3. IRF3 and NF-κB are activated and then translocate into the nucleus to induce the expression of type I IFNs and pro-inflammatory cytokines, thus establishing an antiviral state and directing the following adaptive immune response (Li et al. 2020).

African swine fever (ASF) is a highly pathogenic swine disease with up to 100% morbidity and mortality for which there is currently no effective treatment or vaccine (Walczak et al. 2020, Yu et al. 2022). African swine fever virus (ASFV) is the causative agent of ASF. The viral genome is a double-stranded DNA, and mainly replicates in the cytoplasm, which may be detected by cGAS to induce the expression of type I IFN-β genes (Franzoni et al. 2020). It has been well documented that ASFV expresses various proteins to evade host immune response, especially to manipulate type I IFN pathway (Wang et al. 2021, Yang et al. 2021, Zhuo et al. 2021, Li et al. 2022, Sun et al. 2022). Our previous report has revealed that ASFV DP96R can block both the induction of IFN-β and the activation of NF-κB by inhibiting the cGAS-STING signaling pathway via downregulation the expression and phosphorylation of TBK1 (Wang et al. 2018). Other publications showed that recombinant ASFVs with partial deletions of MGF360 and MGF505/530 were attenuated *in vitro* and *in vivo*, indicating that MGF360-12L may play a crucial role in maintaining high levels of virus replication in macrophages (Afonso et al. 2004, O'Donnell et al. 2015, Rathakrishnan et al. 2022, Xie et al. 2022). Therefore, in the current study we analyzed whether certain MGF360 and MGF505/530 genes could inhibit the cGAS-STING- mediated type I IFN responses and then selected MGF360-12L for further investigation.

## Materials and Methods

### Antibodies and reagents

Antibodies against HA-tag, GAPDH, human TBK1, p-IRF3 (Ser396), p-TBK1 (Ser172) and IRF3 were from Cell Signaling (USA). Rabbit polyclonal antibody anti-IRF3, mouse monoclonal antibody anti-His and anti-GFP were obtained from Proteintech (USA). Human IFN-β and TNF-α protein were purchased from PeproTech (USA). 2'3'-cGAMP was from InvivoGen (USA). Enzymes used for cloning procedure were obtained from Takara (Japan). Double-luciferase reporter assay kit was from TransGen (China). JetPRIME kit was from Polyplus Transfection (France).

### Plasmids construction and transfection

The MGF505-1R, -2R, -3R and MGF360-12L, -13L, -14L genes of ASFV-SY18 (GenBank submission No. MH766894) were synthesized and cloned into pcDNA3.1 vector using *BamH* I and *Xho* I restriction enzymes. Codon-optimized MGF360-12L were synthesized and cloned into pcDNA3.1-myc-his with *myc* and *his* tags in the C terminal. Truncated mutants of the MGF360-12L-His, including 12L-F1 (1-131aa), 12L-F2 (122-240aa) and 12L-F3 (225-350aa), were cloned into pcDNA3.1-myc-his. Plasmids including pEGFP-N1-cGAS, pEGFP-N1-STING, TBK1-flag, pcDNA3.1-IRF3-5D, IKKβ-HA, IFN-β-luc, NF-κB -luc, pRL-TK (Renilla luciferase), ISRE-luc and pRDIII-I have been constructed or obtained as described previously(Wang et al. 2018). IRF3-5D was cloned into pcDNA3.1-HA using pcDNA3.1-V5-IRF3-5D as template. ORFs of IRF3, IRF9, STAT1 and STAT2 were amplified from human embryonic kidney (HEK) 293T cDNA and subcloned into pcDNA3.1-HA with HA-tag in the N terminal. Transient transfection was carried out with indicated plasmids using transfection reagent (jetPRIME) at a 2:1 ~ 3:1 jetPRIME/DNA ratio, according to manuscript's protocols.

### Cells and viruses

. All cells were cultured at 37°C with 5% $CO_2$ in a Thermo Forma incubator. HEK293T, baby hamster kidney 21 (BHK21) and porcine kidney 15 (PK15) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (vol/vol) fetal bovine serum (FBS, Gibco) and 1% (vol/vol) penicillin/streptomycin (Gibco). The porcine alveolar macrophage (PAM) cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco) supplemented with 10% FBS and antibiotics. Vesicular stomatitis virus (VSV)-GFP was propagated and amplified in BHK21 cells.

