



Major vault protein inhibits CSFV replication by promoting cell apoptosis

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ABSTRACT

Classical swine fever virus (CSFV) remains a major pathogen posing substantial threats to the global swine industry. CSFV infection modulates the expression of numerous host cell proteins, with certain factors facilitating viral replication while others exert inhibitory effects. This study investigated the influence of CSFV infection on Major Vault Protein (MVP) expression, revealing that CSFV upregulates MVP in PK-15 cells. Functional analyses demonstrated that stable MVP overexpression significantly suppressed CSFV replication, whereas MVP knockout enhanced viral propagation. Furthermore, MVP was found to upregulate pro-apoptotic proteins Bax, caspase-3, and caspase-9 while downregulating the anti-apoptotic protein Bcl-2. These results indicate that MVP promotes apoptosis and that MVP-mediated apoptotic mechanisms contribute to the inhibition of CSFV replication. Collectively, these findings suggest that CSFV-induced MVP upregulation promotes apoptosis, thereby restricting viral replication. MVP may therefore represent a promising molecular target for developing novel therapeutic interventions against CSFV infection.

1. Introduction

Classical swine fever (CSF) is a devastating porcine disease characterized by high fever, anorexia, neurological disorders, and splenic infarctions. The acute form of CSF results in high morbidity and mortality within affected swine populations (Moennig, 2015; Zhou, 2019). The causative agent, Classical swine fever virus (CSFV), belongs to the genus *Pestivirus* within the family *Flaviviridae* (Thiel et al., 1991). CSFV is a positive-sense single-stranded RNA virus with a genome of approximately 12.3 kb, which contains a 5' untranslated region, a 3' untranslated region, and a central open reading frame. Infection with CSFV induces severe lymphopenia in pigs, leading to primary thymic atrophy and eventual death from disseminated intravascular coagulation and circulatory failure (Summerfield et al., 1998; Summerfield et al., 2001; Yuan et al., 2018). In pregnant sows, the virus can cross the placental barrier and infect fetuses, resulting in persistent infection in newborn piglets. The high mortality rate associated with CSF is attributed primarily to the virus's capacity to cause immunosuppression and establish persistent infections.

The Major Vault Protein (MVP) is primarily localized in the

cytoplasm, with a minor fraction associated with the nuclear membrane and nuclear pore complex (van Zon et al., 2003a; van Zon et al., 2003b). MVP is a multifunctional protein implicated in diverse cellular processes including multidrug resistance, immune regulation, nucleocytoplasmic transport, autophagy, and apoptosis (Zhang et al., 2023; Dong et al., 2022; Chung et al., 2005; Kurusu et al., 2023; Wang et al., 2023). With a molecular weight of approximately 100 kDa, MVP is highly conserved among eukaryotes. Notably, during infection with viruses such as hepatitis C virus (HCV), vesicular stomatitis virus (VSV), influenza A virus (IAV), and enterovirus 71 (EV71), MVP expression is induced via activation of the SP1 signaling pathway (Liu et al., 2015). This upregulation of MVP promotes the production of endogenous type I interferon (IFN-I) and the expression of interferon-stimulated genes, thereby initiating innate host defense mechanisms (Wang et al., 2020a). Furthermore, MVP overexpression has been shown to inhibit the replication and proliferation of multiple viruses, including HCV, VSV, IAV, EV71 (Liu et al., 2012), and porcine reproductive and respiratory syndrome virus (PRRSV) (Wu et al., 2021). However, its specific role in CSFV replication remains unclear.

Previous studies have indicated that CSFV infection modulates

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innate host immunity by suppressing apoptosis. For instance, earlier work from our group demonstrated that mitochondrial antiviral signaling protein (MAVS) induces the expression of IFN, IRF3, IL-6, and interferon-stimulated genes (ISGs), and promotes apoptosis through the caspase pathway, thereby inhibiting CSFV replication. In contrast, the CSFV N^{Pro} protein counteracts MAVS-mediated apoptosis by degrading IRF3 (Dong et al., 2018). MVP has been implicated in the regulation of apoptosis across various cell types. For example, MVP expression in osteoclasts decreases with age in mice. Wang et al. (2023) reported that MVP knockout inhibits osteoclast apoptosis both in vivo and in vitro, and demonstrated that MVP interacts with the death receptor Fas to promote apoptosis, thereby attenuating osteoporosis. Additionally, MVP has been identified as a target of N-(3-oxododecanoyl) homoserine lactone (C12), which reduces programmed cell death (PCD) via modulation of the p38 kinase pathway (Rayo et al., 2021). Furthermore, studies have shown that MVP cleavage frequently occurs during apoptosis in human primary keratinocytes and fibroblasts induced by diverse stimuli. In particular, caspase-1 and caspase-9 mediate MVP cleavage, leading to the inactivation of this cytoprotective protein; coordinated action of these caspases promotes apoptosis in epithelial cells following MVP inactivation (Grossi et al., 2020). Collectively, these findings highlight MVP as an important regulator of cellular apoptosis. Nevertheless, its potential role in modulating CSFV replication through apoptotic pathways remains to be elucidated.

In this study, we demonstrate that CSFV infection upregulates MVP expression in PK-15 cells. We further show that stable overexpression of MVP significantly inhibits CSFV replication, whereas MVP knockout enhances viral replication. Moreover, we found that MVP promotes apoptosis, and subsequent analyses confirmed that apoptosis suppresses CSFV replication. These results indicate that MVP modulates CSFV replication through the regulation of apoptosis, suggesting MVP as a potential therapeutic target for the development of anti-CSFV strategies in swine health management and antiviral drug design.

2. Materials and methods

2.1. Pig infection experiments

Four male Dutch Landrace pigs (4 weeks old) were obtained from the Experimental Animal Center of Northwest A&F University. All animals were healthy with no prior history of CSF outbreaks or vaccination. Virus-specific RT-qPCR confirmed that the pigs were free from CSFV, PRRSV, and porcine circovirus type 2 (PCV2). The animal study protocol was reviewed and approved by the Ethical Committee on Laboratory Animal Management and Animal Welfare of Northwest A&F University (Ethical Review Number: XN2024-0420). All experimental procedures were in line with the ethical standards stipulated in the “Laboratory Animal Welfare Ethics Review Guide GB/T35892” jointly issued by the National Quality Supervision and Inspection of the People’s Republic of China and the National Standardization Management Committee of China. Pigs were divided into two experimental groups: a CSFV inoculation group (n = 3) and a sham-inoculated control group (n = 1). After a 3-day acclimatization period, pigs in the CSFV group were challenged intranasally (1 mL per nostril) with 10^{4.0} TCID₅₀ / mL of the virus. Control pigs received an equivalent volume of cell culture medium. Following inoculation, all animals were monitored daily for clinical signs throughout the experimental period. Rectal temperature was recorded daily post-inoculation, and blood samples were collected from all pigs. Serum was subsequently separated by centrifugation. On days 6 and 7 post-challenge, two pigs in the CSFV-inoculated group succumbed to the infection. All remaining pigs, including those in the CSFV-infected group and the sham-inoculated control group, were humanely euthanized on day 8 post-challenge. During necropsy, tissue samples were collected from multiple organs, including the submandibular, mesenteric, and inguinal lymph nodes, as well as the heart, lungs, liver, spleen, kidneys, duodenum, jejunum, ileum, and colon. Lesion sites were

Table 1
Primers used for PCR in this study.

Primer name	Sequence (5'-3')
CMV-flag-MVP-F	GCTCTAGAAATGGCAACTGAAGAGTCCATCATCCGCAT
CMV-flag-MVP-R	CGGAATTTCAGGCACAATCTGGTTGCCTTGGAGAACC
MVP-sgRNA-F1	CACCGACCGAAGACTTACATCCGGC
MVP-sgRNA-R1	AAACGCCGGATGTAAGTCTTCGGTC
MVP-sgRNA-F2	CACCGCAGCAACGTGTCCCGGTGG
MVP-sgRNA-R2	AAACCCACGCGGACACGTGTGCTGC
MVP-sgRNA-F3	CACCGTTGGACCGAAGACTTACATC
MVP-sgRNA-R3	AAACGATGTAAGTCTTCGGTCCAAC

dissected, ground, and homogenized, after which RNA was extracted for subsequent analysis.

