

Research Article

Encephalomyocarditis virus 3C protease attenuates type I interferon production through disrupting the TANK–TBK1–IKK ϵ –IRF3 complex

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TRAF family member-associated NF- κ B activator (TANK) is a scaffold protein that assembles into the interferon (IFN) regulator factor 3 (IRF3)-phosphorylating TANK-binding kinase 1 (TBK1)–(I κ B) kinase ϵ (IKK ϵ) complex, where it is involved in regulating phosphorylation of the IRF3 and IFN production. However, the functions of TANK in encephalomyocarditis virus (EMCV) infection-induced type I IFN production are not fully understood. Here, we demonstrated that, instead of stimulating type I IFN production, the EMCV-HB10 strain infection potently inhibited Sendai virus- and polyI:C-induced IRF3 phosphorylation and type I IFN production in HEK293T cells. Mechanistically, EMCV 3C protease (EMCV 3C) cleaved TANK and disrupted the TANK–TBK1–IKK ϵ –IRF3 complex, which resulted in the reduction in IRF3 phosphorylation and type I IFN production. Taken together, our findings demonstrate that EMCV adopts a novel strategy to evade host innate immune responses through cleavage of TANK.

Introduction

Encephalomyocarditis virus (EMCV), belonging to the Picornaviridae family, is a small, non-enveloped virus. EMCV infection can cause myocarditis, encephalitis, neurologic diseases, reproductive disorders, and diabetes in many mammalian species [1]. Its genome contains a single-stranded positive-sense RNA, which is ~7.8 kb and flanked by two untranslated regions [1]. Previous studies demonstrated that the EMCV genome encodes a polyprotein and then EMCV 3C protease subsequently cleaves the polyprotein to produce at least 13 mature viral proteins [2]. These proteins are necessary for viral genome replication and assembly. Recently, several mature EMCV proteins were found to participate in the activation of the NLRP3-dependent inflammasome and the regulation of host innate immune responses [3,4].

Rapid induction of type I interferon (IFN) and inflammatory cytokines are central to the host antiviral responses, which are tightly regulated by extracellular and intracellular signals. Retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) are ubiquitously expressed cytoplasmic pattern recognition receptors. Among them, RIG-I and melanoma differentiation-associated gene 5 (MDA5) play different roles in virus recognition [5–7]. RIG-I recognizes most single-stranded positive-sense RNA viruses, such as vesicular stomatitis virus (VSV), paramyxoviruses and influenza virus [6], whereas MDA5 recognizes Picornaviruses, including EMCV [6] and Coxsackievirus B3 (CVB3). Upon ligand recognition, RIG-I or MDA5 interacts with mitochondrial antiviral-signalling protein (MAVS, also designated as VISA, IPS-1, or Cardif) [8–10], a mitochondrion-anchored adaptor molecule, which associates with and activates both the canonical inhibitor of NF- κ B (I κ B) kinase (IKK) complex and

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two IKK-related kinases, TANK-binding kinase 1 (TBK1) and IKK ϵ . Previous studies showed that TBK1 and IKK ϵ are TRAF family member-associated NF- κ B activator (TANK)-binding partners and the tri-complex participates in type I IFN production [11,12]. Activated TBK1 and IKK ϵ can phosphorylate the serine residues in IRF3, causing conformational changes and homogeneous dimerization of IRF3, followed by its nuclear translocation and activation of target gene transcription [13–15].

TANK, also named as I-TRAF, was first identified as a TRAF-binding protein [16,17]. A previous study revealed that TANK can activate NF- κ B signaling in cells transfected with TRAF2 [16]. However, TANK is also found to inhibit the activation of NF- κ B in TNF α -, IL-1-, and CD40-mediated signaling pathways [17,18]. Recently, we found that TANK is cleaved by EMCV 3C at the 197th and 291st glutamine residues, which relieves TANK inhibitory effect on TRAF6-mediated NF- κ B signaling [19]. However, the effect of TANK cleavage on type I IFN signaling is not fully investigated.

It has been reported that the leader protein of Theiler's murine encephalomyelitis virus (TMEV) suppressed IFN- β synthesis at the transcriptional level through interfering with IRF3 dimerization [20–22]. Mechanically, both the zinc-binding domain and phosphorylation of its 47th threonine are essential for this function [20–22]. In this study, we found that EMCV 3C inhibited Sendai virus (SeV) infection- and polyI:C stimulation-mediated IRF3 phosphorylation and type I IFN production in HEK293T cells. To our knowledge, beside the leader protein, EMCV 3C is another antagonist, which disrupted the TANK–TBK1–IKK ϵ –IRF3 complex-mediated phosphorylation of IRF3 and type I IFN production through cleavage of TANK. Therefore, our findings reveal a novel mechanism of EMCV to evade host innate immune responses.

Materials and methods

Cell lines and viruses

HEK293T, BHK-21, HeLa, and Madin–Darby bovine kidney (MDBK) cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT, U.S.A.), 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a 5% CO₂ humidified atmosphere. SeV was kindly provided by Prof. Min Wu (College of life Sciences, Wuhan University, China). EMCV-HB10 strain was propagated in BHK-21 cells as described previously [23]. Viral supernatants were titrated and stored at –80°C. EMCV infection and RT-PCR assay were performed as described previously [23,24].

Plasmids, antibodies and reagents

The plasmids expressing Flag-RIG-I, Flag-MDA5, Flag-MAVS, Flag-TANK, Flag-TBK1, Flag-IKK ϵ , and Flag-IRF3 and pGL3-IFN- β -Luc, pRL-TK and other reporters were gifts from Prof. Hong Tang (Wuhan Institute of Virology, Wuhan, Hubei, China) and Prof. Jianwei Wang (MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China). To construct plasmids encoding HA-tagged EMCV proteins (VP1, VP2, VP3, L-VP4, 2A, 2B, 2C, 3AB, 3C, and 3D), EMCV cDNAs corresponding to these genes were cloned into the pCAGGS-HA (pHA) vector [19]. The cDNAs encoding deletion mutants of TANK, including 197N (1–197 amino acids, aa) and 291N (1–291 aa), were cloned into the pCAGGS-Flag (pFlag) vector. The cDNA encoding TANK with two amino acid mutants (also named as TANK-DM [19]) was cloned into pCAGGS-Myc (pMyc) vector. The cDNA encoding EMCV 3C protease was cloned into the pHA vector and its double-mutant H46A/C159A was constructed using site-directed mutagenesis [19]. The primer sequences used in this study are available upon request. All constructs were validated by DNA sequencing.

Antibodies against Flag, HA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), goat anti-mouse or goat anti-rabbit secondary antibodies, and goat anti-mouse fluorescein isothiocyanate (FITC)-labeled secondary antibody were purchased from Sigma–Aldrich Corporation (St. Louis, MO, U.S.A.). Anti-IRF3, anti-STAT-1 polyclonal antibodies (pAb), and anti-IRF3 and anti-STAT-1 phosphorylation antibodies were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Monoclonal antibodies (mAbs) against EMCV 3C were prepared in mice using recombinant EMCV 3C protein as immunogens. Anti-FLAG[®] M2 agarose affinity gel and polyI:C were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.).

Transfection and luciferase reporter assays

Reporter assay was performed as described previously [25,26]. In brief, HEK293T (2.5×10^5) cells were transfected with plasmids as indicated, in combination with 100 ng of a reporter plasmid and 5 ng of the pRL-TK plasmid (Promega Corporation, Madison, WI, U.S.A.). In each experiment, the total amount of DNA in each sample was kept constant by supplementation with the pFlag empty vector. At 24 h post transfection (hpt), the activities of Firefly and Renilla luciferase were determined using the Dual-Luciferase Reporter Assay System (Promega Corporation) according to the manufacturer's instructions. The data represented relative Firefly luciferase activity normalized to Renilla luciferase activity. All data are presented as means \pm standard deviation (SD) of three independent experiments.