Table 1. Primers for Real-time qPCR used in this study.

| Genes | Primer | Sequence (from 5′ to 3′) |
|---|---|---|
| ISG15 | hISG15-F | GGGACCTGACGGTGAAGATG |
| | hISG15-R | CGCCGATCTTCTGGGTGAT |
| ISG54 | hISG54-F | CACCTCTGGACTGGCAATAGC |
| | hISG54-R | GTCAGGATTCAGCCGAATGG |
| ISG56 | hISG56-F | CAAAGGGCAAAACGAGGCAG |
| | hISG56-R | CCCAGGCATAGTTTCCCCAG |
| IRF3 | hIRF3-F | GTGGCCTGGGTGAACAAGAG |
| | hIRF3-R | GGTTGGCAGGTCTGGCTTAT |
| TBK1 | hTBK1-F | ACCCTTTGAAGGGCCTCGTA |
| | hTBK1-R | ACAGGCATGTCTCCACTCCA |
| IKKβ | hIKKβ-F | GTGGTCCTGTCAGTGGAAGC |
| | hIKKβ-R | GTGCTTCAGCCACCAGTTCT |
| β-actin | hβ-actin-F | CGAGAGAAGATGACCCAGATC |
| | hβ-actin-R | GCCAGAGGCGTACAGGGATA |

### Real-time qPCR analysis

Total cellular RNA isolation and real-time quantitative PCR assay were performed as described previously (Wang et al. 2018). Total RNA was isolated and reverse-transcribed into cDNA, and qPCR was performed using SYBR green Super mix (Takara) as follows: 95℃ for 1 min, followed by 40 amplification cycles of 95℃ for 10 s and 60℃ for 30 s. Relative mRNA expression levels were normalized to β-actin. Primers used in the research were listed in Table 1.

### Dual-luciferase reporter assay

Dual-luciferase reporter (DLR) assays were performed as described in our previous study (Wang et al. 2018). Briefly, HEK293T cells seeded in 48-well plates were cultured overnight to 60~80% confluency and then transfected with indicated plasmids. Afterwards, cells were lysed and luciferase assays were performed with the dual luciferase assay kit. Relative luciferase activities were analyzed by normalizing firefly luciferase activity to renilla luciferase activity.

### Western blot analysis

Western blot analyses (WB) were performed as described (Wang et al. 2018). Briefly, HEK293T cells were transiently transfected with indicated expression plasmids. Then, cells were lysed and equal volume of samples were separated by SDS-PAGE and analyzed by WB using specific primary antibodies: anti-TBK1, anti-p-TBK1-Ser172, anti-IRF3, or anti-pIRF3 at 1:1000, anti-HA, anti-His, anti-GFP or anti-GAPDH at 1:2000~1:5000. GAPDH was used as a loading control.

### Antiviral activity assay

HEK293T cells were transfected with MGF360-12L-His or empty plasmid for 24 h and then treated with IFN protein. After 12 h, the cells were infected with VSV-GFP (MOI=1) and incubated at 37℃ for 24 h. Cells were fixed with 4% paraformaldehyde and green fluorescence was examined using fluorescence microscopy. The expression of MGF360-12L, GFP and GAPDH was analyzed by WB.

### Statistical analysis

All assays were performed at least 3 times with similar results. The data shown were from representative results from single experiment. Data were presented as means ± standard deviations (SDs) and analyzed using Student's t test with GraphPad Prism 5.0 software (* $p<0.05$, ** $p<0.01$, *** $p<0.00$, ns indicates no significance).