2.2. Reagents and antibodies

Mouse anti-CSFV E2 antibody (PC10765) was obtained from Abmart (Shanghai, China). Rabbit monoclonal antibodies against Bcl-2 (CY6717), Bax (CY5059), Caspase 3 (CY5501), and β-actin (AB0035) were sourced from ABways (Shanghai, China). Rabbit anti-MVP (16478-1-AP), mouse anti-Caspase 9 (66169-1-Ig), CoraLite594-conjugated Goat Anti-Rabbit IgG (H + L) (SA00013-1), and CoraLite488-conjugated Goat Anti-Mouse IgG (H + L) (SA00013-4) were procured from Proteintech (Wuhan, China). Z-VAD-FMK (abs812078) was purchased from Absin (Shanghai, China), and etoposide (SC0173-5mg) was acquired from Beyotime (Shanghai, China). The Annexin V-FITC/PI Apoptosis Detection Kit (BL110A) and CCK-8 reagent (BS3508) were manufactured by Biosharp (Hefei, China). Fetal bovine serum (Z7186TBS-500) was obtained from ZETA (USA).

2.3. Cell culture

PK-15 (ATCC: CCL-33) and HEK-293T cells (ATCC: CRL-11268) were cultured in high-glucose DMEM medium (CGM102.05, Cellmax) supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin solution (Gibco). Cells were maintained at 37 °C in a 5 % CO₂ atmosphere. The CSFV Shimen strain (GenBank: AF092448) was obtained from the China Institute of Veterinary Drug Control. Viral propagation was performed in PK-15 cells, and the viral titer was determined using the TCID₅₀ method.

2.4. Plasmid construction

The coding sequence (CDS) region of MVP (Gene ID: 100622738) was amplified from PK-15 cells and cloned into the pCDH-CMV-MCS-EF1-GreenPuro plasmid to generate the pCMV-MVP lentiviral expression vector. sgRNAs targeting MVP were designed using the online tool available at <https://www.zlab.bio/guide-design-resources>. The annealed double-stranded DNA fragments were ligated into the lentiCRISPR-V2 backbone, resulting in the construction of three lentiviral knockout vectors: plenti-V2-sg1MVP, plenti-V2-sg2MVP, and plenti-V2-sg3MVP. The sequences of all PCR primers used in this study are provided in Table 1.

2.5. Generation of lentivirus and construction of stable cell line

The constructed overexpression or knockout vectors (pCMV-MVP or plentiCRISPR-V2-sgMVP) and their corresponding helper plasmids were co-transfected into HEK-293T cells in the logarithmic growth phase. For the overexpression vector pCDH-CMV-MCS-EF1-GreenPuro, the helper plasmids pVSV-G, pRev, and pGag/Pol were used; for the knockout vector lentiCRISPR-V2, psPAX2 and pMD2.G were employed. After 10 h of incubation, the medium was replaced with Advanced DMEM supplemented with 2 % FBS, cholesterol (Sigma-Aldrich, C8667) (0.01 mM), egg yolk lecithin (Sigma-Aldrich, P443) (0.01 mM), L-

Table 2

Primers used for RT-qPCR in this study.

Primer name	Sequence (5'-3')
MVP-F	ACACTTCTGGATACAGGTGAGC
MVP-R	AGTCTTCGGTCCAACCTCCA
CSFV-F	GATCCTCATACTGCCCACTTAC
CSFV-R	GTATACCCCTTACCAGCTTG
β -actin-F	CAAGGACCTCTACGCCAACAC
β -actin-R	TGGAGGCGGATGATCTT

glutamine (Sigma-Aldrich, G7513) (4.0 mM), and chemically defined lipid concentrate (1:1000) (Invitrogen, 11905031). The recombinant lentivirus was harvested from the supernatant 48 h post-transfection, filtered, and used to infect PK-15 cells at a multiplicity of infection (MOI) of 1. At 24 h post-infection (hpi), the medium was replaced with complete medium containing puromycin (Sigma-Aldrich, P8833) (final concentration: 6 μ g/mL) to select positively transduced cells. The efficiency of MVP overexpression or knockout was assessed using quantitative real-time qPCR and Western blot analysis.

2.6. Real-time quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted from PK-15 cells using TRIzol reagent (AG21102, Agbio). Complementary DNA (cDNA) was synthesized from the extracted RNA using the PrimeScript RT kit (AG11728, Agbio) according to the manufacturer's instructions. Relative mRNA abundance was quantified by quantitative PCR (qPCR) with Fast qPCR Master Mix (DN2055-05, China) and the SYBR Green detection system (Bio-Rad, USA) to assess MVP mRNA expression and viral genomic RNA levels. The relative expression of target genes was evaluated using the $2^{-\Delta\Delta CT}$ method, with β -actin serving as the endogenous reference. All primers used for RT-qPCR are listed in Table 2.

2.7. Western blot analysis

PK-15 cell samples were washed twice with cold phosphate-buffered saline (PBS) and lysed on ice for 10 min using RIPA buffer (DXS1515, dongxisw) containing 1 mM PMSF (P0100, solarbio). The lysates were centrifuged at 12,000 rpm at 4 $^{\circ}$ C for 15 min. The resulting supernatant was mixed with 5 $\times$ protein loading buffer and boiled for 10 min. Proteins were separated by 12 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. After blocking with 5 % skim milk for 2 h at room temperature, the membrane was incubated with the appropriate primary antibody at 4 $^{\circ}$ C overnight. Following three washes with TBST (Tris-buffered saline containing 0.05 % Tween 20), the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse secondary antibodies for 2 h at room temperature. Protein bands were visualized using an ECL detection system and imaged with a gel documentation system (Cytiva, USA). Relative target protein expression levels were normalized to β -actin and quantified using ImageJ software.

2.8. Indirect immunofluorescence analysis and virus titer

PK-15 cells were fixed with 4 % paraformaldehyde for 15 min and permeabilized with 1 % Triton X-100 at room temperature for 10 min. After blocking with 5 % skim milk for 3 h at room temperature, the cells were incubated overnight at 4 $^{\circ}$ C with anti-E2 antibody diluted 1:800 in PBS, followed by incubation with CoraLite488-conjugated anti-mouse secondary antibody (diluted 1:500) at room temperature for 1 h. Nuclei were counterstained with DAPI. Immunofluorescence images were acquired using an inverted fluorescence microscope (Zeiss, Germany). The titer of CSFV was determined by TCID₅₀ using the Reed–Muench method.