RNA extraction and quantitative real-time PCR

Total RNA isolated from HEK293T cells (cRNA), total RNA isolated from EMCV-infected HEK293T cells (iRNA), and genomic RNA from EMCV virions (vRNA) were extracted using TRIzol[®] Reagent (Invitrogen) according to the manufacturer's instructions. HEK293T cells were transfected with the cRNA, iRNA, vRNA, or polyI:C using Lipofectamine[®] 2000 (Invitrogen). For quantitative real-time PCR (qRT-PCR), the total RNAs from stimulated cells were extracted using TRIzol[®] reagent (Invitrogen). Total RNA (160 ng) was transcribed into cDNA using PrimeScript[™] RT Master Mix (Takara Bio, Inc., Otsu, Shiga, Japan). The resulting cDNAs were used as a template for quantitative PCR using the SYBR[®] Green method (Takara Bio, Inc.) with specific primers. The primer for IFN- β , IFN- β -F: 5'-ATGACCAACAAGTGTCTCCTCC-3'; IFN- β -R: 5'-GCTCATGGAAAGAGCTGTAGTG-3'. The primers for β -actin, β -actin-F: 5'-CCTTCCTGGGCATGGAGTCCTG-3'; β -actin-R, 5'-GGAGCAATGATCTTGATCTTC-3'.

IFN production

HEK293T cells were transfected with 1 μ g/ml polyI:C using Lipofectamine[®] 2000 or infected with SeV for 24 h. IFN- β levels in the cell supernatants were detected using the IFN- β Enzyme-Linked Immunosorbent Assay (ELISA) Kit according to the manufacturer's instructions (PBL Assay Science, Piscataway, NJ, U.S.A.).

IFN sensitivity assay

HEK293T cells were transfected with empty vector or an increasing amount of a plasmid expressing EMCV 3C, or infected with different doses of EMCV-HB10 or stimulated with polyI:C. The cell supernatants were collected and inactivated with a 254 nm ultraviolet (UV) light for 15 min. The UV-inactivated cell supernatants were diluted 1:10 in DMEM and added to MDBK cells. After 24 h, MDBK cells were then infected with VSV-expressing green fluorescent protein (VSV-GFP) at an MOI of 1.0 for 24 h. VSV replication was determined by fluorescence microscopy.

Co-immunoprecipitation and Western blot analysis

Co-immunoprecipitation (Co-IP) and Western blot analysis were performed as described recently [19]. In brief, the cells were lysed with cell lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 5 mM MgCl₂, 1 mM EDTA, and 10% glycerol] containing 1 mM PMSF and 1 \times protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, U.S.A.). For Co-IP, the cell lysates were incubated with anti-FLAG agarose on a roller overnight at 4°C. For Western blot analysis, equal amounts of cell lysates or the immunoprecipitants were resolved by 10–12% SDS-polyacrylamide gel and then transferred to polyvinylidene difluoride membrane (EMD Millipore Corporation, Billerica, MA, U.S.A.). After incubation with the indicated antibodies, the membranes were visualized using chemiluminescence (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, U.K.).

Statistical analysis

All experiments were performed at least three times and the statistical analyses were performed using Student's *t*-test and one-way analysis of variance via the SPSS 16.0 software package (version 16.0, SPSS, Inc., Chicago, IL, U.S.A.). Data was presented as mean \pm SD. A *P*-value (*P* < 0.05) was considered statistically significant.

Results

EMCV-HB10 infection inhibits type I IFN signaling in HEK293T cells

EMCV has been widely used in immunology to study the functions of Toll-like receptors (TLRs) and cytosolic receptors. It has been reported that EMCV infection-mediated pathogenesis is viral strain- and host-specific [1]. The EMCV-HB10 strain was first isolated from a dead piglet with acute myocarditis in northern China [24]. However, the biological characteristics of EMCV-HB10 are not fully investigated. We found that BALB/c mice infected with EMCV-HB10 began to die at 5 days post infection (Supplementary Figure S1A) and 70% mice died with myocarditis and encephalitis (Supplementary Figure S1B).

To test whether the EMCV-HB10 infection can activate the host innate immune responses, HEK293T cells were either mock-infected or infected with different doses of EMCV-HB10 as indicated. As shown in Figure 1A, B, EMCV-HB10 infection did not induce IFN- β mRNA expression, IFN- β production, and IRF3 phosphorylation in HEK293T cells (Figure 1C). Additionally, IFN sensitivity assay revealed that EMCV-HB10 infection did not produce enough IFNs to inhibit VSV-GFP replication (Figure 1D). To further investigate whether EMCV-HB10 infection activates different reporters' activities involved in IFN signaling pathway, HEK293T cells were transfected with different reporters as indicated and the cells were infected with EMCV-HB10. We found that EMCV-HB10 infection barely activated IFN- α (Figure 1E), IFN- β (Figure 1F), IRF7 (Figure 1G), ISRE (Figure 1H), or ISG56 (Figure 1I) reporters in HEK293T cells. Consistent with these results, we also found that EMCV-HB10 infection barely activated IFN- β promoter (Supplementary Figure S1C) and did not induce IRF3 phosphorylation (Supplementary Figure S1D) and STAT-1 phosphorylation (Supplementary Figure S1E) in HeLa cells, although IFN- β production was enhanced when peritoneal macrophages from mouse were infected with EMCV. All these data suggest that EMCV-HB10 infection activated IFN- β production is dependent on different cell types. It has been demonstrated that polyI:C, a synthetic mimic of viral dsRNA, can activate type I IFN signaling and induce IFN production [27,28]. To test whether EMCV-HB10 infection inhibits polyI:C-induced IFN- β signaling, HEK293T cells were either mock-infected or infected with EMCV-HB10 and then transfected with polyI:C. We found that EMCV-HB10 infection inhibited polyI:C-induced IFN- β production (Figure 2A) and IRF3 phosphorylation (Figure 2B). In addition, we also found that EMCV-HB10 infection inhibited polyI:C-induced IFN- α (Figure 2C), IFN- β (Figure 2D), IRF7 (Figure 2E), ISRE (Figure 2F), ISG54 (Figure 2G), and ISG56 (Figure 2H) reporters' activities. Taken together, our findings reveal that EMCV-HB10 infection blocks polyI:C-mediated type I IFN signaling.

EMCV-HB10 infection inhibits its RNA-mediated IFN signaling

Previous reports showed that total RNA isolated from EMCV-infected HEK293T cells (iRNA) and EMCV genomic RNA isolated from EMCV virions (vRNA) induced IFN- β promoter activation and IFN- β production [29,30]. We also noticed that the mRNA levels of IFN- β from the iRNA- or vRNA-stimulated cells were higher than those of the cells infected with EMCV-HB10. Consistent with previous results, we found that transfection of iRNA or vRNA activated IFN- β reporter (Figure 3A) and enhanced the mRNA levels of IFN- β in a dose-dependent manner (Figure 3B). To confirm these results, HEK293T cells were transfected with the IFN- β reporter, along with total RNA isolated from HEK293T cells (cRNA), iRNA, or vRNA as indicated. To test whether EMCV-HB10 infection inhibits different RNA-mediated type I IFN responses, HEK293T cells were infected with EMCV-HB10 and then transiently transfected with cRNA, iRNA, vRNA, or polyI:C, along with the IFN- β reporter. As shown in Figure 3C and 3D, EMCV-HB10 infection inhibited vRNA- or iRNA-induced IFN- β reporter's activity and IFN- β mRNA expression. Based on these results, we proposed that several viral proteins encoded by EMCV-HB10 might be responsible for inhibition of viral RNA-mediated type I IFN production.