## Results

### ASFV MGF360-12L inhibits the cGAS-STING signaling pathway

Several field and laboratory ASFV strains lacking the MGF360 and MGF505 genes, mostly MGF360-12L, -13L, -14L and MGF505-1R, -2R, -3R were attenuated both in *vitro* and in *vivo* (O'Donnell et al. 2015, Golding et al. 2016, Xie et al. 2022). To investigate whether MGF505-1R, -2R, -3R and MGF360-12L, -13L, -14L of ASFV-SY18 strain could inhibit the induction and response of type I IFN, plasmids expressing MGF505-1R, -2R, -3R and MGF360-12L, -13L, -14L were constructed and transfected into PK-15 and PAM
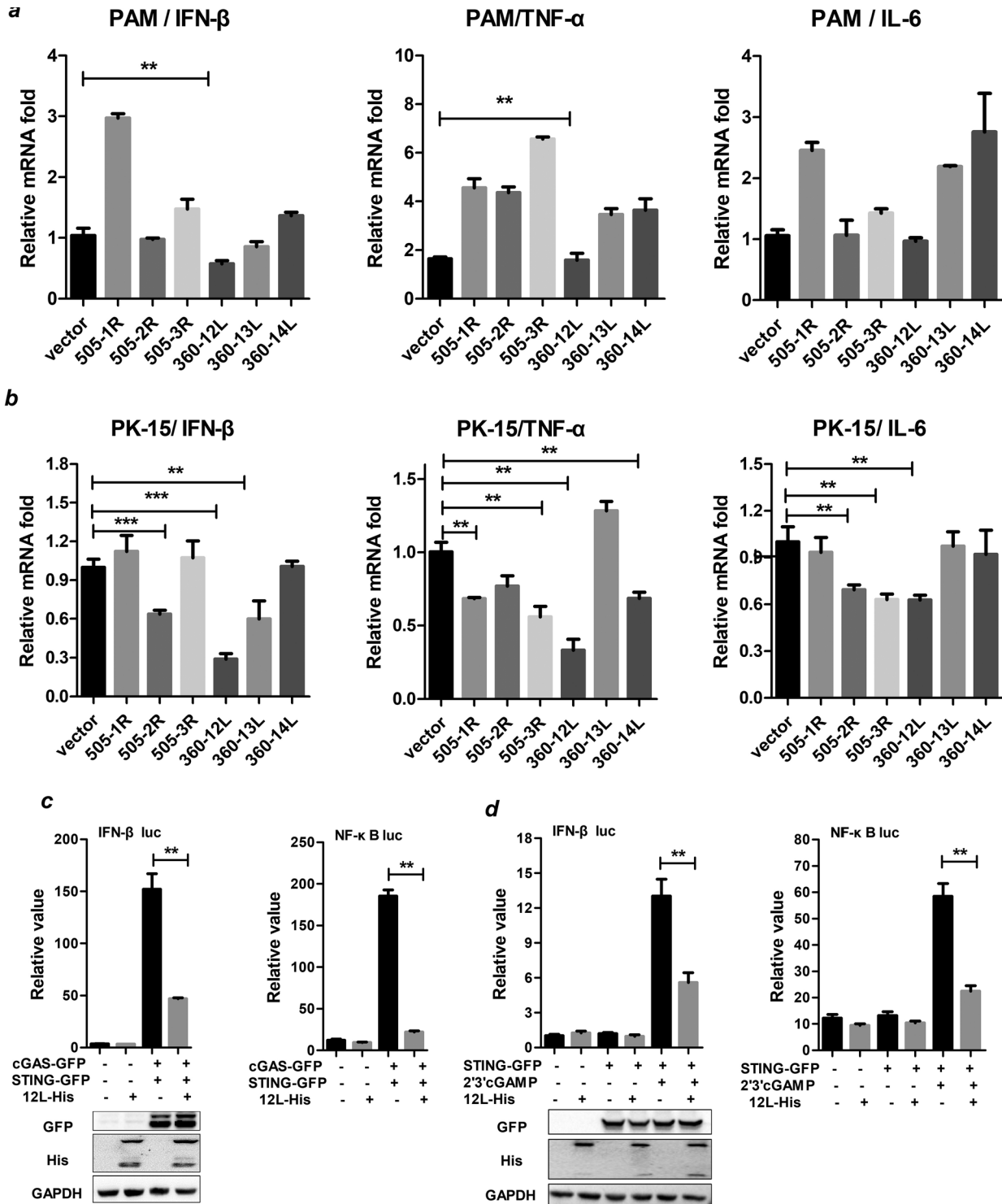
Fig. 1. Inhibition of cGAS-STING signaling by ASFV MGF360-12L. (a-b) ASFV MGF360-12L inhibited the mRNA expression level of IFN-β and TNF-α. PAM cells (a) and PK-15 cells (b) were transfected with identical amounts of plasmids, MGF505-1R, MGF505-2R, MGF505-3R, MGF360-12L, MGF360-13L, MGF360-14L or empty vector (pcDNA3.1) for 24 h, then harvested for RNA extraction. The mRNA expression levels of IFN-β, TNF-α, IL-6 and β-actin were measured by real-time PCR. (c) HEK293T cells were co-transfected with cGAS-GFP and STING-GFP, plus IFNβ-luc or NF-κB-luc and pRL-TK plasmid, along with MGF360-12L-His or empty vector. After 24 h transfection, the luciferase activities were measured using DLR assay kits. (d) HEK293T cells were co-transfected with STING and MGF360-12L-His or empty vector, plus IFNβ-luc or NF-κB-luc and pRL-TK plasmid. After 24 h, the cells were treated or untreated with 2'3'-cGAMP (100 nM) for 12 h. The luciferase activities were measured using DLR assay kits. The expression of cGAS-GFP, STING-GFP, MGF360-12L-his and GAPDH were assessed by WB using anti-GFP, anti-His and GAPDH monoclonal antibodies. ** p<0.01, *** p<0.001.

cells. The results showed that the MGF360-12L significantly suppressed the mRNA levels of IFN-β in both PK-15 and PAM cells (Fig. 1a and b). Besides, MGF360-12L also inhibited the mRNA levels of TNF-α and IL-6 in PK-15 cells (Fig. 1a and b).