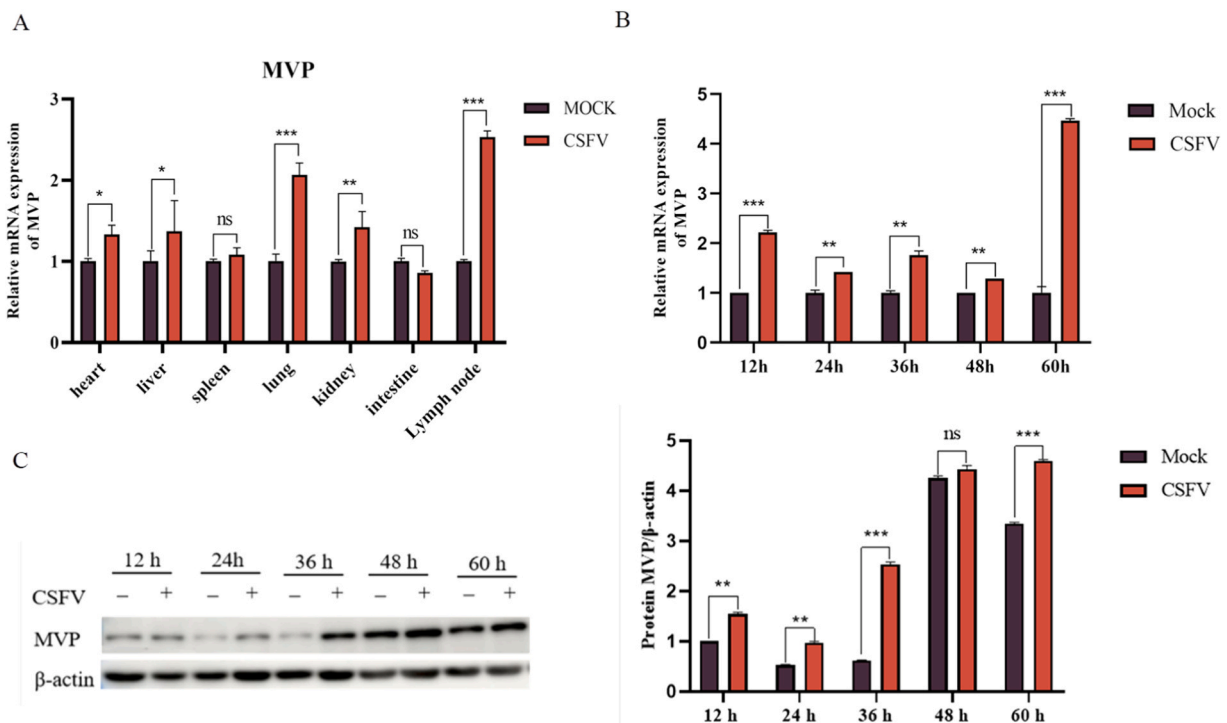


Fig. 1. CSFV infection upregulates MVP expression. (A) Heart, liver, lung, kidney, and lymph node tissues were collected from 4-week-old Dutch Landrace pigs following CSFV infection. MVP transcript levels were assessed by RT-qPCR. (B) PK-15 cells were infected with CSFV, and samples were harvested at 12, 24, 36, 48, and 60 hpi. MVP transcript levels were measured using RT-qPCR. (C) PK-15 cells infected with CSFV were lysed at 12, 24, 36, 48, and 60 hpi, and MVP protein expression was analyzed by Western blot, with β -actin serving as a loading control. Data are presented as mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001; ns, not significant (P > 0.05); Student's t -test.

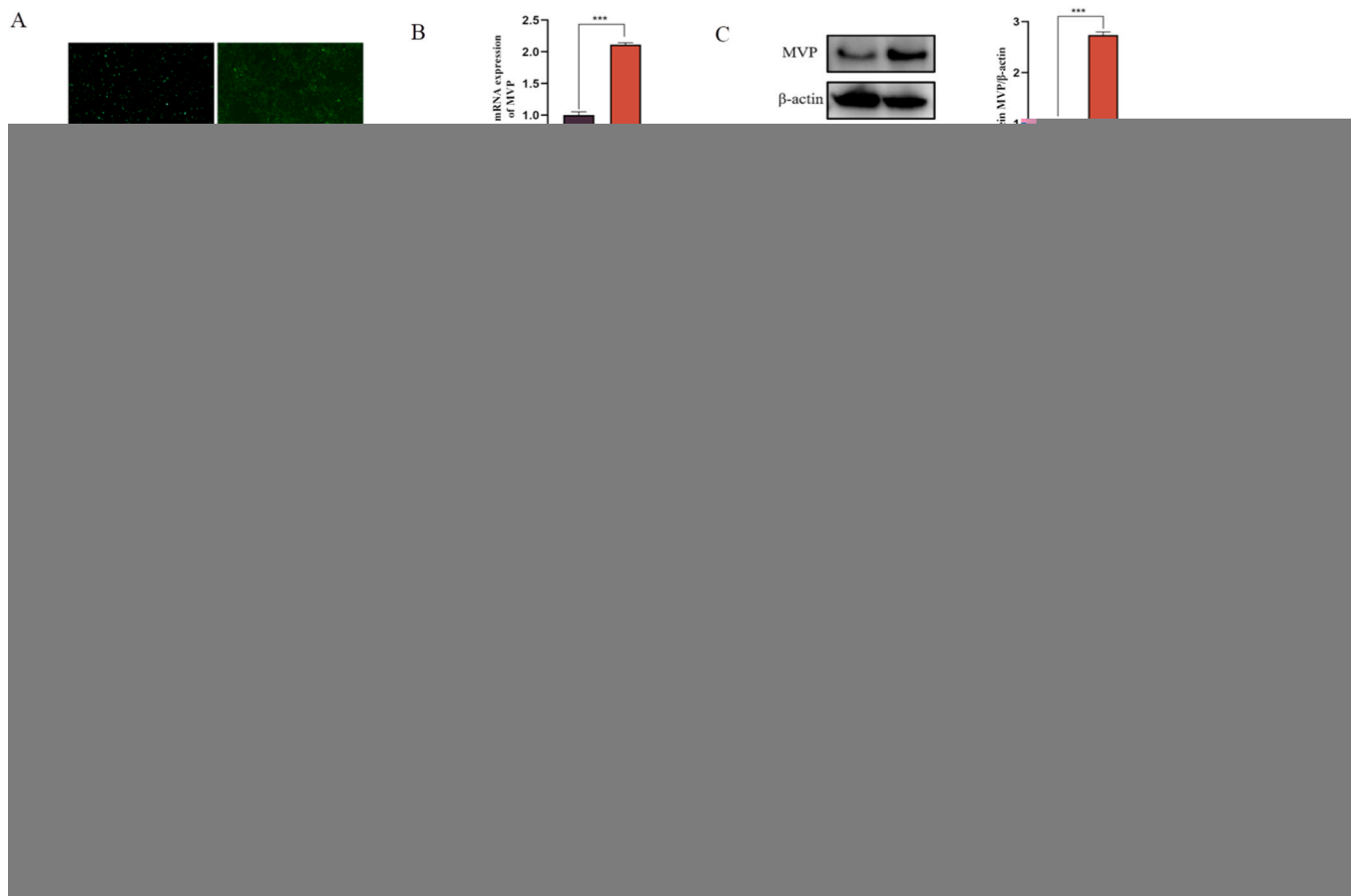


Fig. 2. Overexpression of MVP inhibits CSFV replication. (A–C) MVP-overexpressing (pCMV-MVP) and control (CMV) cell lines were generated using a recombinant lentiviral system and validated by RT-qPCR and Western blot analysis. Data are presented as mean $\pm$ SD; *P* values were determined by Student's *t*-test. (D–G) PK-15 cells were infected with CSFV at an MOI of 1 and harvested at 12, 24, and 48 hpi. Viral RNA levels, progeny virus titers, and viral infectivity were assessed (scale bar: 200 μ m). Infectivity was assessed based on E2 immunofluorescence signal intensity and positive cell count. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; ns, not significant (*P* > 0.05).

2.9. Detection of apoptosis by flow cytometry

The constructed MVP-knockout cell line (plenti-V2-sgMVP) and the control cells (plenti-V2) were seeded in cell culture dishes. At 24 hpi with CSFV at a MOI of 1, the cells were trypsinized, harvested, and washed twice with PBS. Apoptosis was assessed using an Annexin V-FITC/PI Apoptosis Detection Kit (BL110A, Biosharp) according to the manufacturer's instructions. Briefly, cells were resuspended in binding buffer and incubated with 5 μ L of Annexin V-FITC for 10 min at room temperature in the dark, followed by incubation with 10 μ L of propidium iodide (PI) for 5 min under the same conditions. Apoptotic cells were detected using a flow cytometer (BD, USA), and data were analyzed with FlowJo software (Tree Star, USA).

2.10. CCK-8 assay

Cell viability and cytotoxicity in response to apoptosis modulators were assessed using the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instructions. Briefly, PK-15 cells were seeded into 96-well plates. Upon reaching 70 % confluence, the cells were treated with various concentrations of apoptosis inhibitors or inducers and incubated for 24 h. Then, 10 μ L of CCK-8 reagent was added to each well, and the plates were incubated for an additional 2 h. The absorbance values were recorded at 450 nm using an Infinite M200pro system (Tecan, Männedorf, Switzerland).

2.11. Statistical analysis

All data were derived from three independent replicates. All data were expressed as mean $\pm$ standard deviation (SD). The statistical significance between groups was determined using the *t*-test with GraphPad Prism v.8.0 (San Diego, CA, USA). Differences were considered statistically significant at **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and non-significant at *P* > 0.05.

3. Results

3.1. CSFV infection increases MVP expression in PK-15 cells

We initially observed a marked upregulation of MVP mRNA levels across various tissues of infected pigs, including the heart, liver, lung, kidney, and lymph nodes (Fig. 1A). To further characterize the effect of CSFV infection on MVP expression, we assessed both transcriptional and translational levels at multiple time points post-infection using RT-qPCR and Western blot analysis. The results indicated that CSFV infection consistently increased MVP transcription in PK-15 cells (Fig. 1B). At the protein level, MVP expression was elevated at 12, 24, 36, and 60 hpi, although no significant change was detected at 48 h (Fig. 1C). Collectively, these findings demonstrate that CSFV infection significantly upregulates MVP expression.