EMCV 3C inhibits SeV- and polyI:C-mediated type I IFN signaling

EMCV encodes at least 13 mature structural and nonstructural proteins. To evaluate the abilities of these EMCV proteins to inhibit viral RNA-activated IFN- β transcription, HEK293T cells were transiently transfected with the IFN- β reporter and a plasmid encoding one of the 13 EMCV proteins. At 24 hpt, the cells were infected with SeV. We found that different EMCV proteins had different effects on the IFN- β reporter. Among them, L-VP4 (L fused with VP4), 2C, and 3C inhibited IFN- β promoter activation in SeV-infected cells (Supplementary Figure S2). Here, we focused on EMCV 3C because several 3C proteases from other Picornaviruses had been reported to inhibit type I IFN production through cleaving critical molecules in the RLR-mediated type I IFN signaling pathway [25,31]. To further test whether EMCV 3C inhibits the type I IFN signaling, HEK293T cells

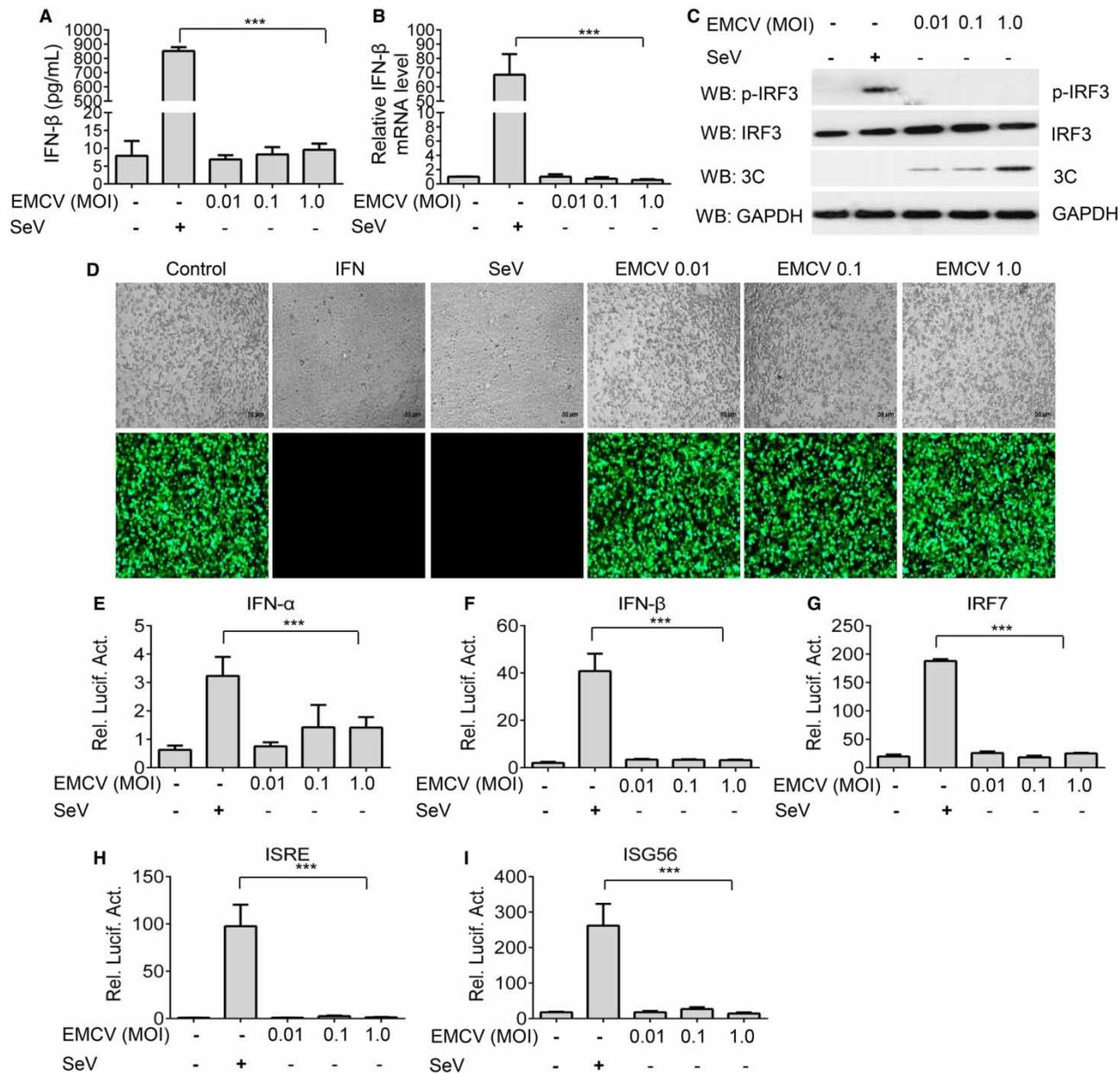


Figure 1. EMCV-HB10 infection inhibits SeV-mediated type I IFN production.

(A–C) HEK293T cells were either mock-infected or infected with different doses of EMCV-HB10. At 12 h post infection (hpi), the IFN-β production in cell supernatants was detected by ELISA (A) and the mRNA levels of IFN-β were evaluated by qRT-PCR (B). The cell lysates were analyzed by Western blotting with indicated antibodies (C). SeV infection was used as a positive control. The results were representative data from three independent experiments. (D) MDBK cells were non-treated (control), pretreated with IFN-α (1000 U) or incubated with the cell supernatant from panel A and then infected with VSV-GFP (MOI = 1) for 24 h. Bright field images (top) indicated growing cells. Green fluorescence (bottom) indicated viral replication. (E–I) HEK293T cells were transfected with 100 ng of IFN-α (E), IFN-β (F), IRF7 (G), ISRE (H), or ISG56 (I) reporter, along with 5 ng of pRL-TK. At 24 hpt, the cells were either mock-infected or infected with different doses of EMCV-HB10 (MOI: 0.01, 0.1, and 1.0) or infected with SeV as a control. At 12 hpi, the cells were lysed and luciferase assays were performed. All the results represent three independent experiments. *** represents $P < 0.001$. Rel. Lucif. Act. represents relative luciferase activation.

were transfected with the IFN-β reporter in combination with different amounts of a plasmid encoding EMCV 3C, and then the cells were transfected with polyI:C. We found that EMCV 3C inhibited polyI:C-mediated IFN-β promoter activation (Figure 4A) and its mRNA expression (Figure 4B). Furthermore, IFN sensitivity

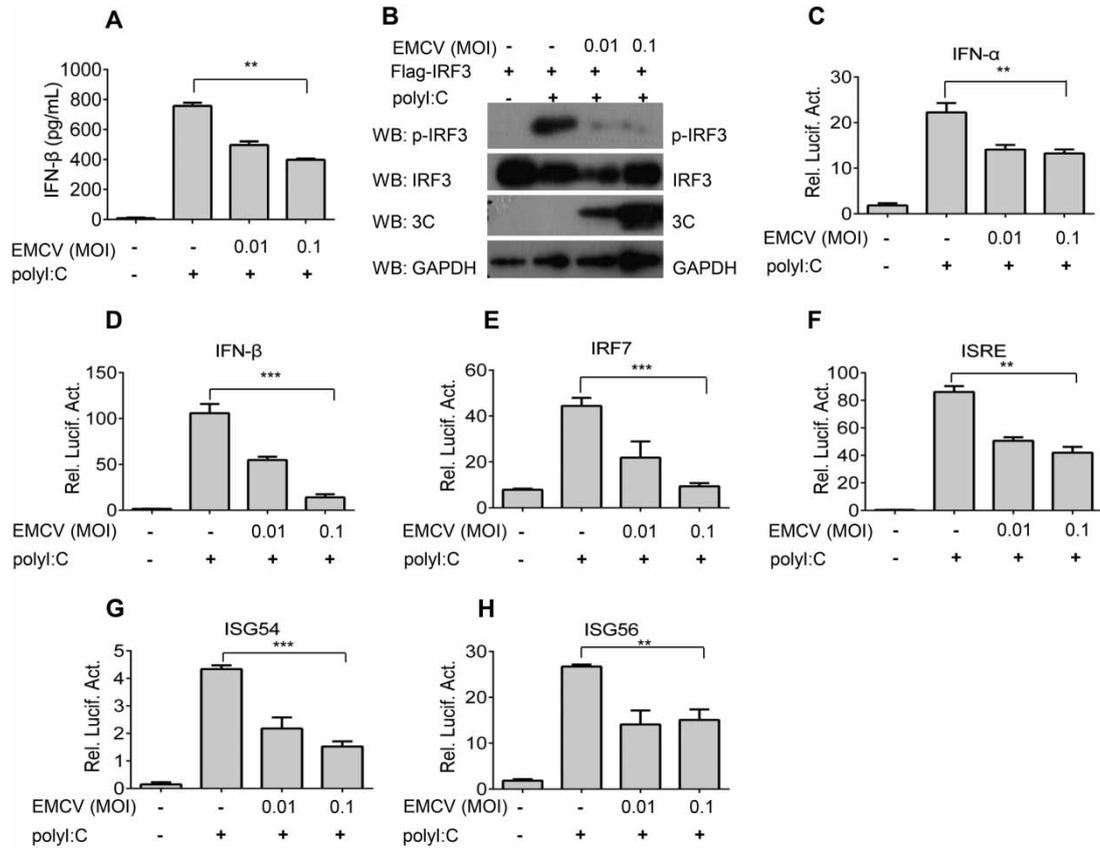


Figure 2. EMCV-HB10 infection inhibits PolyI:C-mediated type I IFN production.