To further investigate the roles of MGF360-12L in cGAS-STING-mediated DNA sensing signaling pathway, MGF360-12L-his was transfected into HEK293T cells in combination with plasmids expressing porcine cGAS and human STING, IFN-β-luc or NF-κB-luc and pRL-TK. Then, cells were harvested and the promoter activities of IFN-β and NF-κB were detected by DLR. As shown in Fig. 1c, the expression of MGF360-12L significantly inhibited the activation of IFN-β and NF-κB promoters induced by cGAS and STING co-transfetion. Besides, MGF360-12L could also suppress the activation of IFN-β-luc and NF-κB-luc mediated by STING when stimulated with 2'3'-cGAMP (Fig. 1d). These data suggest that MGF360-12L may act as a potent IFN-β induction antagonist in cGAS-STING- mediated immune signaling.

### MGF360-12L blocked TBK1 and IRF3-5D-induced IFN-β activation

To investigate how MGF360-12L down-regulates the expression of IFN-β, MGF360-12L was co-transfected with corresponding plasmids into HEK293T cells. Results showed that MGF360-12L expression significantly blocked IFN-β, ISRE, and pRDIII-I promoters activation induced by TBK1 or IRF3-5D (Fig. 2a). When co-transfected with MGF360-12L, the exogenous expressions of TBK1, IRF3, IRF3-5D were decreased, while the endogenous protein levels of TBK1 and IRF3 were not changed (Fig. 2b). Furthermore, the activation of ISRE and pRDIII-I promoters mediated by IRF3-5D was decreased in a dose-dependent manner when co-transfected with MGF360-12L (Fig. 2c). When MGF360-12L was co-transfected with cGAS and STING, there was no significant change in the endogenous protein levels of TBK1, IRF3, pTBK1 and pIRF3 (Fig. 2d). Meanwhile, when MGF360-12L was co-transfected with TBK1 or IRF3-5D, the mRNA level of TBK1 was reduced significantly (Fig. 2e), while no significant change was observed for IRF3 transcription (Fig. 2f).

### MGF360-12L inhibited NF-κB signaling pathway at the TBK1 and IKKβ level

NF-κB plays a critical role in antiviral and pro-inflammatory immune responses. As shown in Fig. 3a and Fig. 1(c-d), MGF360-12L significantly inhibited NF-κB signaling activation induced by TNF-α or overexpression of cGAS-STING. TBK1 and IKKβ were

downstream effector proteins of the cGAS-STING pathway, which can activate NF-κB signaling pathway through canonical as well as non-canonical ways (Abe and Barber 2014, Fang et al. 2017). To determine at which level MGF360-12L could inhibit the activation of the NF-κB pathway, co-transfection was performed with MGF360-12L, TBK1 or IKKβ in HEK293T cells. It was observed that MGF360-12L robustly blocked TBK1 or IKKβ directed NF-κB promoter activation in a dose-dependent manner (Fig. 3b-c). Moreover, the mRNA levels of TBK1 (Fig. 2e) and IKKβ (Fig. 3d) were decreased significantly when co-expressed with MGF360-12L. These findings indicated that MGF360-12L might regulate negatively in cGAS-STING-mediated NF-κB activation at the level of TBK1 and IKKβ.

### MGF360-12L suppresses IFN-β-stimulateded immune responses and antiviral effects

Type I IFNs, specifically IFN-α and IFN-β, are important for host anti-viral immune response through inducing the transcription of ISGs. To determinate the role of MGF360-12L in IFN-β-directed pathway responses, HEK293T cells were transfected with MGF360-12L and then treated with IFN-β. As shown in Fig. 4a and 4b, MGF360-12L exerted significant negative effect on the mRNA expression levels of ISG15, ISG54 and ISG56.The activation of ISRE promoter stimulated by IFN-β was also down-regulated. IRF9 is an important transcription factor, and together with transducers and activators of transcription 1 (STAT1) and STAT2, IRF9 forms the ISG factor3 (ISGF3) complex in response to type I IFNs activation (Blaszczyk et al. 2015). We found that MGF360-12L repressed the activation of ISRE promoter induced by IRF9 and IFN-β stimulation in a dose-dependent manner (Fig. 4c). Besides, MGF360-12L inhibited the exogenous expression level of IRF9 while had no effects on the exogenous expression of STAT1 and STAT2 (Fig. 4d). Moreover, MGF360-12L expression could inhibit IFN-β mediated antiviral immune response. As shown in Fig. 4e, the VSV-GFP related immunofluorescence intensity and protein level in HEK293T cells treated with IFN-β were enhanced in the MGF360-12L transfected group. In summary, these results indicated that the MGF360-12L could inhibit both IFN-β-stimulated immune responses and IFN-β triggered antiviral response, which might function at the level of IRF9.

