Encephalomyocarditis Virus Structural Protein VP3 Interacts with MAVS and Promotes its Autophagic Degradation to Interfere with the Type I Interferon Signaling Pathway

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Abstract

Background: Understanding the mechanisms through which interferon (IFN) signaling is negatively regulated is crucial for preserving the equilibrium of innate immune reactions, as the innate immune system functions, such as the original barrier, combat threats to the host. Although the function of the encephalomyocarditis virus (EMCV) viral proteins in antagonizing innate immunity has been related to earlier studies, the precise mechanism underlying the role of viral protein 3 (VP3) in type I IFN has yet to be fully illuminated. Methods: VP3 expression and many other adaptor molecules belonging to type I IFN pathway expression levels were evaluated using Western blotting. The IFN and other antiviral genes, such as interferon-stimulated genes (ISGs) 15 and 56, were assessed by real-time quantitative polymerase chain reaction (RT-qPCR). A 50% tissue culture infectious dose (TCID₅₀) assay was utilized to explore the effect of VP3 on EMCV proliferation in human embryonic kidney (HEK293) cells. Co-immunoprecipitation (Co-IP) assays and confocal microscope analysis were used to investigate the underlying mechanisms mediated by VP3. Results: We discovered that the VP3 of EMCV acts as a suppressor of innate immune reactions. Increased levels of VP3 enhance viral reproduction through modulation of innate immune signaling pathways and suppression of antiviral responses. Additional information indicated that during viral infection, the VP3 of EMCV enhances autophagy and interacts specifically with mitochondrial antiviral signaling protein (MAVS), leading to its degradation in an autophagy pathway that relies on p62. Conclusions: Our findings showed that EMCV developed a tactic to combat host antiviral defenses by using autophagy to break down a protein that controls the innate immune response following a viral infection of the host. Notably, VP3 plays an important role in this process. Overall, these discoveries may provide a novel therapeutic target for EMCV.

Keywords: EMCV viral protein 3; interferon; mitochondrial antiviral signaling protein; autophagy

1. Introduction

Encephalomyocarditis virus (EMCV) is a RNA virus belonging to the Picornaviridae family [1]. The EMCV genetic material comprises approximately 7861 nucleotides and comprehends a single open reading frame responsible for encoding a polyprotein [2]. Virus-encoded proteases post-translationally process this polyprotein into four structural proteins, viral protein 1 (VP1) to VP4, along with eight nonstructural proteins: L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D [3]. EMCV 2C, 3C, and VP2 are crucial in inhibiting interferon (IFN) production and subsequent antiviral signaling [4–7]. However, the mechanism through which EMCV viral protein VP3 restricts the natural immune signaling pathway remains poorly understood.

The innate immunity system contributes to the host’s initial defense against invading microorganisms. Recognition receptors such as retinoic acid-inducible gene-I (RIG)-like receptors (RLRs), (RIG-I, melanoma differentiation-associated protein 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2)), cyclic GMP-AMP synthase (cGAS), and nucleotide-binding oligomerization domain (NOD)-like receptors (NOD2) are essential in innate immunity by interacting with pathogen-associated molecular patterns (PAMPs) to produce IFN that is effective against viruses [8]. Viruses must overwhelm the host’s innate immunologic reaction to build a constructive infection and counteract the host’s antiviral defenses for sustained viral replication. Following the identification of virus-related RNA, RIG-I brings in the adapter protein mitochondrial antiviral signaling protein (MAVS) to trigger TANK-binding kinase 1 (TBK1) and the inhibitor of nuclear factor-κB (IkB) kinase family (IKK) complex, leading to the stimulation of interferon regulatory factor 3/7 (IRF3/7) and nuclear factor NF-kappaB (NF-κB), ultimately causing the generation of IFN α/β [9]. Subsequently, the released
IFN-I inspires the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway, leading to the phosphorylation of STAT1/2. Phosphorylated STAT1/2 binds with IRF9 to create a tripartite transcriptional factor (ISGF3) complex, which attaches to the interferon-stimulated response element (ISRE) to trigger the transcription of IFN-induced genes (ISGs), resulting in an anti-virus status within the cell [10,11].