(A) HEK293T cells were either mock-infected or infected with different doses of EMCV-HB10 (MOI: 0.01 or 0.1) and then transfected with polyI:C. The IFN-β production in the cell supernatants was determined by ELISA. (B) HEK293T cells were transfected with a plasmid encoding Flag-IRF3 for 24 h and then mock-infected or infected with EMCV-HB10 (MOI: 0.01 or 0.1) for another 12 h. The cells were stimulated with polyI:C or not and the cell lysates were analyzed by Western blotting. (C–H) HEK293T cells were transfected with 100 ng of IFN-α (C), IFN-β (D), IRF7 (E), ISRE (F), ISG54 (G), or ISG56 (H) reporter, along with 5 ng of pRL-TK plasmid. At 24 hpi, the cells were either mock-infected or infected with different doses of EMCV (MOI: 0.01 and 0.1). At 12 hpi, the cells were stimulated with polyI:C and luciferase assays were performed. The results are representative of three independent experiments. ** represents $P < 0.01$. *** represents $P < 0.001$.

assay revealed that EMCV 3C inhibited polyI:C-mediated IFN production (Figure 4C) and the phosphorylation of IRF3 (Figure 4D). Consistent with these results, we also found that EMCV 3C inhibited other reporters' activities, including IFN-α (Figure 4E), IRF7 (Figure 4F), ISRE (Figure 4G), ISG54 (Figure 4H), and ISG56 (Figure 4I) reporters. In agreement with these findings, we also found that EMCV 3C significantly inhibited SeV-mediated promoter activation (Supplementary Figure S3A), mRNA expression (Supplementary Figure S3B), and protein level (Supplementary Figure S3C) of IFN-β. Moreover, the phosphorylation levels of IRF3 (p-IRF3; Supplementary Figure S3D) and STAT-1 (p-STAT-1) were also reduced (Supplementary Figure S3E). As expected, EMCV 3C antagonized SeV-induced IFN production, resulting in inhibition of VSV-GFP infection and replication (Supplementary Figure S3F). Taken together, our findings reveal that EMCV 3C inhibits polyI:C and SeV-induced type I IFN signaling.

EMCV 3C inhibits TBK1- and IKKε-mediated type I IFN signaling

EMCV infection is dependent on the MDA5-MAVS signaling pathway, but not RIG-I-MAVS [32]. To further determine the key step at which EMCV 3C acts, HEK293T cells were transfected with a plasmid encoding EMCV 3C and a plasmid encoding RIG-I, MDA5, MAVS, TBK1, or IKKε, respectively. As shown in Figure 5A–E, EMCV 3C suppressed IFN-β promoter activation induced by RIG-I, MDA5, MAVS, TBK1, and

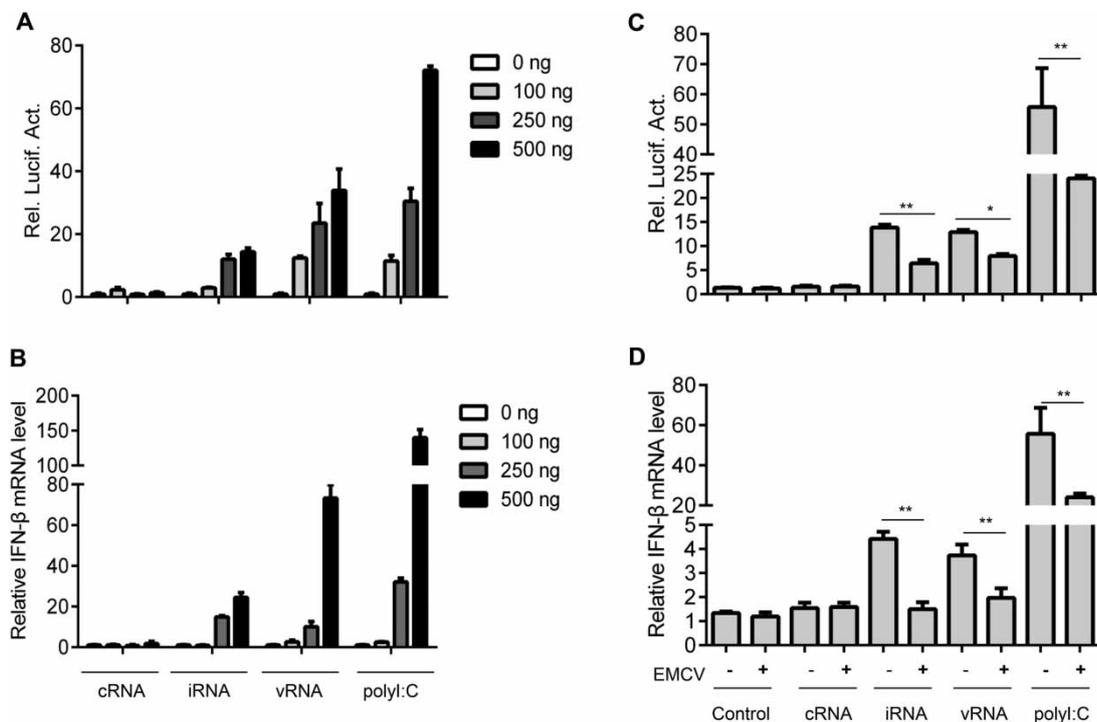


Figure 3. EMCV-HB10 infection inhibits viral RNA-mediated type I IFN signaling.

(A and B) HEK293T cells were transfected with 100 ng of IFN- β -luc and 5 ng of pRL-TK. At 24 hpt, the cells were transfected with different doses of cRNA, iRNA, vRNA, or polyI:C, and then the cells were collected to measure the luciferase activities (A) or the mRNA levels of IFN- β were detected by qRT-PCR (B). (C and D) HEK293T cells were either mock-infected or infected with EMCV-HB10 (MOI: 0.1) for another 12 h and then the cells were transfected with 100 ng of IFN- β reporter, along with cRNA, iRNA, or vRNA or polyI:C. After 12 h, the cells were collected to measure the luciferase activity (C) or test the mRNA levels of IFN- β (D). Data were representative of three independent experiments performed in triplicate. * represents $0.01 < P < 0.05$. ** represents $P < 0.01$.

IKK ϵ in a dose-dependent manner. These results suggest that EMCV 3C might act in the downstream of RLR signaling.

It has been demonstrated that the protease activity of EMCV 3C is mainly dependent on the 46th histidine and 159th cysteine residues, and its double-mutant H46A/C159A (named as EMCV 3C-DM) completely abolishes its protease activity [19]. We then tested whether EMCV 3C protease activity is required for its inhibitory effect on RLR-induced type I IFN signaling. As shown in Figure 5F, G, EMCV 3C, but not EMCV 3C-DM, inhibited TBK1- and IKK ϵ -mediated IFN- β promoter activation. Additionally, EMCV 3C inhibited TBK1- and IKK ϵ -mediated IFN- β mRNA expression (Supplementary Figure S4A,E) and IRF3 phosphorylation in a dose-dependent manner (Supplementary Figure S4B, F). However, EMCV 3C-DM did not block TBK1- and IKK ϵ -mediated IFN- β mRNA expression (Supplementary Figure S4C, G) and IRF3 phosphorylation (Supplementary Figure S4D,H). We also noticed that EMCV 3C inhibited IRF3-5D (an IRF3 mutant that is constitutively active) induced IFN- β promoter activity in a dose-dependent manner (Supplementary Figure S5A), and the inhibition ability was dependent on its protease activity (Supplementary Figure S5B), suggesting that EMCV 3C may also exercise its function in the downstream of IRF3. Taken together, our findings reveal that the protease activity of EMCV 3C is required for its inhibition of RLR-induced type I IFN signaling.