### Regions of ASFV MGF360-12L responsible for inhibitory activity

Three expression plasmids, 12L-F1 (1-131aa), 12L-F2 (122-240aa) and 12L-F3 (225-350aa), were constructed to determine the regulatory elements of

Fig. 2. ASFV MGF360-12L interrupted IFN-β induction via TBK1 and IRF3. (a-b) HEK293T cells were co-transfected with TBK1-flag or IRF3-HA or IRF3-5D-HA, plus IFNβ-luc or ISRE-luc or pRDIII-I-luc and pRL-TK plasmid, along with MGF360-12L-His or empty vector. After 24 h, cells were analyzed by DLR (a) and WB (b). (c) Increasing amounts (50, 100, and 200 ng) of MGF360-12L-His and IRF3-5D-HA plasmids were co-transfected into HEK293T cells with pRL-TK and ISRE-luc or pRDIII-I-luc plasmid. After 24 h, cells were analyzed by DLR and WB. (d) HEK293T cells were co-transfected with MGF360-12L-His together with cGAS-GFP and STING-GFP plasmids for 24 h, then cells were lysed and detected by antibodies against pTBK1, TBK1, pIRF3, IRF3, His and GAPDH by WB. (e-f) HEK293T cells were co-transfected with MGF360-12L-His together with TBK1-flag or IRF3-5D-HA plasmids for 24 hours, and then the mRNA level of TBK1 (e), IRF3 (f) and β-actin were determined by qRT-PCR. ns, no significant difference; * p<0.05, ** p<0.01, *** p<0.001.
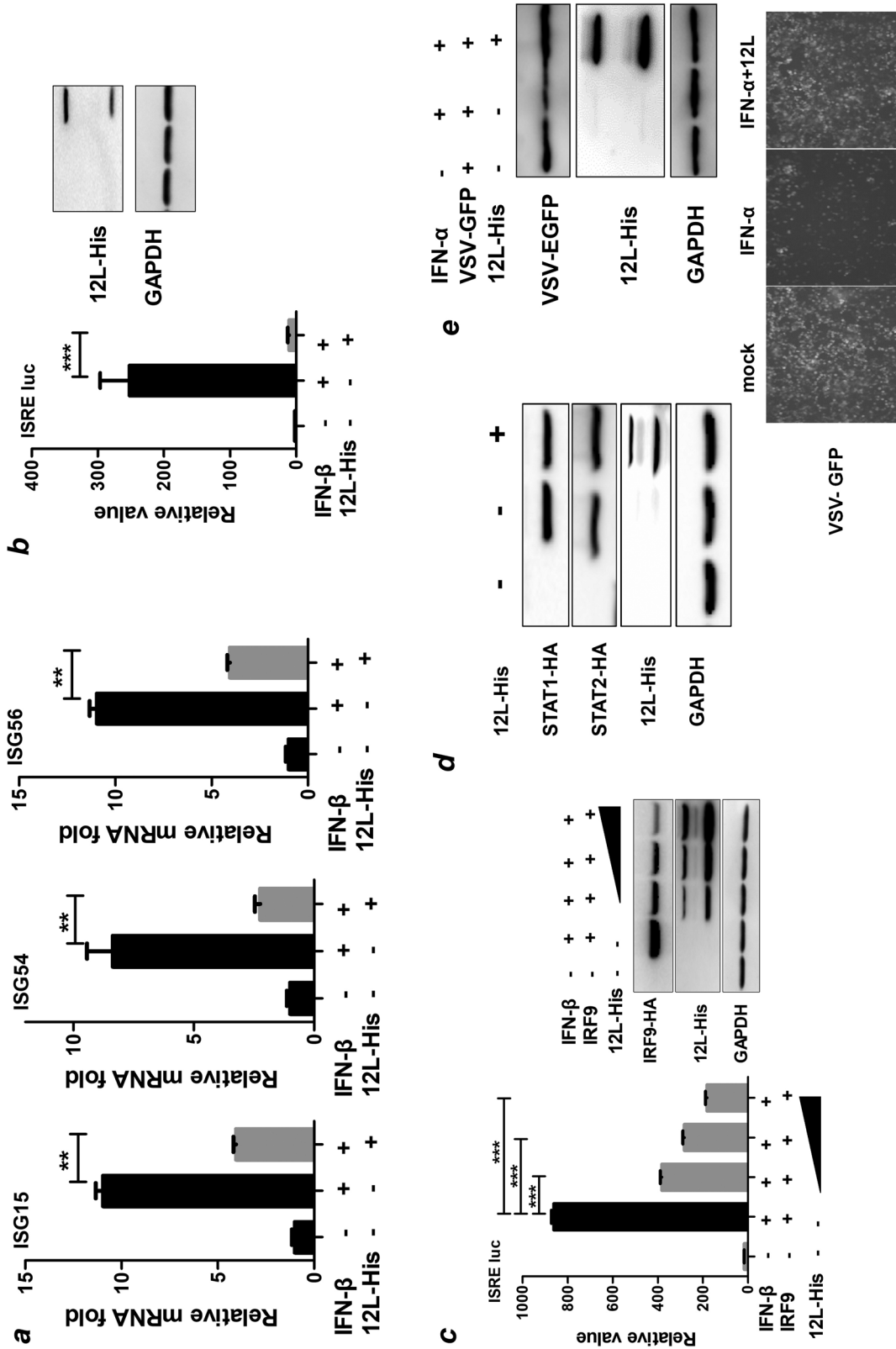
Fig. 3. MGF360-12L blocked NF-κB activation. (a) HEK293T cells were co-transfected with MGF360-12L-His or empty vector, NF-κB-luc and pRL-TK plasmids for 24 h, and treated or untreated with TNF-α (20 ng/ml) for 6 h. (b-c) HEK293T cells were co-transfected with TBK1-flag. (b) or IKKβ-HA (c), increasing amounts of MGF360-12L-His (50, 100, 200 ng), NF-κB-luc and pRL-TK plasmids for 24 h. Cells were