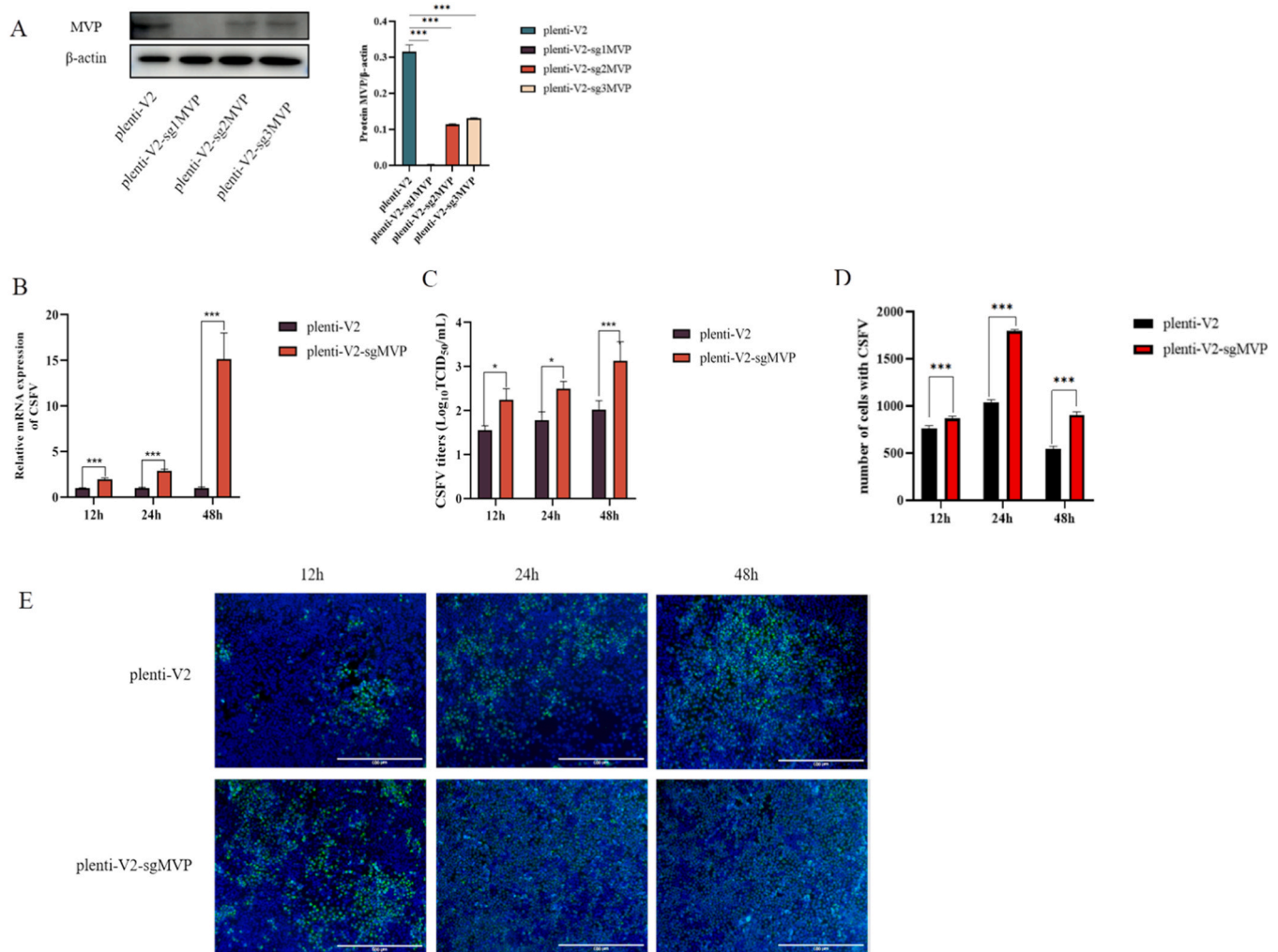


Fig. 3. Knockout of MVP promotes CSFV replication. (A) Three pairs of MVP-specific sgRNAs were designed and synthesized. Recombinant lentivirus was used to deliver sgRNAs into PK-15 cells, and knockout efficiency was evaluated by Western blot, with β -actin serving as a loading control. Data are presented as mean $\pm$ SD; P values were determined by Student's *t*-test. (B–D) The plenti-V2-sg1MVP construct, which exhibited the highest knockout efficiency in (A), was selected for further experiments. plenti-V2-sg1MVP and control plenti-V2 cells were infected with CSFV at an MOI of 1 and harvested at 12, 24, and 48 hpi. CSFV RNA levels were quantified by RT-qPCR, while viral titers and infectivity were assessed using TCID₅₀ and IFA, respectively (scale bar: 200 μ m). Infectivity was assessed based on E2 immunofluorescence signal intensity and positive cell count. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; ns, not significant (*P* > 0.05).

3.2. Overexpression of MVP inhibits CSFV replication

To evaluate the role of MVP in CSFV infection, we established MVP-overexpressing PK-15 cells using a lentiviral delivery system (Fig. 2A–C). These cells were subsequently infected with CSFV at an MOI of 1. Viral RNA levels, progeny virus titers, and viral infectivity were assessed at 12, 24, and 48 hpi. The results revealed that MVP overexpression significantly reduced CSFV RNA accumulation at all time points examined (Fig. 2D). Moreover, both progeny virus production and viral infectivity were markedly decreased in MVP-overexpressing cells compared to controls (Fig. 2E–G). These results indicate that MVP overexpression suppresses CSFV replication in PK-15 cells.

3.3. MVP knockout promotes CSFV replication

To further investigate the role of MVP in CSFV replication, we generated MVP-knockout PK-15 cells using CRISPR/Cas9. Western blot analysis confirmed that plenti-V2-sg1MVP yielded the highest knockout efficiency (Fig. 3A). Following infection with CSFV at an MOI of 1, MVP knockout significantly increased viral mRNA levels at 12, 24, and 48 hpi compared to control cells (Fig. 3B). Immunofluorescence assay (IFA) and

TCID₅₀ results further demonstrated that MVP knockout enhanced both viral titers and infectivity (Fig. 3C–E). These findings indicate that MVP deficiency promotes CSFV replication and propagation.

3.4. MVP expression promotes apoptosis in PK-15 Cells

Our results indicated that CSFV infection upregulates MVP expression, which subsequently suppresses viral replication. To investigate the mechanistic basis of this effect, we focused on the potential involvement of apoptosis. CSFV has been reported to establish persistent infection by inhibiting host apoptosis; for example, the viral NP^{ro} protein degrades IRF3 to attenuate MAVS-mediated apoptosis (Dong et al., 2018). Since MVP has been shown to promote apoptosis—such as by interacting with the Fas receptor to induce osteoclast apoptosis (Wang et al., 2023), we hypothesized that MVP may inhibit CSFV replication through pro-apoptotic mechanisms.

To evaluate this hypothesis, we assessed apoptotic rates in MVP-knockout PK-15 cells using flow cytometry. Both MVP knockout and CSFV infection significantly reduced apoptosis compared to control cells (Fig. 4A, B). We further analyzed the expression of key apoptosis-related proteins by Western blot following MVP overexpression or knockout.