MAVS, also called VISA, is primarily found within the cell’s mitochondria. In addition to being distributed at mitochondrion, MAVS is found locally at peroxisomes and mitochondria-associated membranes (MAM) [12,13]. Furthermore, the aggregation of MAVS is facilitated by its localization to the membrane. Specifically, proper activation of the protein necessitates mitochondrial localization of MAVS; upon activation, MAVS forms a functional prion-like structure on mitochondria through aggregation [14]. The platform is essential for assembling the MAVS signalosome, then initiates the rousing of TNF receptor and IKKε [15,16]. This process drives the downstream molecules IRF3, IRF7, and NF-κB enlivening, triggering anti-virus reactions, including producing IFNs [17,18].

EMCV has developed various immune escape strategies to prevent IFN-I from spreading quickly and effectively at the initial infection site. Numerous EMCV proteins are involved in evading the host’s immune system. EMCV proteases 3Cpro hindered the ability of TANK to block TNF receptor-associated factor 6 (TRAF6)-induced NF-κB signaling [5]. Furthermore, 3Cpro cleaved TANK and dismantled the TANK–TBK1–IKKε–IRF3 complex, leading to the decreased phosphorylated format of IRF3 and the type I IFN generation [6]. VP3 breaks down MDA5, a pattern recognition receptor in interferon signaling that detects virus RNA. It also breaks down the TBK1–IRF3 complex to facilitate viral replication [7]. EMCV protein 2C effectively blocks the IFN-β signal pathway through interplay with MDA5, acting as a potent antagonist of IFN-β [4]. Proteins 2C and 3A are involved in the degradation of heat shock protein 27 (HSP27), enabling EMCV proliferation, according to a previous study [19]. EMCV VP3 and 3C are accountable for the suppression of IRF3 phosphorylation through DEAD (Asp–Glu–Ala–Asp)-box RNA helicases 56 (DDX56) [20].

This research aimed to investigate the impact of VP3 on the natural immune system reaction. VP3 negatively regulated EMCV-elicted innate immunity response and strengthened viral replication in vitro. Detailed research discovered that VP3 repressed IFN production by interacting with MAVS and promoting its autophagic degradation. The discovery revealed a novel role of VP3 in suppressing natural immunity reactions.

2. Materials and Methods

2.1 Cells and Viruses

Baby Hamster Kidney (BHK-21) and Human Embryonic Kidney 293 (HEK-293) cells were acquired from ATCC (Manassas, VA, USA). All cell lines were validated by STR profiling and tested negative for mycoplasma. Cells were cultured in high glucose Dulbecco’s modified Eagle medium (DMEM, CMG101.05, Bailing, Lanzhou, China) with 10% newborn bovine serum (NBS, SA301.02.V, Cellmax, Beijing, China) at 37 °C in a 5% CO2 incubator (BB150, Thermofisher, Waltham, MA, USA). The EMCV PV21 strain (GenBank Accession no. X74312) was propagated in BHK-21 cells and preserved at −80 °C in a freezer.

2.2 Antibodies and Chemicals

All antibodies were bought from specified suppliers. Sangon Biotech (Shanghai, China) provided horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (D110058) and HRP-conjugated goat anti-mouse IgG (D110087). Antibodies for MAVS (14341-1-AP), HA-tag (S1064-2-AP), and IRF3 (11312-1-AP) were bought from Proteintech (Wuhan, China). Cell Signaling Technology (Beverly, MA, USA) provided the antibodies for STING (13647S), TBK1 (3013S), and Myc-tag (2276S). Beyotime Biotechnology (Shanghai, China) provided the monoclonal antibody for β-actin (AA128). Green conjugated Donkey anti-mouse IgG (128-545-003) or Red conjugated Donkey anti-rabbit IgG (128-165-160) were purchased from Jackson Immuno Research (Bar Harbor, ME, USA).

In-house construction of plasmids containing VP3 (Myc-tag) was completed. The SYBR Green Pro Taq HS qPCR kit (ROX plus) (AG11718) and Pro Taq HS Premix Probe qPCR kit (AG11704) were bought from Accurate Biotechnology (Shanghai, China). Invitrogen sold Lipofectamine® 3000 (L3000008, Invitrogen, Carlsbad, CA, USA).