harvested and analyzed by DLR. The protein levels of TBK1-flag, IKKβ-HA, MGF360-12L-His and GAPDH were determined by WB. (d) HEK293T cells were co-transfected with MGF360-12L-His and IKKβ-HA plasmids for 24 h, and then the mRNA levels of IKKβ and β-actin were determined by qRT-PCR. *** p<0.001.
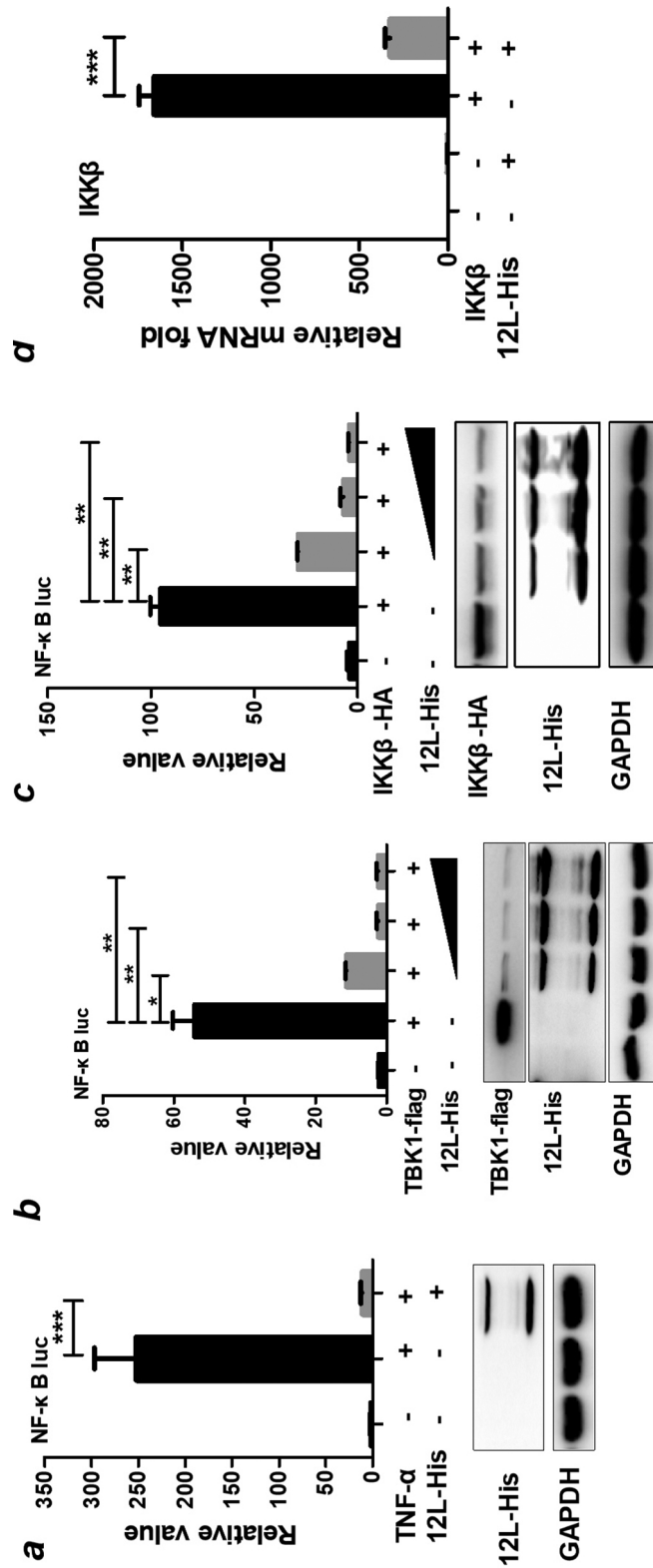
Fig. 4. MGF360-12L suppressed IFN-β induced immune responses and antiviral effects. (a) HEK293T cells were co-transfected with MGF360-12L-His or empty vector for 24 h, followed by treated or untreated with human IFN-β for 12 h. The mRNA level of ISG15, ISG54, ISG56 and β-actin were determined by qRT-PCR. (b) HEK293T cells were co-transfected with MGF360-12L-His or empty vector and along with ISRE-luc and pRL-TK plasmids for 24 h, followed by treated or untreated with human IFN-β protein for 12 h. (c) HEK293T cells were co-transfected and increased amounts of the plasmid MGF360-12L-His (50, 100, 200 ng) together with IRF9-HA or empty vector for 24 h. Then cells were incubated with IFN-β protein for 12 h and analyzed by DLR. The expression of IRF9-HA and MGF360-12L-His were detected by WB using anti-HA and anti-His antibodies. (d) HEK293T cells were co-transfected with MGF360-12L-His or empty vector, together with STAT1-HA, STAT2-HA plasmids for 24 h, then proteins were detected using antibodies against HA, His and GAPDH by WB. (e) HEK293T cells were transfected with MGF360-12L-His or empty vector. After 24 h, the cells were mock treated or treated with IFN-β for 12 h and then infected with VSV-GFP (MOI=1) for an additional 24 h. The GFP expression was quantitated by fluorescence microscopy and WB. ** p<0.01, *** p<0.001.