Cleavage of TANK by EMCV 3C inhibits IFN production

Kawagoe et al. [33] demonstrated that TBK1 activity is reduced and IKK ϵ activity is abolished in *tank*^{-/-} macrophages. We then tested whether knockdown of TANK expression affects TBK1- and IKK ϵ -mediated IFN promoter activity. As shown in Figure 6A–C, we found that knockdown of TANK

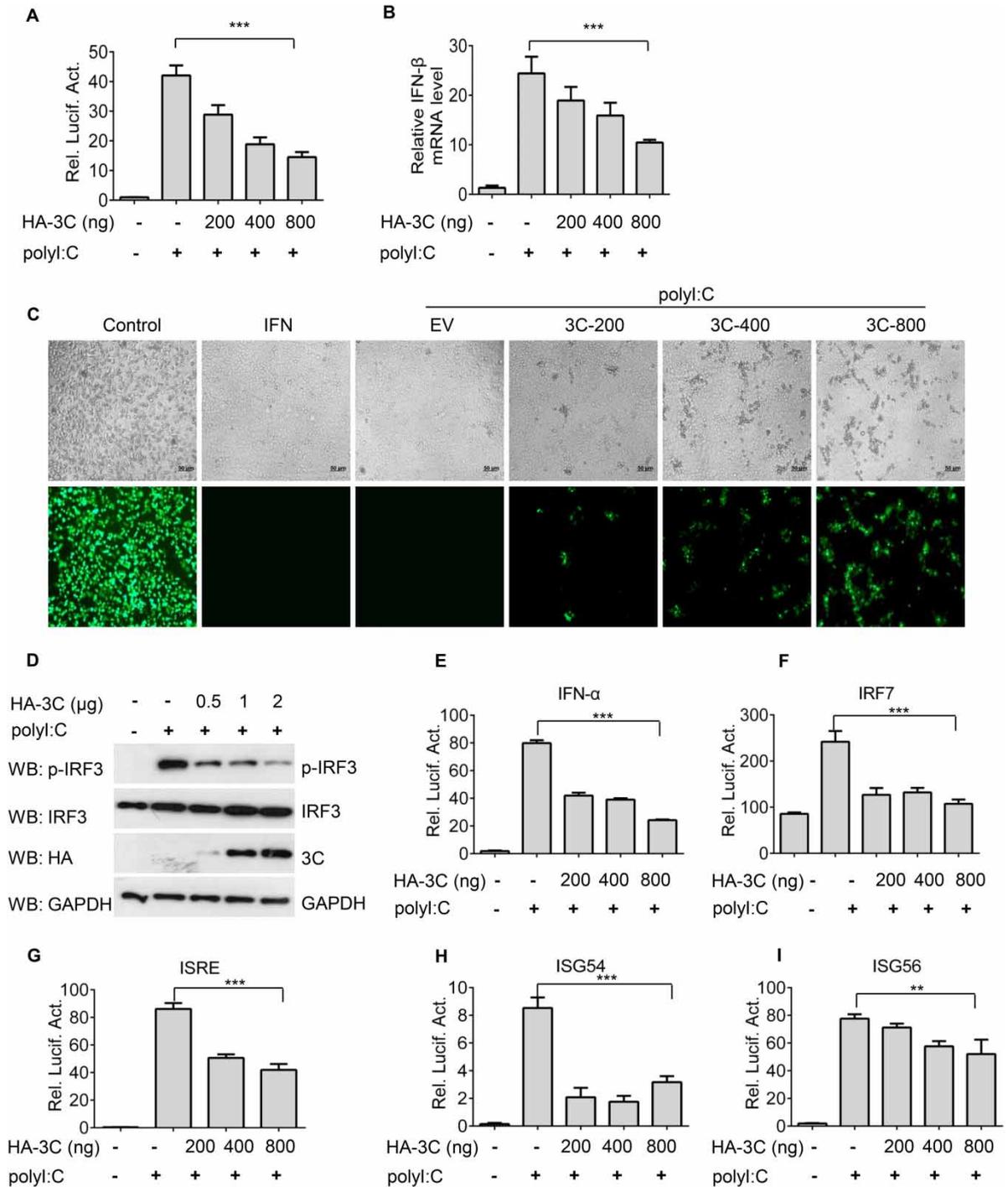


Figure 4. EMCV 3C inhibits polyI:C-mediated type I IFN production.

Part 1 of 2

(A) HEK293T cells were transfected with a plasmid expressing EMCV 3C and IFN- β promoter reporter for 24 h and then the cells were transfected with polyI:C. At 12 hpt, the cells were collected and the luciferase activities were measured. (B) HEK293T cells were transfected with an increasing amount of a plasmid expressing EMCV 3C for 24 h and then transfected with polyI:C. At 12 hpt, the cells were collected and the mRNA levels of IFN- β were evaluated by qRT-PCR. (C) The cell supernatants from panel B were inactivated and added to the MDBK cells and then infected with VSV-GFP (MOI: 1.0). At 24 hpi, the MDBK cells were pretreated with IFN- α (1000 U) as a positive control. Bright field images (top) indicated growing cells. Green fluorescence (bottom) indicated viral replication. EV: empty vector. (D) The cell lysates from panel B were analyzed by Western blotting with the indicated antibodies. (E–I) HEK293T cells were transfected with 100 ng of IFN- α (E), IRF7 (F), ISRE

Figure 4. EMCV 3C inhibits polyI:C-mediated type I IFN production.

Part 2 of 2

(G), ISG54 (H), or ISG56 (I) reporter and 5 ng of pRL-TK plasmids, along with an increasing amount of a plasmid encoding EMCV 3C. At 24 hpt, the cells were stimulated with polyI:C for another 12 h and the cells were collected and the luciferase assay was performed. The results were representative of data from three independent experiments. ** represents $P < 0.01$. *** represents $P < 0.001$.

expression significantly reduced TBK1- and IKK ϵ -mediated IFN promoter activation. Consistent with these results, we found that overexpressed TANK enhanced TBK1- and IKK ϵ -mediated IFN mRNA transcription (Figure 6D,E). We previously found that of several tested key molecules in type I IFN signaling, only TANK was cleaved by EMCV 3C [19]. We then tested whether EMCV 3C and the cleaved products of TANK affected TBK1 and IKK ϵ -mediated IFN- β promoter activity. As shown in Figure 6D,E, we found that EMCV 3C, but not EMCV 3C-DM, inhibited TBK1-TANK and IKK ϵ -TANK-mediated IFN- β mRNA expression. Moreover, we found that, unlike intact TANK, the TANK-197N and TANK-291N did not affect TBK1- and IKK ϵ -mediated IFN- β promoter activity (Figure 6F,G). These results indicate that intact TANK is required for TBK1- and IKK ϵ -mediated type I IFN production.