2.3 Western Blotting

The Myc-VP3 plasmid was introduced into HEK293 cells through Lipofectamine® 3000 reagent following the guidelines provided by the manufacturer. Radio-Immunoprecipitation Assay Lysis Buffer (RIPA) from Solarbio (Beijing, China) was added to collected cells to prepare whole-cell extracts. Cell lysates underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and polyvinylidene fluoride (PVDF) membranes from Millipore were used for protein trans-blotting. PBS/Tween® 20-diluted skim milk (P1033, Solarbio, Beijing, China) was utilized to prevent non-specific antibody binding, and then unambiguous primary and secondary antibodies labeled with HRP were applied. Proteins were detected using an electrochemiluminescence (ECL) reagent from BioRad in the Hercules, CA, USA, with β-actin applied as the loading control.

2.4 Viral Infection

Transfection of HEK293 cells was carried out using Lipofectamine® 3000 following the producer’s guidelines. A total of 100 50% tissue culture infectious dose (TCID50) EMCV was used for cell infection. Two hours later, cells
were cultured with 3% NBS DMEM. Supernatants and lysates from cell-free cultures were collected 24 hours after infection to assess how VP3 impacts EMCV replication.

2.5 RNA Extraction and RT-qPCR

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed for IFN-β mRNA detection. Cellular total RNA was extracted as previously described [7]. The primer sequences of IFN-β and GAPDH are listed as follows: IFN-β: (forward: 5′-TTGGTGAAGACCTCCTGGCT-3′, reverse: 5′-TGACTATGGTCCAGGCACAG-3′); GAPDH: (forward: 5′-GTCTCCTCTGACTTCAACAGCG-3′, reverse: 5′-ACCACCCCTGTGCTGTAGCCAA-3′).

2.6 Detection and Quantitation of the EMCV Genome

Viral RNA was isolated using the Viral Genomic RNA Extraction kit (TIANGEN, Beijing, China) and performed as previously described [7]. The standard curve method was used to quantify EMCV genomic numbers. The 3D gene was used to design primers and probes targeted for RT-qPCR assay. The primer sequences of EMCV 3D gene and viral probe are listed as follows: 3D: (forward: 5′-GTGATCTGATGAGAAGCTTGG-3′, reverse: 5′-CATCTCTGACTTCAACAGCG-3′); probe: 5′-FAM/CCTCAGTACATGCTTTGCCAGTT (Eclipse)-3′.

2.7 Virus Titration

BHK-21 cells were used in these experiments. Five replicates of BHK-21 cells were infected with 10-fold serial dilutions of EMCV, and fresh DMEM was added after 1 h at 37 °C. The virus titers were determined after 72 h at 37 °C using the Reed–Muench method.

2.8 Co-Immunoprecipitation Assay

Cells were lysed using NP40 (Beyotime, Shanghai, China) and phenylmethylsulfonyl fluoride (PMSF; Beyotime, Shanghai, China) and phenylmethylsulfonyl fluoride (PMSF; Beyotime, Shanghai, China) and phenylmethylsulfonyl fluoride (PMSF; Beyotime, Shanghai, China) and then exposed to specified antibodies at 4 °C for 20 minutes. Next, the cells were treated with 2% BSA (A8020, Solarbio, Beijing, China) in 1 × PBS at room temperature for 1 hour. This was followed by exposure to specific primary antibodies (ProteinTech, Wuhan, China) at 4 °C overnight. Following that, the cells underwent three washes with 1 × PBS-T before exposure to a suitable secondary antibody (Jackson Immuno Research, Bar Harbor, ME, USA) at an ambient temperature for 1 hour. After that, the cells were treated with 4′,6-diamidino-2-phenylindole (DAPI) at room temperature for 10 minutes. Images were taken under a Zeiss laser scanning confocal microscope (LSM 900) (Zeiss, Oberkochen, Germany), and the fluorescent intensity was read using ZEN Blue software version 3.10 (Zeiss, Oberkochen, Germany). The graph was drawn using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA).

2.10 Statistical Analysis

GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA) was used to analyze the data. Data were expressed as the mean ± standard deviation (SD) of at least three independent experiments. Asterisks indicate a significant difference (**p < 0.001, ***p < 0.01, and *p < 0.05).