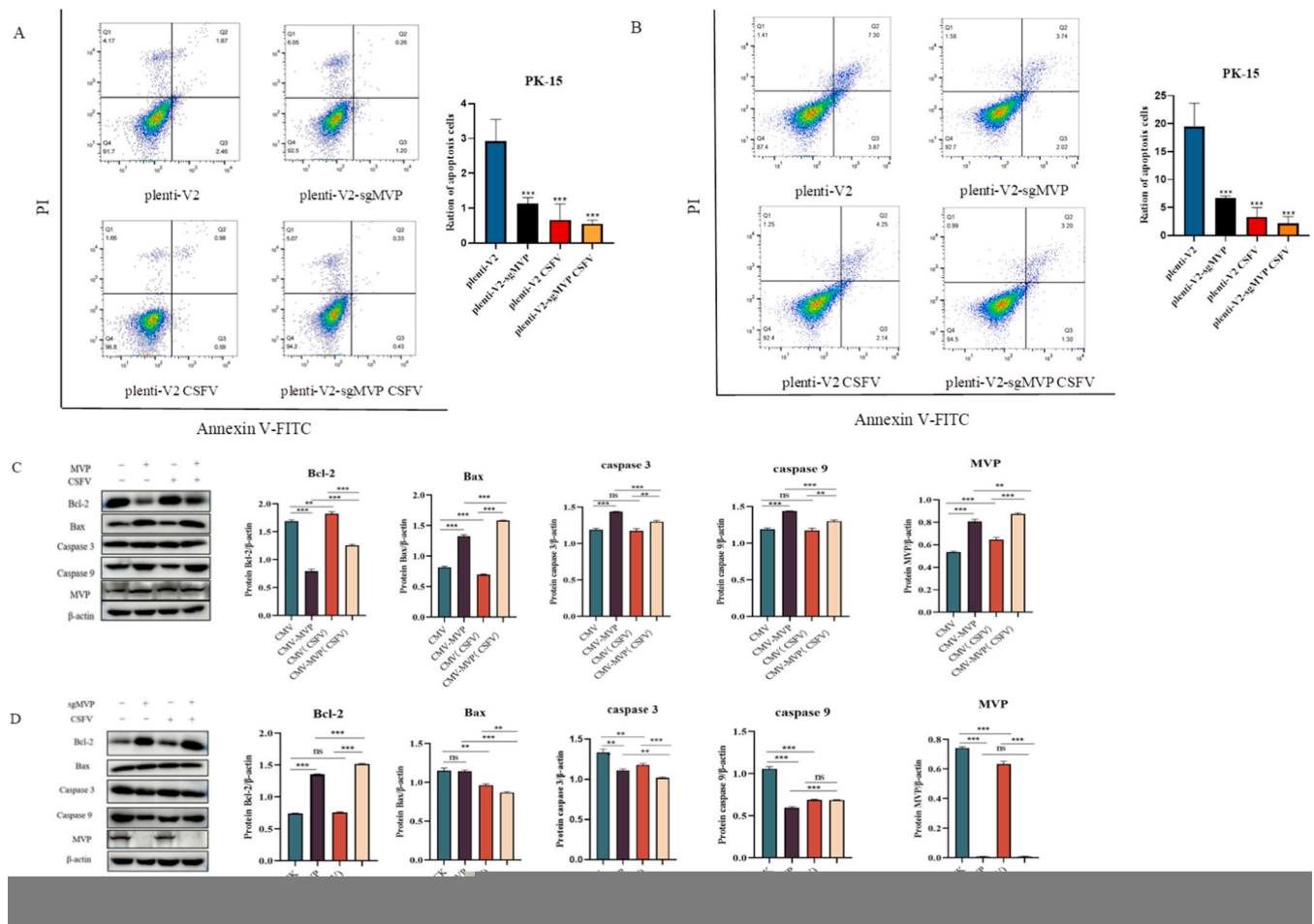


Fig. 4. MVP expression promotes apoptosis in PK-15 cells. (A, B) Apoptosis rates in MVP-knockout and CSFV-infected PK-15 cells were analyzed by flow cytometry at (A) 12 hpi and (B) 24 hpi. (C, D) Western blot analysis of apoptosis-related proteins following MVP overexpression or knockout. β -actin was used as a loading control. Data are presented as mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001; ns, not significant (P > 0.05; Student's t -test).

Overexpression of MVP led to decreased Bcl-2 levels and increased expression of Bax, caspase-9, and caspase-3. In contrast, MVP knockout resulted in the opposite effects (Fig. 4C, D). Consistent with previous reports, our data confirmed that CSFV infection suppresses apoptosis in PK-15 cells. Together, these findings indicate that MVP promotes apoptosis, whereas CSFV inhibits it.

3.5. Inhibition of apoptosis enhances CSFV replication

Having established the pro-apoptotic role of MVP in PK-15 cells, we next investigated whether MVP-induced apoptosis contributes to the inhibition of CSFV replication. To this end, we employed the apoptosis inhibitor Z-VAD-FMK, which was confirmed not to affect cell viability at the concentration used (Fig. 5A, B). Apoptosis was pharmacologically inhibited during CSFV infection under MVP-overexpressing conditions. RT-qPCR and IFA conducted at 24 hpi showed that while MVP overexpression reduced CSFV transcriptional levels, progeny virus titers, and viral infectivity, these antiviral effects were significantly attenuated upon treatment with Z-VAD-FMK (Fig. 5C-F).

3.6. Induction of apoptosis inhibits CSFV replication

To further elucidate the role of apoptosis in CSFV replication, we treated cells with etoposide to induce apoptosis (Fig. 6A), which did not affect cell viability within the concentration range used (Fig. 6B). We then evaluated CSFV replication in control (plenti-V2) and MVP-knockout (plenti-V2-sgMVP) cells following etoposide treatment. RT-

qPCR analysis revealed that MVP knockout enhanced CSFV transcription, but this increase was attenuated upon apoptosis induction (Fig. 6C). Similarly, MVP knockout elevated both progeny virus titers and viral infectivity; however, these effects were counteracted by etoposide-induced apoptosis (Fig. 6D-F). Together, these findings further support the conclusion that MVP inhibits CSFV replication through the induction of apoptosis.

4. Discussion

In this study, we elucidated the mechanism through which MVP confers resistance against CSFV infection. We found that CSFV infection triggers MVP upregulation in host cells, which subsequently suppresses viral replication by promoting apoptosis (Fig. 7). It is well established that CSFV infection modulates the expression of numerous host proteins. For example, CSFV has been shown to upregulate Allograft Inflammatory Factor 1 (AIF1) and components of the ESCRT machinery, while downregulating proteins such as claudin-1 and Receptor for Activated C Kinase 1 (RACK1) (Liu et al., 2022; Wang et al., 2021a; Wang et al., 2020b). Such alterations in host protein expression can lead to two opposing outcomes: either the host restricts viral replication through innate defense mechanisms, or the virus hijacks cellular pathways to facilitate its own propagation. Here, we demonstrated that CSFV infection of PK-15 cells significantly upregulates MVP expression at both the mRNA and protein levels, indicating that MVP serves as a host factor involved in the antiviral response against CSFV.