Cleavage of TANK by EMCV 3C disrupts the formation of the TANK tetramer complex and IRF3 phosphorylation

It is well known that the TANK-TBK1-IKK ϵ -IRF3 complex is responsible for the phosphorylation of IRF3, and we previously demonstrated that EMCV 3C cleaved TANK and EMCV 3C inhibited IRF3 phosphorylation. However, EMCV 3C did not affect polyI:C-mediated phosphorylation of TBK1 and IKK ϵ (Supplementary

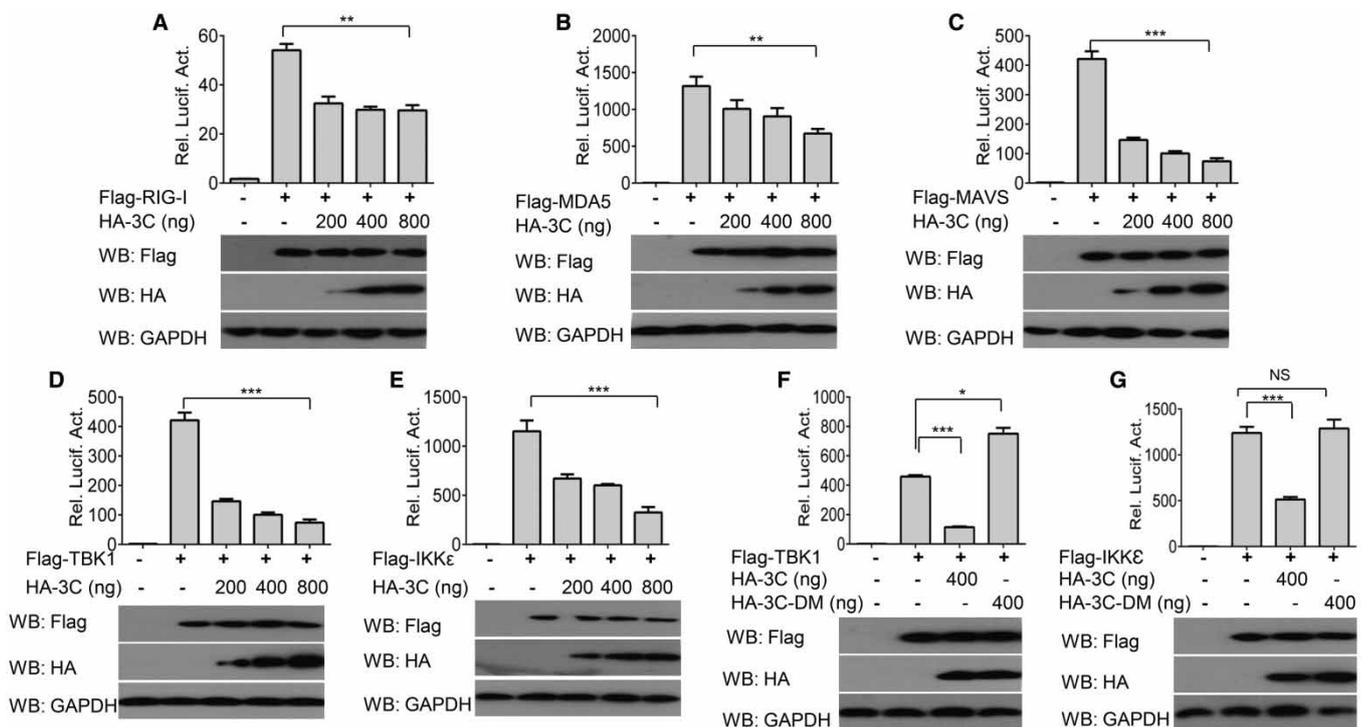


Figure 5. EMCV 3C inhibits the TBK1- and IKK ϵ -mediated type I IFN signaling.

(A–E) HEK293T cells were transfected with 100 ng of IFN- β -luc and 5 ng of pRL-TK plasmids, along with different amounts (0, 200, 400, or 800 ng) of a plasmid encoding EMCV 3C and 250 ng of a plasmid encoding RIG-I (A), MDA5 (B), MAVS (C), TBK1 (D), and IKK ϵ (E). (F and G) HEK293T cells were transfected with 100 ng of IFN- β -Luc and 5 ng of pRL-TK, along with a plasmid encoding EMCV 3C or EMCV 3C-DM and a plasmid encoding TBK1 or IKK ϵ as indicated. The luciferase assay was performed to evaluate the activities of the different promoters. The results represent three independent experiments. * represents $0.01 < P < 0.05$. ** represents $P < 0.01$. *** represents $P < 0.001$. NS represents non-statistically significant. The expression levels of indicated proteins were analyzed by Western blotting.

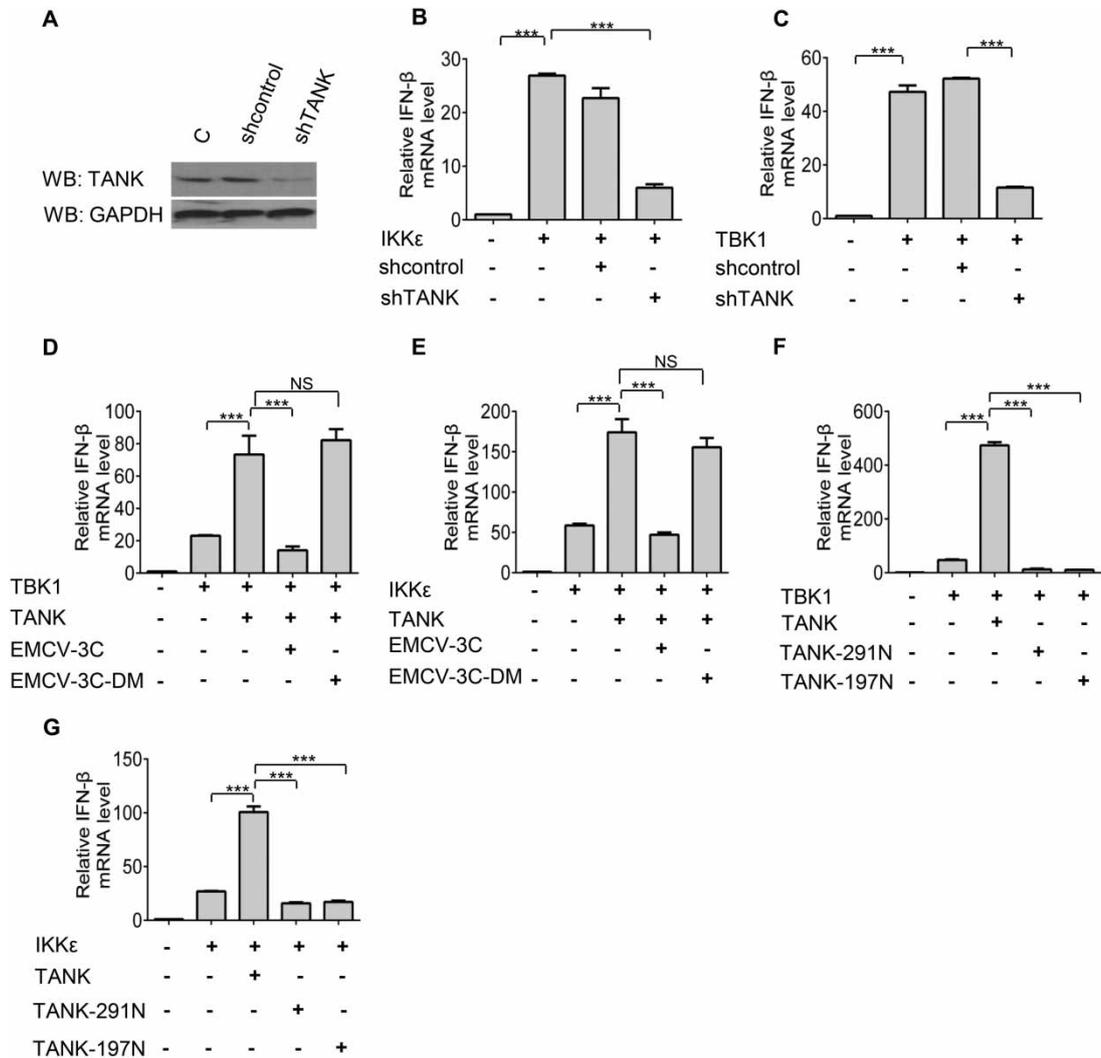


Figure 6. Intact TANK is required for TBK1- and IKKε-induced type I IFN signaling.