3. Results

3.1 VP3 Promotes Viral Proliferation In Vitro

The impact of VP3 on EMCV replication was investigated by transfecting a Myc-tagged VP3 expression plasmid into HEK293 cells (Fig. 1A), followed by EMCV infection for 24 hours. The findings indicated that VP3 overexpression notably boosted EMCV copy numbers (Fig. 1B) and titers (Fig. 1C), indicating an ability of VP3 to enhance EMCV proliferation within host cells.

3.2 VP3 Restrains EMCV-Mediated IFN Signaling and Anti-Virus Gene Transcription

To further discuss the effect of EMCV VP3 on the type I IFN signaling cascade, we monitored the IFN-β, ISG15, and ISG56 transcription. For this purpose, HEK293 cells were transfected with VP3 or control plasmid and then treated with EMCV. Samples were collected at specified time intervals. Cellular RNA was extracted and used for RT-qPCR detection. Fig. 2 shows that mRNA levels for IFN, while IFN-related anti-virus genes were decreased in HEK293 cells with VP3 expression, demonstrating that the diminished anti-virus reaction was due to the repression of IFN-β, ISG15, and ISG56 gene expression by EMCV VP3.

3.3 VP3 Contrastively Regulates the Antiviral Responses

The structural protein VP3 of EMCV has a crucial function in inhibiting antiviral reactions, yet the specific molecular mechanism remains unknown. Host sensor molecules are known to recognize a viral infection and trigger the IFN pathway, leading to the initiation of antiviral
Fig. 1. VP3 overexpression promotes EMCV replication. (A) HEK293 cells were seeded in 6-well plates and transfected with Myc-VP3 (1 µg). After 24h, samples were collected for Western blot analysis. (B,C) HEK293 cells were cultured in 6-well plates, followed by Myc-VP3 (1 µg) or empty vector (1 µg) transfection for 24h. Cells were infected with EMCV (100 TCID₅₀) for another 24h. RT-qPCR was performed to determine the viral copy number (B). EMCV titer was observed by TCID₅₀ assay (Reed–Muench method) (C). Data were listed as the mean ± standard deviation (SD) of three independent experiments. ***, p < 0.001, **, p < 0.01. HEK293, human embryonic kidney 293 cells; VP3, viral protein 3; EV, empty vector; EMCV, encephalomyocarditis virus; RT-qPCR, real-time quantitative polymerase chain reaction; TCID₅₀, 50% tissue culture infectious dose.

Fig. 2. VP3 inhibits EMCV-induced type I IFN signaling activation. (A) HEK293 cells were transfected with Myc-tagged VP3 plasmid (1 µg) or pCMV-Myc empty plasmid (EV, 1 µg) for 24h and then stimulated with EMCV (100 TCID₅₀) for 12h. Cellular RNA was extracted, and the mRNA levels of IFN-β (A), ISG15 (B), and ISG56 (C) were analyzed by RT-qPCR. Data were presented as the mean ± SD of three independent experiments. **, p < 0.01. IFN-β, interferon beta; ISG15, interferon-stimulated gene 15; ISG56, interferon-stimulated gene 56; SD, standard deviation.

defenses. We assessed the impact of EMCV VP3 on innate immune reactions by examining the expression levels of components associated with the IFN signaling pathway. In the case of VP3 overexpression, only the expression level of MAVS was affected, regardless of EMCV infection (Fig. 3A–C). Additionally, we investigated how VP3 impacts the transcription of IFN triggered by Polynosinic-polycytidylic acid (poly(I:C)). Following transfection of HEK293 cells with varying amounts of the VP3 plasmid, the suppressive impact of VP3 on IFN production exhibited a correlation with dosage (Fig. 3D). These findings indicate that EMCV VP3 inhibits antiviral reactions, leading us to believe that VP3 may target MAVS signaling to suppress IFN responses.