Fig. 5. Regions of ASFV MGF360-12L responsible for the inhibitory effect. (a–b) HEK293T cells were co-transfected with IFN-β-luc, ISRE-luc or NF-κB-luc, pRL-TK, and the plasmids encoding his-tagged MGF360-12L or its truncated plasmids MGF360-12L-F1, MGF360-12L-F2 and MGF360-12L-F3, together with TBK1 (a) or IRF3-5D (b) for 24 h. Cells were collected for DLR and WB. (c) HEK293T cells were co-transfected with NF-κB-luc, pRL-TK, and the plasmids encoding his-tagged MGF360-12L or its truncated plasmids together with IKKβ-HA or empty vector. After 24 h, cells were collected for DLR and WB. (d) HEK293T cells were co-transfected with ISRE-luc, pRL-TK, and the plasmids encoding his-tagged MGF360-12L or its truncated plasmids together with IRF9-HA or empty vector for 24 h. Cells were stimulated with IFN-β for an additional 12 h, and analyzed by DLR and WB. The expression of TBK1, IRF3-5D-HA, IKKβ-HA and MGF360-12L-His were detected using anti-TBK1, anti-IRF3, anti-HA and anti-His by WB.

MGF360-12L. We found that both 12L-F2 and 12L-F3 blocked TBK1 mediated IFN-β, ISRE and NF-κB promoters activation (Fig. 5a) and IKKβ mediated NF-κB activation (Fig. 5c). 12L-F3 slightly inhibited IRF3-5D-mediated IFN-β, ISRE and pRDIII-I promoters activation (Fig. 5b). Besides, 12L-F3 could also inhibit the ISRE activation mediated by IRF9 and IFN-β (Fig. 5d).