(A–C) HEK293T cells were transfected with a control shRNA or a TANK shRNA for 24 h and then the knockdown efficiency of TANK was analyzed by Western blotting (A) or the cells were transfected with a plasmid expressing IKKε (B) or TBK1 (C). At 24 hpt, the mRNA levels of IFN-β were analyzed by qRT-PCR. (D and E) HEK293T cells were transfected with a plasmid encoding Flag-tagged TBK1 or IKKε alone or along with a plasmid expressing HA-tagged EMCV 3C or EMCV 3C-DM, respectively. The mRNA levels of IFN-β were analyzed by qRT-PCR. (F and G) HEK293T cells were transfected with a plasmid expressing TBK1 (F) or IKKε (G), along with 2 μg of a plasmid expressing Flag-tagged full-length TANK, TANK-291N, or TANK-197N. At 24 hpt, the cells were collected and the mRNA levels of IFN-β were analyzed by qRT-PCR. The results represent three independent experiments. *** represents $P < 0.001$. NS represents non-statistically significant.

Figure S6). Therefore, we proposed that EMCV 3C might inhibit IRF3 phosphorylation through disrupting the formation of the TANK–TBK1–IKKε–IRF3 complex. To test the hypothesis, TANK and IRF3, TBK1 and IRF3, or IKKε and IRF3 were co-expressed with EMCV 3C or EMCV 3C-DM in HEK293T cells. The interaction between TANK and IRF3 was demolished because TANK was cleaved by EMCV 3C (Figure 7A). In addition, EMCV 3C expression attenuated the TBK1–IRF3 interaction (Figure 7B) and IKKε–IRF3 interaction (Figure 7C). In agreement with these results, we found that both TBK1 and IKKε interacted with the full-length TANK, TANK-197N, and TANK-291N (Supplementary Figure S7A,B), while IRF3 interacted with intact TANK, but not TANK-197N and TANK-291N (Supplementary Figure S7C). These findings reveal that intact TANK interacts with TBK1 and IKKε via its N-terminus and interacts with IRF3 via its C-terminus, which forms a tetramer. Therefore, cleavage of TANK by EMCV 3C disrupts the TANK–IRF3 interaction, thus

impairing IRF3 phosphorylation. Further results showed that both TANK and TANK-DM enhanced polyI:C-mediated IRF3 phosphorylation. EMCV infection decreased TANK-mediated IRF3 phosphorylation, but not TANK-DM (Figure 7D). These results reveal that TANK cleavage by 3C protease blocks IRF3 activation in EMCV-infected HEK293T cells.

Discussion

EMCV encodes multiple antagonists to restrict the host antiviral responses

It has been demonstrated that a double-stranded RNA structure called the replication form (RF) is produced during EMCV replication [34]. EMCV RF could be sensed by MDA5 as a potent and specific activator of type I IFN signaling [29]. However, we found that, like other members [such as Foot-and-mouth disease virus (FMDV) and enterovirus 71 (EV71)] of Picornaviruses, EMCV-HB10 infection barely induced type I IFN production in HeLa cells (Supplementary Figure S1) and HEK293T cells (Figure 1). Of note, the genomic RNA of EMCV-HB10 induced robust IFN- β mRNA expression and IFN production, which could be inhibited by EMCV-HB10 infection (Figure 3). Consistent with these results, transfection of iRNA or vRNA induced more IFN- β expression than that of the cells infected with EMCV infection [29,30], suggesting that some viral proteins may negatively regulate IFN- β production induced by viral RNA.

Previous studies demonstrated that the (L) protein of TMEV suppresses IFN- α/β synthesis at the transcriptional level [21,35]. Hato et al. [20] demonstrated that expression of the L protein interfered with the transactivation function of IRF3 by disturbing its dimerization. The zinc-binding domain in its N-terminus is essential for the function of L protein [20,21]. It has been shown that phosphorylation of threonine-47 [22] and mutation of the zinc-binding domain [21,36] impair the function of the L protein. These results suggest that the leader protein, by suppressing IRF3-mediated IFN- α/β production, plays an important role in replication and dissemination of mengovirus in its host. In the present study, L-VP4 fused protein was used to test EMCV L protein's function because the EMCV L protein is unstable and VP4 enhanced L protein stability, although VP4 itself did not inhibit IFN signaling (data not shown). Consistent with others results, we also found that EMCV L protein could inhibit IFN- β promoter activity (Supplementary Figure S2). In addition, we also found that EMCV 2C and 3C also had potent inhibitory effect on type I IFN signaling (Supplementary Figure S2). These data suggest that EMCV encodes multiple antagonists to synergistically restrict the host antiviral responses. Consistent with these findings, at least three nonstructural proteins of FMDV were found to be IFN antagonists [37–39].

EMCV 3C cleaves TANK and disrupts TANK–TBK1–IKK ϵ –IRF3-mediated type I IFN signaling

TANK was firstly identified as a co-inducer with TRAF2 of TNF- and CD40L-mediated NF- κ B activation [16]. A previous study showed that overexpressed TANK itself promotes type I IFN activation [40]. However, we found that overexpressed TANK alone did not induce IFN- β promoter activation and type I IFN production in HEK293T cells (data not shown). It has been demonstrated that TBK1 and IKK ϵ are essential components of the IRF3 signaling pathway [41], and TANK is required for TBK1- and IKK ϵ -mediated IRF3 phosphorylation and IFN production during TLR stimulation or viral infection [40,42]. Upon virus infection, TANK interacts with TBK1 and IKK ϵ with its N-terminus and IRF3 with its C-terminus, and thus bridges the kinases and the target IRF3, which participates in type I IFN production by assembling the TANK–TBK1–IKK ϵ –IRF3 complex.

EMCV 3C is the only cysteine protease encoded by the viral genome, and it exhibits a high degree of substrate specificity. We found that EMCV 3C attenuated IFN- β production induced by SeV infection, polyI:C or vRNA stimulation (Figures 1, 2, and 4). Additionally, we found that EMCV 3C reduced IFN- β reporter activity induced by RLRs (Figure 7), and EMCV 3C inhibited TBK1- or IKK ϵ -mediated IRF3 phosphorylation (Figure 7D,E), but not EMCV 3C-DM (Supplementary Figure S4D,E), suggesting that EMCV 3C may inhibit type I IFN signaling through cleavage of RLRs. Recently, we found that TANK is a novel substrate of EMCV 3C. EMCV 3C cleaves TANK at Q291 to yield two N-terminal fragments (TANK-291N and TANK-291C) in EMCV-infected cells [19]. However, whether cleavage of TANK by EMCV 3C affects IFN signaling is not fully investigated.

Interestingly, we found that both TANK and TANK-DM enhanced polyI:C-mediated IRF3 phosphorylation. EMCV infection decreased TANK-mediated IRF3 phosphorylation (Supplementary Figure S4), suggesting that intact TANK is necessary for TBK1- and IKK ϵ -mediated IFN- β signaling. Consequently, we found that overexpressed TANK enhanced IFN- β mRNA expression, whereas the cleaved fragments of TANK blocked TBK1-