The host response to viral infection involves a complex interplay of

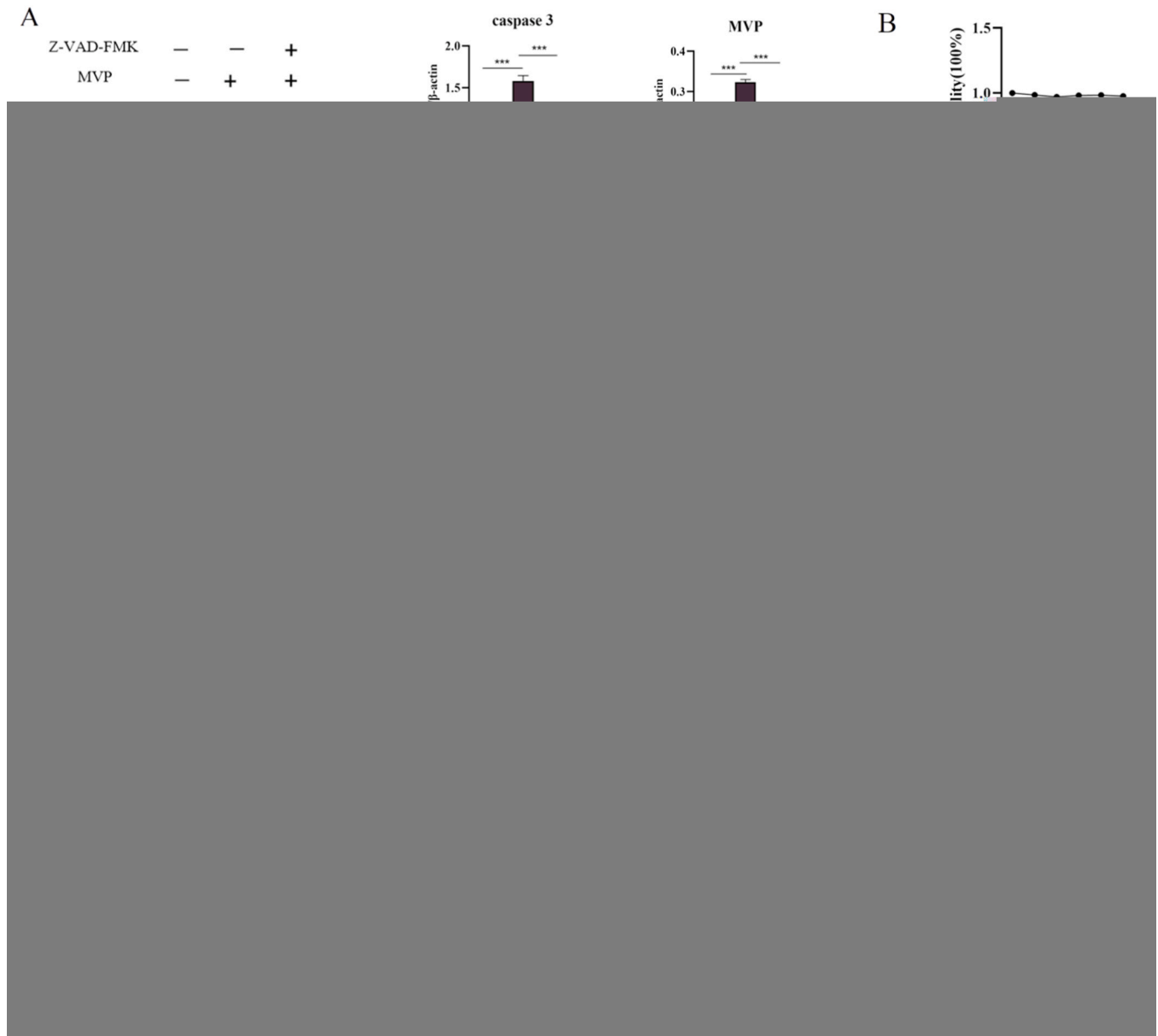


Fig. 5. Inhibition of apoptosis promotes CSFV replication. (A, B) pCMV and pCMV-MVP cells were infected with CSFV. Three experimental groups were established: pCMV, pCMV-MVP, and pCMV-MVP + Z-VAD-FMK (pre-treated for 1 h). At 24 hpi, RNA, supernatant, and protein samples were collected. MVP and caspase-3 expression levels were analyzed by Western blot. The optimal concentration range of Z-VAD-FMK that did not affect PK-15 cell viability was determined using a CCK-8 assay. (C-F) CSFV RNA levels were quantified by RT-qPCR. Viral titers and infectivity were assessed using TCID₅₀ and IFA, respectively (scale bar: 200 μm). Infectivity was assessed based on E2 immunofluorescence signal intensity and positive cell count. Data are presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant (P > 0.05; Student's *t*-test).

mechanisms. Pattern recognition receptors (PRRs) in host cells recognize specific viral components, thereby activating downstream signaling cascades that regulate cytokine production and facilitate viral clearance. It is well established that IFN production constitutes a critical step in the innate immune response. Previous studies have shown that MVP expression is induced via activation of the SP1 signaling pathway during infection with HCV, VSV, IAV, and EV71 (Liu et al., 2015). This upregulation of MVP facilitates the nuclear translocation of IRF7 and NF-κB, thereby enhancing the production of endogenous type IFN-I and the expression of interferon-stimulated genes (ISGs), which collectively contribute to antiviral activity. However, viruses have evolved various immune evasion strategies. For instance, during hepatitis B virus (HBV) infection, Toll-like receptors (TLRs) recruit and activate myeloid differentiation primary response 88 (MyD88) (Iwasaki et al., 2011). MVP interacts with MyD88 and integrates into MyD88-mediated signaling

complexes, promoting IFN-I production by facilitating the nuclear translocation of IRF7 and NF-κB (Liu et al., 2015; Liu et al., 2012). In contrast, HBV surface antigen (HBsAg) and e antigen (HBeAg) suppress IFN-I production by disrupting the interaction between MVP and MyD88 (Wang et al., 2020a). Additional evidence indicates that HBV employs multiple mechanisms to evade host immunity. HBV polymerase (Pol) inhibits MyD88 induction in response to IFN-α stimulation (Chen et al., 2013), HBeAg impairs TLR-induced IFN-β production (Wu et al., 2009), and HBsAg blocks the IRF-7-mediated IFN-α production pathway (Xu et al., 2009). Whether the upregulation of MVP during CSFV infection is primarily mediated by the host cell or facilitated by the virus itself requires further investigation. To evaluate the effect of MVP on CSFV replication, we generated MVP-overexpressing and MVP-knockout cell lines and employed RT-qPCR, IFA, and TCID₅₀ assays. The results demonstrated that MVP overexpression significantly decreased CSFV

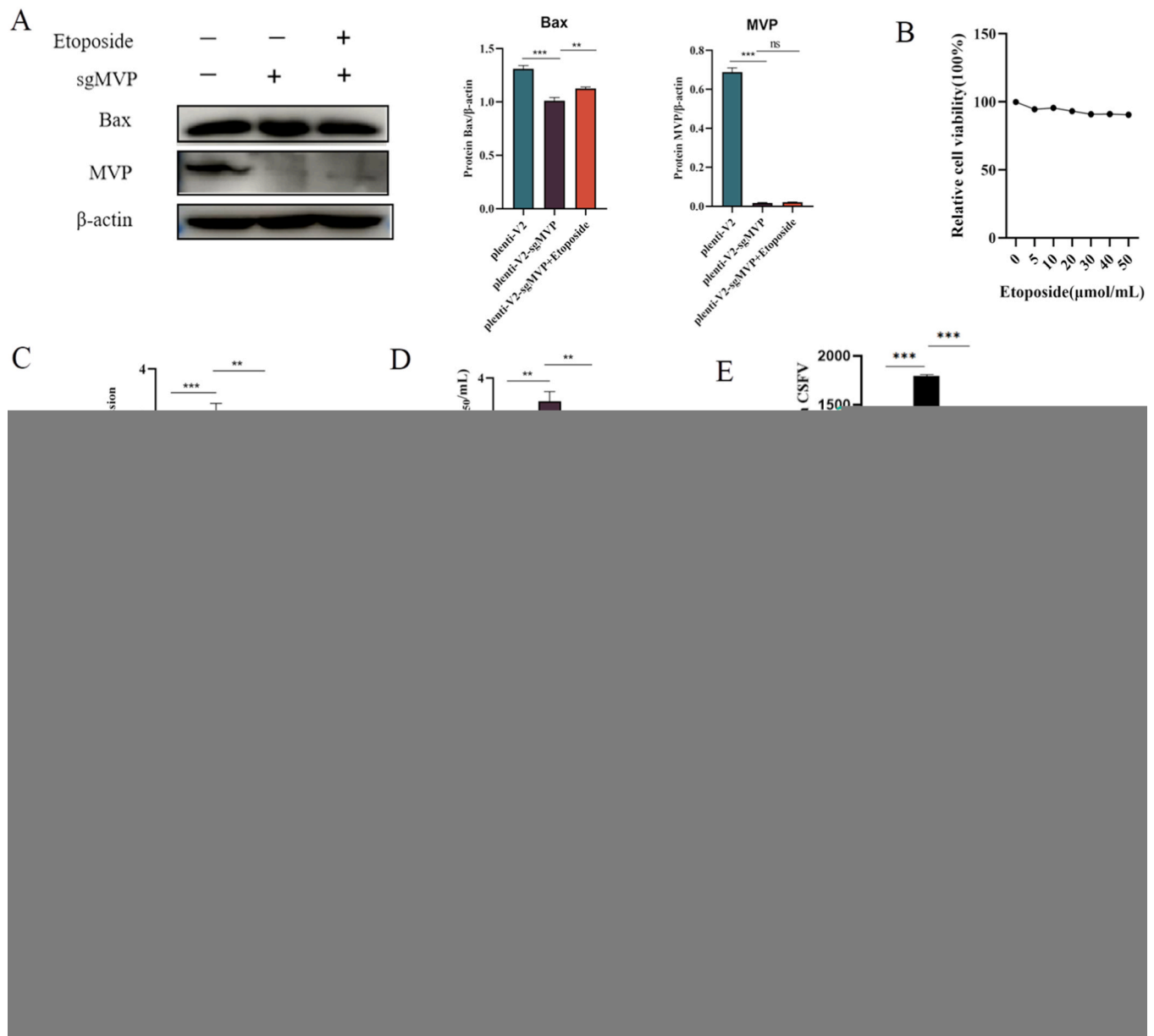


Fig. 6. Induction of apoptosis inhibits CSFV replication. (A, B) plenti-V2 and plenti-V2-sgMVP cells were infected with CSFV. Three experimental groups were included: plenti-V2, plenti-V2-sgMVP, and plenti-V2-sgMVP + etoposide (pre-treated for 1 h). RNA, supernatant, and protein samples were collected at 24 hpi. Expression levels of MVP and the pro-apoptotic protein Bax were analyzed by Western blot. The concentration range of etoposide that did not affect PK-15 cell viability was determined using a CCK-8 assay. Data are presented as mean $\pm$ SD; P values were determined by Student's *t*-test. (C-F) CSFV RNA levels were quantified by RT-qPCR. Viral titers and infectivity were assessed using TCID₅₀ and IFA, respectively (scale bar: 200 μm). Infectivity was assessed based on E2 immunofluorescence signal intensity and positive cell count. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; ns, not significant (*P* > 0.05).

transcriptional levels, viral titers, and infectivity at 12, 24, and 48 hpi. Conversely, MVP knockout enhanced viral replication. These findings indicate that MVP exerts an antiviral effect during CSFV infection and suggest that its upregulation represents a host defense mechanism against CSFV.