3.4 VP3 Aims at MAVS to Inhibit IFN-β Production

MAVS was confirmed as the target of EMCV VP3 by co-transfecting VP3 and MAVS expression plasmids into
Fig. 3. VP3 inhibits type I IFN signaling by targeting MAVS. (A,B) HEK293 cells were transfected with pCMV-Myc (1 µg) or Myc-VP3 (1 µg) for 24 h; then cells were collected for p50, p65, IκBα, MDA5, MAVS, TBK1, IRF3 and Myc-tagged VP3 detection. For all experiments, β-actin served as the loading control. The grayscale protein expression analysis is displayed in the lower panel. (C) HEK293 cells were transfected with Myc-tagged VP3 (1 µg) or pCMV-Myc (1 µg) for 24 h, followed by EMCV (100 TCID<sub>50</sub>) infection for another 24 h. The STING, MAVS, p65, and Myc-tagged VP3 expression levels were determined. β-actin was used as the loading control. (D) HEK293 cells were transfected with pCMV-Myc (1 µg) or different concentrations of Myc-tagged VP3 plasmid (0.5 µg, 1 µg, 1.5 µg) for 24 h and stimulated with poly(I:C) (1 µg/mL) for another 12 h. Cellular RNA was extracted for IFN-β mRNA analysis. Data are shown as the mean ± SD of three independent experiments. ***, p < 0.001; ns, no significant difference. The VP3 expression was also confirmed by immune blotting. β-actin served as the loading control. IκBα, an inhibitor of kappa B alpha; MDA5, melanoma differentiation-associated protein 5; MAVS, mitochondrial antiviral signaling protein; TBK1, TANK-binding kinase 1; IRF3, interferon regulatory factor 3; STING, stimulator of interferon genes; SD, standard deviation; poly(I:C), Polyinosinic-polycytidylic acid.
HEK293 cells and detecting the $IFN-\beta$ mRNA levels. The findings indicated that VP3 effectively suppressed the activation of $IFN-\beta$ by MAVS, suggesting that MAVS is the specific target of VP3, as demonstrated in Fig. 4A. The protein level alterations additionally validated that VP3 successfully suppressed the MAVS expression in a dosage-dependent manner (Fig. 4B).

### 3.5 VP3 Binds with MAVS

Based on previous findings indicating the role of VP3 in MAVS degradation, we hypothesized a potential interaction between VP3 and MAVS. As anticipated, an interaction was detected in HEK293 cells between EMCV VP3 and endogenous MAVS. Following immunoprecipitation using an anti-MAVS antibody, VP3 can be detected by an anti-Myc antibody (Fig. 5A). Subsequently, after immunoprecipitation with an anti-Myc antibody, MAVS was identified via Western blotting (Fig. 5B). Furthermore, we verified the co-localization of VP3 and MAVS using confocal microscopy (Fig. 5C).