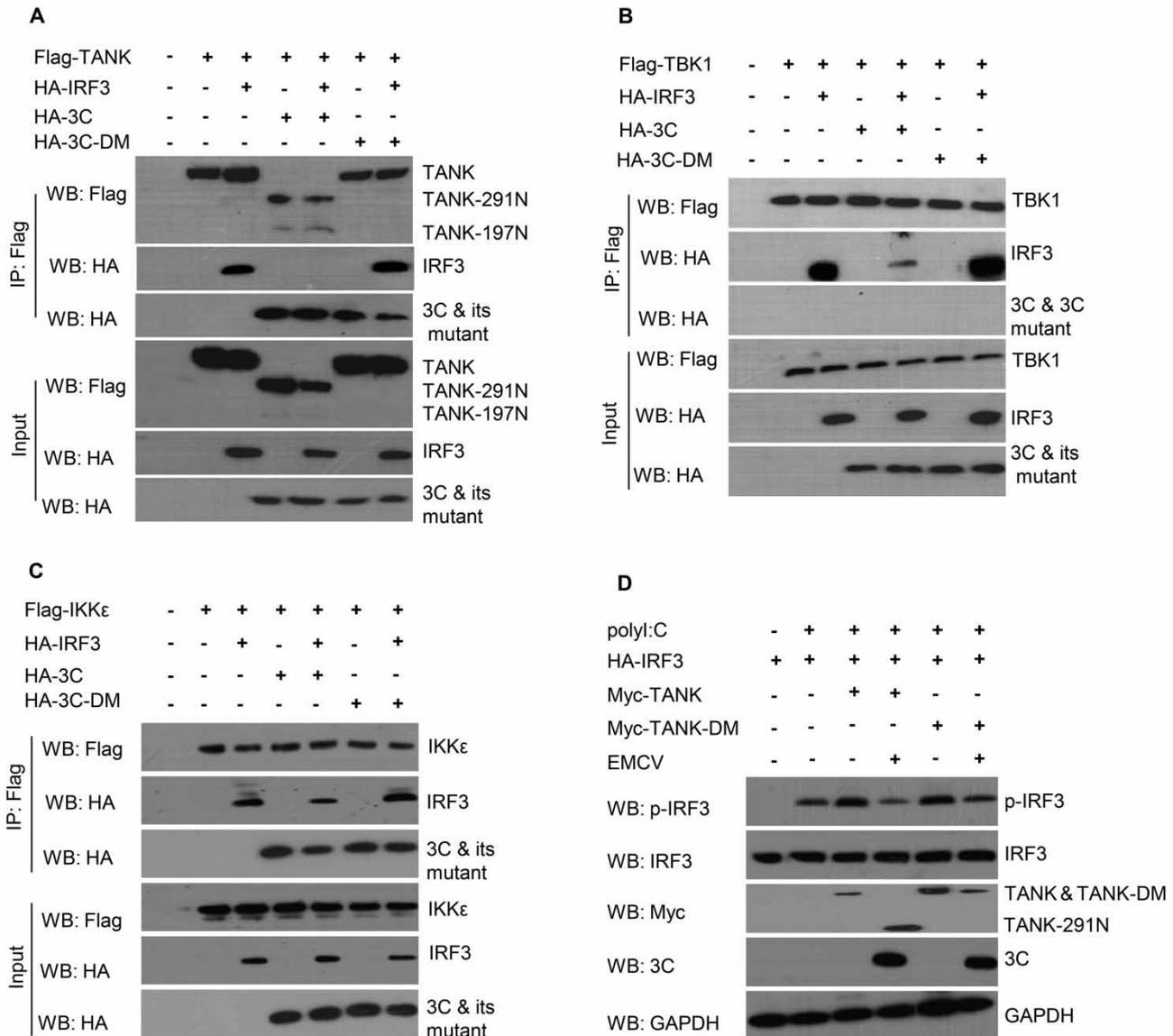


Figure 7. EMCV 3C inhibits type I IFN signaling through disrupting the TANK–TBK1–IKKε–IRF3 complex.

(A–C) HEK293T cells were transfected with 2 μg of a plasmid expressing HA-tagged IRF3, along with a plasmid expressing Flag-tagged TANK (A), TBK1 (B), or IKKε (C) and in combination with a plasmid expressing EMCV 3C or EMCV 3C-DM as indicated. The cell lysates were immunoprecipitated with anti-FLAG antibody. The cell lysates and the immunoprecipitants were analyzed by Western blotting. (D) HEK293T cells were transfected with 2 μg of a plasmid expressing HA-tagged IRF3, along with a plasmid expressing Myc-tagged TANK or TANK-DM. At 24 hpt, the cells were infected with EMCV or not, and 4 h later, the cells were transfected with polyI:C for another 8 h. The cell lysates were analyzed by Western blotting.

and IKKε-mediated IFN-β mRNA expression (Figure 6), although the cleaved fragments still bound IRF3 (Supplementary Figure S7). These data are consistent with a previous study showing that the activity of TBK1 kinase is reduced and the activity of IKKε is abolished in TANK^{-/-} macrophages [15]. Taken together, these results suggest that TANK enhances type I IFN production by acting as an adaptor protein that bridges the IKK-related TBK1–IKKε kinase complex and IRF3 downstream, and EMCV 3C cleaves TANK and disrupts TANK–TBK1–IKKε–IRF3-mediated type I IFN signaling.

As shown in Figure 5, we also found that EMCV 3C inhibited IRF3-5D induced IFN-β promoter activity, which was dependent on the protease activity of 3C. This result suggests that EMCV 3C may inhibit IRF3 or STAT1 binding to IFN-β promoter or inhibit JAK–STAT signaling through cleavage of other key proteins.

Cleavage of key molecules in the RLR-mediated type I IFN signaling pathway is a common mechanism of evasion in the Picornaviruses

Many Picornaviruses have evolved different strategies to evade host innate immune responses by interfering the type I IFN signaling pathway. It has been demonstrated that the proteases encoded by different members of Picornaviruses can inhibit type I IFN production through cleavage of receptors, adaptors, and regulators involved in the signaling pathway [25,31,43]. Previous studies reported that RIG-I and MDA5 are cleaved in poliovirus-infected cells [44,45]. Recently, Feng et al. [46] also found that MDA5, MAVS, and RIG-I all undergo proteolytic degradation in CVB3-infected cells in a caspase- and proteasome-independent manner. Furthermore, their data convincingly showed that cleavage of MDA5 and MAVS are both mediated by CVB3 2A, whereas RIG-I is cleaved by 3C. EV71 genome encodes two viral proteases: 2A and 3C. It has been demonstrated that MAVS was cleaved by EV71 protease 2A [26], and TRIF and IRF7 were cleaved by EV71 3C to inhibit Toll-like receptor 3-mediated antiviral responses [25,47]. Recently, the TAK1/TAB1/TAB2/TAB3 complex was found to be cleaved by EV71 3C, which inhibits the expression of several cytokines [48]. In addition, NEMO, an adaptor that bridges the NF- κ B and type I IFN signaling pathways [49], is also identified as a substrate of FMDV 3C protease, and the cleavage of NEMO impairs its ability to induce IFN production and acts as a signaling adaptor in the RIG-I/MDA5 pathway [31,50].

In summary, we demonstrated that EMCV-HB10 infection did not induce type I IFN production in HEK293T cells, although the viral genomic RNA could induce robust type I IFN production. Mechanistically, EMCV 3C attenuated type I IFN production by cleaving TANK, which disrupted the TANK-TBK1-IKK ϵ -IRF3 tetramer and decreased TBK1- and IKK ϵ -mediated IRF3 phosphorylation and IFN production.

Abbreviations

Co-IP, co-immunoprecipitation; DM, double mutant; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; EMCV, encephalomyocarditis virus; FMDV, Foot-and-mouth disease virus; hpt, hours post transfection; I-TRAF, TNF receptor associated factor-interacting protein; IFN, interferon; IKK, I κ B kinase; IL-1, Interleukin-1; IRF3, interferon regulator factor 3; L-VP4, L fused with VP4; mAbs, monoclonal antibodies; MAVS, mitochondrial antiviral-signalling protein; MDA5, melanoma differentiation-associated gene 5; MDBK, Madin-Darby bovine kidney; MOI, multiplicity of infection; NEMO, NF- κ B essential modulator; NLRP3, nucleotide-binding domain (NOD)-like receptor protein 3; pFlag, pCAGGS-Flag; pHA, pCAGGS-HA; qRT-PCR, quantitative real-time PCR; RIG-I, retinoic acid-inducible gene-I; RLRs, retinoic acid-inducible gene-I-like receptors; SeV, Sendai virus; STAT1, signal transducer and activator of transcription 1; TANK, TRAF family member-associated NF- κ B activator; TBK1, TANK-binding kinase 1; TLRs, Toll-like receptors; TNF, tumor necrosis factor alpha; UV, ultraviolet; VSV, vesicular stomatitis virus.

Author Contribution

C.J.W. and L.H. conceived and co-ordinated the study and wrote the paper. H.B.Y. and X.J.H. designed, performed, and analyzed the experiments shown in Supplementary Figure S1. L.H. and C.Y.L. designed, performed, and analyzed the experiments shown in Figures 1 and 3. L.H., T.X., S.N.W., Q.Z., Q.F.L., and J.N.L. designed, performed, and analyzed the experiments shown in Figures 2–7. X.H.C. and Y.F.Z. prepared anti-EMCV 3C mAb. S.J.C. and Z.G.B. provided EMCV-HB10 and recombinant VSV-expressing GFP.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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