Apoptosis represents a critical defense mechanism by which the host counters viral infections. It disrupts the intracellular environment necessary for viral replication and facilitates the exposure and clearance of viral particles by immune cells such as macrophages (Nordén et al., 2013). Interestingly, CSFV has evolved strategies to inhibit apoptosis, thereby promoting its own persistence. For instance, the viral N^{Pro} protein degrades IRF3, suppressing MAVS-mediated apoptosis (Dong et al., 2018). In this study, we confirmed that CSFV infection inhibits apoptosis in PK-15 cells. MVP is also known to play a complex role in apoptosis

regulation. Previous studies have reported that MVP can inhibit apoptosis through suppression of STAT3 and Akt signaling (Das et al., 2016), yet it can also promote apoptosis, as demonstrated in osteoclasts via interaction with the death receptor Fas (Wang et al., 2023). Our flow cytometry analysis revealed that MVP knockout reduced the apoptosis rate in PK-15 cells, an effect that was further enhanced by CSFV infection. Western blot analysis of key apoptotic markers-Bax, Bcl-2, caspase-3, and caspase-9-supported the pro-apoptotic function of MVP in this context. Overexpression of MVP significantly decreased levels of the anti-apoptotic protein Bcl-2 and increased those of the pro-apoptotic proteins Bax, caspase-3, and caspase-9. Conversely, MVP knockout elevated Bcl-2 expression and reduced levels of Bax and caspase-3, though caspase-9 remained largely unchanged. To further examine the functional link between apoptosis and CSFV replication, we employed

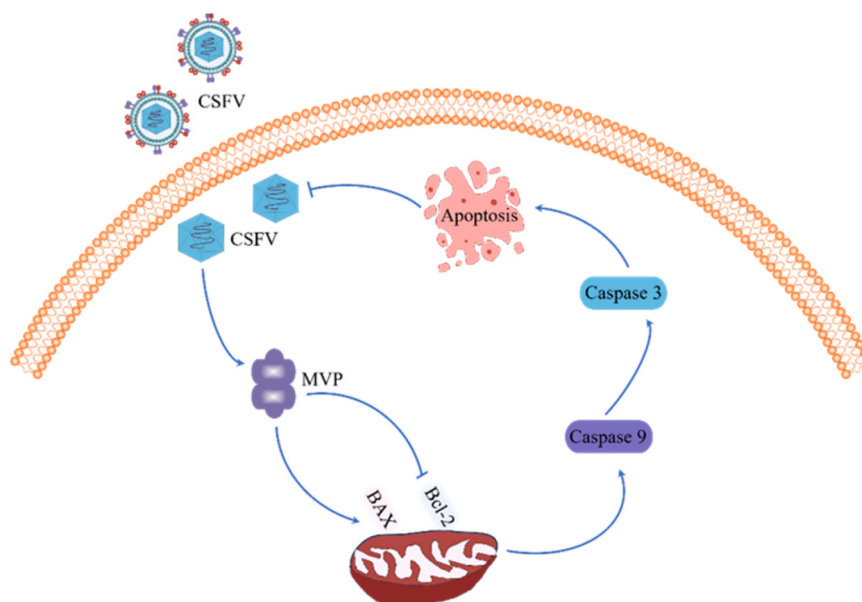


Fig. 7. Schematic model of the antiviral mechanism of MVP against CSFV infection. Following CSFV infection, the host upregulates MVP expression. This leads to the dissociation of Bcl-2 from Bax, subsequent activation of caspase-3, and induction of apoptosis, ultimately resulting in the inhibition of CSFV replication.

the apoptosis inhibitor Z-VAD-FMK and the inducer etoposide. Our results showed that pharmacological inhibition of apoptosis attenuated the antiviral effect of MVP overexpression, whereas induction of apoptosis counteracted the enhanced viral replication observed in MVP-knockout cells.

In conclusion, this study provides the first evidence that MVP exerts an antiviral effect against CSFV infection. We demonstrated that CSFV infection upregulates MVP expression, which in turn promotes apoptosis in PK-15 cells and thereby suppresses viral replication. These findings enhance our understanding of host defense mechanisms against CSFV and suggest that MVP may represent a promising target for the development of novel antiviral strategies.

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CRediT authorship contribution statement

Yuehan Quan: Writing – original draft, Validation, Resources, Investigation, Data curation. **Ning Li:** Validation, Resources, Investigation, Data curation. **Chen Chen:** Methodology, Investigation. **Liang Zhang:** Supervision, Resources, Project administration, Writing – review & editing. **Lei Xu:** Writing – review & editing, Supervision, Methodology. **Kangkang Guo:** Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation. **Bihao Luo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Investigation, Data curation. **Na Su:** Writing – original draft, Software, Methodology, Investigation, Data curation.

Declaration of Competing Interest

The authors report no conflict of interest.

References

- Chen, J., Wu, M., Zhang, X., Zhang, W., Zhang, Z., Chen, L., He, J., Zheng, Y., Chen, C., Wang, F., Hu, Y., Zhou, X., Wang, C., Xu, Y., Lu, M., Yuan, Z., 2013. Hepatitis b virus polymerase impairs interferon- α -induced STAT activation through inhibition of importin- α 5 and protein kinase C- δ . *Hepatology* 57 (2), 470–482. <https://doi.org/10.1002/hep.26064>.
- Chung, J.H., Ginn-Pease, M.E., Eng, C., 2005. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) has nuclear localization signal-like sequences for nuclear import mediated by major vault protein. *Cancer Res.* 65 (10), 4108–4116. <https://doi.org/10.1158/0008-5472.CAN-05-0124>.
- Das, D., Wang, Y.H., Hsieh, C.Y., Suzuki, Y.J., 2016. Major vault protein regulates cell growth/survival signaling through oxidative modifications. *Cell. Signal.* 28 (1), 12–18. <https://doi.org/10.1016/j.cellsig.2015.10.007>.
- Dong, W., Lv, H., Li, C., Liu, Y., Wang, C., Lin, J., Wang, Y., Qian, G., Guo, K., Zhang, Y., 2018. MAVS induces a host cell defense to inhibit CSFV infection. *Arch. Virol.* 163 (7), 1805–1821. <https://doi.org/10.1007/s00705-018-3804-z>.
- Dong, X., Akueteh, P.D.P., Song, J., Ni, C., Jin, C., Li, H., Jiang, W., Si, Y., Zhang, X., Zhang, Q., Huang, G., 2022. Major vault protein (MVP) associated with *braf*V600E mutation is an immune Microenvironment-Related biomarker promoting the progression of papillary thyroid cancer via MAPK/ERK and PI3K/AKT pathways. *Front. Cell Dev. Biol.* 9, 688370. <https://doi.org/10.3389/fcell.2021.688370>.
- Grossi, S., Fenini, G., Kockmann, T., Hennig, P., Di Filippo, M., Beer, H.D., 2020. Inactivation of the cytoprotective major vault protein by Caspase-1 and -9 in epithelial cells during apoptosis. *J. Invest. Dermatol.* 140 (7), 1335–1345.e10. <https://doi.org/10.1016/j.jid.2019.11.015>.
- Iwasaki, H., Takeuchi, O., Teraguchi, S., Matsushita, K., Uehata, T., Kuniyoshi, K., Satoh, T., Saitoh, T., Matsushita, M., Standley, D.M., Akira, S., 2011. The I κ B kinase complex regulates the stability of cytokine-encoding mRNA induced by TLR-IL-1R by controlling degradation of regnase-1. *Nat. Immunol.* 12 (12), 1167–1175. <https://doi.org/10.1038/ni.2137>.
- Kurusu, R., Fujimoto, Y., Morishita, H., Noshiro, D., Takada, S., Yamano, K., Tanaka, H., Arai, R., Kageyama, S., Funakoshi, T., Komatsu-Hirota, S., Taka, H., Kazuno, S., Miura, Y., Koike, M., Wakai, T., Waguri, S., Noda, N.N., Komatsu, M., 2023. Integrated proteomics identifies p62-dependent selective autophagy of the supramolecular vault complex. *Dev. Cell* 58 (13), 1189–1205.e11. <https://doi.org/10.1016/j.devcel.2023.04.015>.
- Liu, C., Zhao, W., Su, J., Chen, X., Zhao, F., Fan, J., Li, X., Liu, X., Zou, L., Zhang, M., Zhang, Z., Zhang, L., Fan, S., Li, Y., Zhao, M., Chen, J., Yi, L., 2022. HSP90AA1 interacts with CSFV NS5A protein and regulates CSFV replication via the JAK/STAT and NF- κ B signaling pathway. *Front. Immunol.* 13, 1031868. <https://doi.org/10.3389/fimmu.2022.1031868>.
- Liu, S., Hao, Q., Peng, N., Yue, X., Wang, Y., Chen, Y., Wu, J., Zhu, Y., 2012. Major vault protein: a virus-induced host factor against viral replication through the induction of type-I interferon. *Hepatology* 56 (1), 57–66. <https://doi.org/10.1002/hep.25642>.
- Liu, S., Peng, N., Xie, J., Hao, Q., Zhang, M., Zhang, Y., Xia, Z., Xu, G., Zhao, F., Wang, Q., Han, T., Zhu, Y., 2015. Human hepatitis b virus surface and e antigens inhibit major vault protein signaling in interferon induction pathways. *J. Hepatol.* 62 (5), 1015–1023. <https://doi.org/10.1016/j.jhep.2014.11.035>.
- Moennig, V., 2015. The control of classical swine fever in wild boar. *Front. Microbiol.* 6, 1211. <https://doi.org/10.3389/fmicb.2015.01211>.