## Discussion

cGAS is a widely-expressed cytosolic DNA sensor, and plays key roles in detecting invading DNA viruses and initiating a series of antiviral signaling pathways (Xia et al. 2016). Previous study has shown that ASFV virulent strain Armenia/07 inhibits the cGAS-STING-mediated synthesis of IFN-β by inhibiting IRF3 activation and STING phosphorylation, while attenuated strain NH/P68 does not (Garcia-Belmonte et al. 2019). Deleting different combinations of MGF360 and MGF505 genes, such as MGF360-12L, -13L, -14L, MGF505-1R and K145R or MGF360-12L, MGF505-1R, and K145R, reduced virus replication in macrophages and virulence in pigs, while the combination of MGF360-13L, -14L, and MGF505-2R, -3R, and K145R failed in virus attenuation (Rathakrishnan et al. 2022). Moreover, the deletion of only MGF360-12L, combined with K145R, reduced virus replication in macrophages, indicating that MGF360-12L may be a key viral immune evasion protein.

It has been reported that MGF360-12L could significantly inhibit the mRNA transcription and promoter activity of IFN-β and NF-κB in Hela cells, accompanied by decreasing levels of IRF3, STING, TBK1, ISG54, ISG56 and AP-1 mRNA transcription after poly (I: C) or TNF-α stimulation. MGF360-12L can competitively inhibit the interaction of NF-κB with nuclear transport proteins (Zhuo et al. 2021). Our results correspond to previous study by Zhuo et al. (2021), however, instead of Hela cells and poly (I: C)/TNF-α stimulation were adopted in that study, HEK293T cells were used and cGAS-STING related innate immune activation was investigated in the current research. We found that MGF360-12L could inhibit the induction of IFN-β and activation of NF-κB induced by cGAS-STING. Moreover, MGF360-12L inhibits the activation of IFN-β and ISRE promoter induced by TBK1 and IRF3-5D in a dose-dependent manner, and blocks the exogenous expression of TBK1 and IRF3. Although MGF360-12L down-regulates the mRNA level of TBK1, it doesn't affect IRF3 transcription. MGF360-12L doesn't inhibit the phosphorylation of endogenous TBK1 and IRF3 stimulated by cGAS-STING, but significantly inhibits

IRF3-5D-induced activation of IFN-β and ISRE promoter. We speculated that MGF360-12L might fulfill its function by blocking the entry of pIRF3 from entering the nucleus or the binding of pIRF3 dimer to the promoter.

Moreover, we have identified several host proteins that interact with ASFV MGF360-12L by yeast two-hybrid system (data not shown). One of these proteins is the SERTA domain containing protein 1 (SERTAD1), which is reported to play an important role the stimulation of the transcriptional activities of different host genes (Lai et al. 2007, Vuono et al. 2020). Whether the interaction between MGF360-12L and SERTAD1 inhibits the broad transcription scenario in the current study needs to be further investigated.

Activated IKKβ is required for TBK1 and NF-κB activation in cGAS-STING pathway, forming a positive feedback loop with TBK1 to activate NF-κB and IRF3 (Balka et al. 2020). Our data revealed that MGF360-12L was involved in inhibition of NF-κB activation triggered by ectopic expression of TBK1 and IKKβ in 293T cells via down-regulating the mRNA levels of TBK1 and IKKβ.

In the canonical IFN-β signaling pathway, STAT1 and STAT2 are activated to form p-STAT1/p-STAT2 heterodimers and bind to IRF9. The activated ISGF3 proteins translocate to the nucleus, and then activate the transcription of ISGs (Blaszczyk et al. 2016). In our experiment, overexpression of MGF360-12L enhanced VSV-GEP replication in HEK293T compared to mock-treated group with IFN-β. Furthermore, we found that MGF360-12L inhibited IRF9 expression while showing no inhibitory effect on the expression of STAT1 and STAT2. And truncated mutation analysis demonstrated that the C-terminal domain of MGF360-12L was responsible for inhibiting TBK1 and IKKβ related immune response, while its inhibitory effects on IRF3-5D and IRF9 may require the complete sequence of MGF360-12L.

In summary, our results demonstrate that ASFV MGF360-12L is a potent and multifunctional immune-evasion protein. MGF360-12L may exert its immuno-suppressive function through a variety of mechanisms, including inhibition of the expression of TBK1, IRF3, IKKβ and IRF9 to block the IFN production and interruption of the JAK/STAT signaling. However, the molecular mechanisms of MGF360-12L in down-regulating the expression of TBK1, IRF3, IKKβ and IRF9 need to be further investigated.

## Acknowledgements

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