### 3.6 VP3 Degrades MAVS Dependent on p62-Mediated Autophagy

Various degradation pathway inhibitors were introduced to the cultured cells transfected with VP3 to determine the specific pathway involved in EMCV infection. Fig. 6A–C demonstrates that the presence of chloroquine (CQ), an autophagy–lysosomal pathway inhibitor, resulted in a notable increase in MAVS expression. In contrast, the presence of MG132, an inhibitor of the ubiquitin–proteasome pathway, did not lead to a noticeable improvement in MAVS expression. However, Z-VAD-FMK-treated
Fig. 5. VP3 interacts with MAVS. (A,B) MAVS and VP3 immunoprecipitation (IP) assay. All these experiments were performed in HEK293 cells transfected with Myc-VP3 (1 µg) or pCMV-Myc empty vector (EV, 1 µg). An anti-MAVS or anti-Myc antibody was incubated with protein G agarose, and IgG was used as the control. Input and IP complexes were analyzed using Western blotting. (C) Confocal laser scanning microscopy images of VP3 (anti-Myc (red)) and MAVS (anti-MAVS (green)). Nuclei were stained with DAPI (blue). Scale bars = 10 µm. MAVS, mitochondrial antiviral signaling protein; DAPI, 4′,6-diamidino-2-phenylindole.
Fig. 6. VP3 promotes MAVS degradation via autophagy. MAVS degradation pathway screening was performed in HEK293 cells transfected with Myc-VP3 (1 µg) or pCMV-Myc (1 µg). Proteasomal inhibitor MG132 (7.5 µM) (A), autophagy–lysosomal inhibitor CQ (50 µM) (B), caspase inhibitor Z-VAD-FMK (50 µM) (C), caspase 3 inhibitor Ac-DEVD-CHO (20 nM) (D), caspase 8 inhibitor Z-IETD-FMK (20 nM) (E) or caspase 9 inhibitor Z-LEHD-FMK TFA (20 nM) (F) were used to treat related cells. MAVS and Myc-tagged VP3 expressions were detected. β-actin served as the loading control. (G) Confocal laser scanning microscopy images of VP3 (anti-Myc (red)) and LC3 (anti-LC3 (green)) in A549 cells. Nuclei were stained with DAPI (blue). Scale bars = 10 µm. (H) Confocal laser scanning microscopy images of VP3 (anti-Myc (red)) and p62 (anti-p62 (green)) in A549 cells. Nuclei were stained with DAPI (blue). Scale bars = 10 µm. (I–J) p62 and VP3 immunoprecipitation (IP) assay were conducted in HEK293 cells. Antibodies specific to Myc, p62, NDP52, Tollip, or β-actin were used for input and IP complexes analyses. (K) p62 siRNA was transfected into Myc-VP3 (1 µg) or pCMV-Myc (1 µg) overexpression HEK293 cells. Cells were lysed for MAVS, p62, and Myc-tagged VP3 detection. β-actin served as the control. ***,$p < 0.001$. DMSO, dimethyl sulfoxide; LC3, microtubule-associated protein 1 light chain 3; CQ, chloroquine; DAPI, 4′,6-diamidino-2-phenylindole.
Upon EMCV infection, viral protein VP3 can interact with MAVS by recruiting and interacting with p62, then degrades MAVS via the autophagy-lysosomal pathway to evade the innate antiviral response of the host. EMCV, encephalomyocarditis virus; RLRs, retinoic acid-inducible gene-I (RIG)-like receptors; VP3, viral protein 3; MAVS, mitochondrial antiviral signaling protein; TBK1, TANK-binding kinase 1; IKKs, inhibitor of nuclear factor-κB (IκB) kinase family; IRF3, interferon regulatory factor 3; IFN-β, interferon β; NF-κB, nuclear factor-κB.

The transformation of soluble microtubule-associated protein 1 light chain 3 (LC3) to membrane-bound LC3 plays a crucial role in autophagy. LC3, which is attached to the membrane, regulates various important functions within autophagy, such as the development and enlargement of the phagophore, the gathering of materials, and the merging of autophagosomes with lysosomes [21]. Confocal microscopy analysis was performed to verify the expression...
of LC3 and investigate if VP3 promotes autophagy. As shown in Fig. 6G, the expression of LC3 in VP3-transfected cells transferred from the cytoplasm to the perikaryon and exhibited punctate distribution compared to the control group, implying the formation of autophagosomes. Further, confocal images also directly revealed the obvious colocalization between VP3 and LC3.

In addition to LC3, autophagy can be monitored by assessing p62 levels. p62 is specifically included in autophagosomes by directly binding to LC3 and is effectively broken down by autophagy, leading to an inverse relationship between the overall cellular levels of p62 and autophagic activity. The p62 protein level notably decreases when VP3 is transfected compared to the control group (Fig. 6H). Subsequently, an interaction between VP3 and p62 was identified through immunoprecipitation tests (Fig. 6I,J). Furthermore, siRNA knockdown of p62 alleviated the inhibitory effect of VP3 on MAVS (Fig. 6K). The above data showed that VP3 triggers MAVS autophagic degradation through the p62-mediated autophagy pathway.