- Nordén, R., Nyström, K., Aurelius, J., Brisslert, M., Olofsson, S., 2013. Virus-induced appearance of the selectin ligand sLeX in herpes simplex virus type 1-infected T-cells: involvement of host and viral factors. *Glycobiology* 23 (3), 310–321. <https://doi.org/10.1093/glycob/cws160>.
- Rayo, J., Gregor, R., Jacob, N.T., Dandela, R., Dubinsky, L., Yashkin, A., Aranovich, A., Thangaraj, M., Ernst, O., Barash, E., Malitsky, S., Florea, B.I., Krom, B.P., Wiemer, E. A.C., Kickhoefer, V.A., Rome, L.H., Mathison, J.C., Kaufmann, G.F., Overkleeft, H.S., Cravatt, B.F., Meijler, M.M., 2021. Immunoeediting role for major vault protein in apoptotic signaling induced by bacterial N-acyl homoserine lactones. *Proc. Natl. Acad. Sci. USA* 118 (12), e2012529118. <https://doi.org/10.1073/pnas.2012529118>.
- Summerfield, A., Knötig, S.M., McCullough, K.C., 1998. Lymphocyte apoptosis during classical swine fever: implication of activation-induced cell death. *J. Virol.* 72 (3), 1853–1861. <https://doi.org/10.1128/JVI.72.3.1853-1861.1998>.
- Summerfield, A., Zingle, K., Inumaru, S., McCullough, K.C., 2001. Induction of apoptosis in bone marrow neutrophil-lineage cells by classical swine fever virus. *J. Gen. Virol.* 82 (Pt 6), 1309–1318. <https://doi.org/10.1099/0022-1317-82-6-1309>.
- Thiel, H.J., Stark, R., Weiland, E., Rümenapf, T., Meyers, G., 1991. Hog cholera virus: molecular composition of virions from a pestivirus. *J. Virol.* 65 (9), 4705–4712. <https://doi.org/10.1128/JVI.65.9.4705-4712.1991>.
- van Zon, A., Mossink, M.H., Scheper, R.J., Sonneveld, P., Wiemer, E.A., 2003a. The vault complex. *Cell. Mol. life Sci. CMLS* 60 (9), 1828–1837. <https://doi.org/10.1007/s00018-003-3030-y>.
- van Zon, A., Mossink, M.H., Schoester, M., Houtsmuller, A.B., Scheffer, G.L., Scheper, R. J., Sonneveld, P., Wiemer, E.A., 2003b. The formation of vault-tubes: a dynamic interaction between vaults and vault PARP. *J. Cell Sci.* 116 (Pt 21), 4391–4400. <https://doi.org/10.1242/jcs.00749>.
- Wang, R., Yang, Y., Zhang, Z., Zhao, N., Wiemer, E.A.C., Ben, J., Ma, J., Yuan, L., 2023. Major vault protein (MVP) suppresses aging- and estrogen deficiency-related bone loss through Fas-mediated apoptosis in osteoclasts. *Cell death Dis.* 14 (9), 604. <https://doi.org/10.1038/s41419-023-05928-4>.
- Wang, W., Xiong, L., Wang, P., Wang, F., Ma, Q., 2020a. Major vault protein plays important roles in viral infection. *IUBMB life* 72 (4), 624–631. <https://doi.org/10.1002/iub.2200>.
- Wang, X., Gao, L., Yang, X., Zuo, Q., Lan, R., Li, M., Yang, C., Lin, Y., Liu, J., Yin, G., 2020b. Porcine RACK1 negatively regulates the infection of classical swine fever virus and the NF- κ B activation in PK-15 cells. *Vet. Microbiol.* 246, 108711. <https://doi.org/10.1016/j.vetmic.2020.108711>.
- Wang, X., Yang, Y., Yang, X., Liu, X., Wang, X., Gao, L., Yang, C., Lan, R., Bi, J., Zhao, Q., Yang, G., Wang, J., Lin, Y., Liu, J., Yin, G., 2021. Classical swine fever virus infection suppresses claudin-1 expression to facilitate its replication in PK-15 cells. *Microb. Pathog.* 157, 105012. <https://doi.org/10.1016/j.micpath.2021.105012>.
- Wu, J., Meng, Z., Jiang, M., Pei, R., Trippler, M., Broering, R., Bucchi, A., Sowa, J.P., Dittmer, U., Yang, D., Roggendorf, M., Gerken, G., Lu, M., Schlaak, J.F., 2009. Hepatitis b virus suppresses toll-like receptor-mediated innate immune responses in murine parenchymal and nonparenchymal liver cells. *Hepatology* (Baltim. Md.) 49 (4), 1132–1140. <https://doi.org/10.1002/hep.22751>.
- Wu, X., Fang, J., Huang, Q., Chen, X., Guo, Z., Tian, L., Zhou, E., Chen, J., Mu, Y., Du, T., 2021. Major vault protein inhibits porcine reproductive and respiratory syndrome virus infection in CRL2843CD163 cell lines and primary porcine alveolar macrophages. *Viruses* 13 (11), 2267. <https://doi.org/10.3390/v13112267>.
- Xu, Y., Hu, Y., Shi, B., Zhang, X., Wang, J., Zhang, Z., Shen, F., Zhang, Q., Sun, S., Yuan, Z., 2009. HBsAg inhibits TLR9-mediated activation and IFN- α production in plasmacytoid dendritic cells. *Mol. Immunol.* 46 (13), 2640–2646. <https://doi.org/10.1016/j.molimm.2009.04.031>.
- Yuan, J., Zhu, M., Deng, S., Fan, S., Xu, H., Liao, J., Li, P., Zheng, J., Zhao, M., Chen, J., 2018. Classical swine fever virus induces pyroptosis in the peripheral lymphoid organs of infected pigs. *Virus Res.* 250, 37–42. <https://doi.org/10.1016/j.virusres.2018.04.004>.
- Zhang, J.Z., Ong, S.E., Baker, D., Maly, D.J., 2023. Single-cell signaling analysis reveals that major vault protein facilitates RasG12C inhibitor resistance. *bioRxiv Prepr. Serv. Biol.*, 2023.10.02.560617 <https://doi.org/10.1101/2023.10.02.560617>.
- Zhou, B., 2019. Classical swine fever in China-An update minireview. *Front. Vet. Sci.* 6, 187. <https://doi.org/10.3389/fvets.2019.00187>.