4. Discussion

The initial defense against RNA or DNA viruses is provided by the innate immune response triggered by RLRs, Toll-like receptors (TLRs), NOD-like receptors (NLRs), or cGAS. Following viral infection, the IFN-β pathway is initiated to stimulate IFN-β and ISG transcription, contributing to the initiation of a suitable adaptive immune response. Understanding the processes of the innate immunity system may aid in improving disease management and developing vaccines. Our current research revealed that VP3 controls the antiviral reaction and the activation of immune response genes that aim at MAVS. VP3 degraded MAVS in a p62-dependent autophagic pathway (Fig. 7). The following experiments verify the results. Initially, VP3 enhances EMCV reproduction in host cells and suppresses the activation of type I IFN induced by EMCV and poly(I:C); additionally, transfection of VP3 facilitates the degradation of MAVS through the autophagy and caspase-dependent pathway. Furthermore, VP3 interacts with MAVS, LC3, and p62 to stimulate autophagy. Our data establish a link between autophagy and the immune response during EMCV infection. The ratio of LC3-II/LC3-I is important in evaluating autophagy [22], but relative experiments were not performed. In the present study, we only visually examined the effect of the presence of VP3 on LC3 autophagosome formation using confocal experiments without applying immunoblotting to detect protein expression changes in LC3-II and LC3-I. This also represents a shortcomings of this study.

IFNs produced by cells stimulated by pathogens are an important antiviral factor that the RLR pathway regulates. MAVS is an adapter essential for regulating IFN expression in the RLRs signaling pathway [23]. Multiple research studies have demonstrated the importance of the MAVS post-translational modification in initiating downstream signaling pathways. Viral infection of MAVS knockout mice has been shown to inhibit interferon production in mouse embryonic fibroblasts and dendritic cells, leading to increased mortality in mice [24,25]. Some viral proteins have been found to have negative effects through immune evasion against MAVS molecules; for example, the NS1 protein of duck tambucu virus inhibits the activation of the IFNs signaling pathway by binding to the carboxyl terminus of the host MAVS [26]. Hepatitis B virus (HBV) X protein downregulates IFN production by interacting with the host MAVS protein [27]. The NS3/4A protein of type B GB virus inhibits IFN production by cleaving the host MAVS protein to affect its proper localization [28]. Studies on foot-and-mouth disease virus (FMDV) showed that the structural proteins VP1 and VP3 could block the activation of the host RNA receptors; it may also act as a direct innate immune antagonist within the cytoplasm to antagonize host cell innate immune defense mechanisms [29,30].

EMCV proteins 3C, 2C, and the leading protein L have been reported as interferon antagonists [4,6,31]. EMCV 2C can block the natural defenses by binding with MDA5; the V26 amino acid of the EMCV 2C protein displays an indispensable function in restraining the IFN-β signaling pathway [4]. EMCV 2B protein activates the NLRP3 inflammasome [31]. EMCV 3C protein interferes with the TANK–TBK1–IKKε–IRF3 complex, reducing IFN production [6]. The current work demonstrates that the EMCV VP3 protein interplays with selective autophagy receptor p62 through a stepwise biological process. It has been suggested that p62 can interact with MAVS [32] and that the three may form a VP3–MAVS–p62 complex that is delivered to autophagosomes for MAVS degradation. Based on this, MAVS-mediated signaling pathways are interrupted, and the interferon response is inhibited, thereby enhancing virus replication.

5. Conclusions

Overall, our findings show that EMCV VP3 promotes virus replication by inhibiting the type I IFN signaling pathway. VP3 specifically binds to MAVS and degrades MAVS depending on the p62-mediated autophagy pathway. The discovery of a new method employed by EMCV VP3 to suppress innate immunity responses enhances our understanding of the immune evasion tactics utilized by EMCV to escape the host’s immunity.

Availability of Data and Materials

All data generated or analysed during this study are included in this published article. Uncropped western blot images used for analysis are provided as Supplementary Material.
Author Contributions
Conceptualization and investigation: XZ, ZH, YZ, DM, ZY, SY, JX, RF; methodology and resources: XZ, ZH, YZ, DM, ZY, SY and JX; writing and original draft preparation: XZ, ZH, YZ, DM, ZY and SY; critical review and editing: JX and RF. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate
Not applicable.

Acknowledgment
We would like to thank Dr. Shasha Li for her invaluable assistance in drafting and reviewing this manuscript.

Funding
This research was funded by the College Students Innovative Entrepreneurship Training Program project (state level) of Northwest Minzu University, grant number 31920230162, (state level) of Northwest Minzu University, grant number 31920230162, 31920230160 and 31920240116, and National Natural Science Foundation of China, grant number 32260037.

Conflict of Interest
The authors declare no conflict of interest.

Supplementary Material
Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2907273.

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