SPI-2/CrmA inhibits IFN-β induction by targeting TBK1/IKKε

Yue Qin1, Mi Li2, Sheng-Long Zhou1, Wei Yin1, Zhuan Bian1 & Hong-Bing Shu2,3

Viruses modulate the host immune system to evade host antiviral responses. The poxvirus proteins serine protease inhibitor 2 (SPI-2) and cytokine response modifier A (CrmA) are involved in multiple poxvirus evasion strategies. SPI-2 and CrmA target caspase-1 to prevent apoptosis and cytokine activation. Here, we identified SPI-2 and CrmA as negative regulators of virus-triggered induction of IFN-β. Ectopic expression of SPI-2 or CrmA inhibited virus-triggered induction of IFN-β and its downstream genes. Consistently, knockdown of SPI-2 by RNAi potentiated VACV-induced transcription of antiviral genes. Further studies revealed that SPI-2 and CrmA associated with TBK1 and IKKε to disrupt the MITA-TBK1/IKKε-IRF3 complex. These findings reveal a novel mechanism of SPI-2/CrmA-mediated poxvirus immune evasion.

Poxviruses comprise a large family of linear dsDNA viruses that replicate in the cytoplasm of host cells. The most widely studied genus, Orthopoxvirus (OPXV), is pathogenic to humans, cattle, and zoo animals. OPXVs include vaccinia virus (VACV), cowpox virus (CPXV), ectromelia virus (ECTV), and variola virus (VARV), the causative agent of smallpox. VACV, which was used in the vaccination campaign for smallpox eradication, is now being developed as live vaccines against other infectious diseases and cancer1. CPXV infects a wide range of host species and causes zoonosis, and the outbreaks of CPXV in recent years have caused a public health crisis2.

OPXVs have evolved various mechanisms to evade and suppress host antiviral responses3. They have large genomes with a variety of immunomodulatory genes and thus display a wide range of immune evasion strategies4,5. Type I interferons (IFNs) are critical for both innate and adaptive immune responses against viral infection6. Accordingly, the suppression of type I IFN signaling by viral immunomodulatory proteins is one of the key events in virus immune evasion.

Host germline-encoded pattern-recognition receptors (PRRs) recognize viral nucleic acids and trigger downstream signaling events7. Viral DNA can be recognized by cyclic GMP-AMP synthase (cGAS)8, and viral RNA can be recognized by RIG-I-like receptors (RLRs)7. cGAS and RLRs activate the transcription factors interferon regulatory factor 3 (IRF3) and NF-κB through adaptor proteins, mediator of IRF3 activation (MITA, also termed STING) and virus-induced signaling adaptor (VISA, also termed MAVS), respectively9–11. The signaling pathways of IRF3 and NF-κB activation triggered by cGAS and RLRs converge at the level of TANK-binding kinase 1 (TBK1) and IκB kinases (IKKs). On the one hand, activation of cGAS or RLR leads to the recruitment of kinases (TBK1 and IKKε) by MITA or VISA9–11. MITA or VISA is phosphorylated by the kinases and subsequently recruits IRF314. TBK1 and IKKε then phosphorylate IRF3, leading to the dimerization and nuclear translocation of IRF314. On the other hand, inhibitor of NF-κB (IκB) is phosphorylated by the IKK complex (consisting of IKKα, IKKβ, and IKKγ) and then activated NF-κB is released6. Activated IRF3 and NF-κB respectively bind to interferon-stimulated response element (ISRE) and the κB site, leading to the transcriptional induction of IFN-β7.

Vaccinia virus (VACV) strain Western Reserve (WR) immediate-early gene B13R encodes a 38-kDa cytosolic protein SPI-2, which is highly homologous to its orthologue, CPXV cytokine response modifier A (CrmA). SPI-2 and CrmA are the most extensively studied OPXV serine protease inhibitors. They are non-essential for virus replication and are involved in multiple immunomodulatory events. It has been reported that SPI-2 and CrmA inhibit extrinsic apoptosis and host inflammatory responses15–20. SPI-2 and CrmA target caspase-1 to protect virus-infected cells from TNF- and Fas-mediated apoptosis as well as to prevent the proteolytic activation of interleukin-1β16–19.

1The State Key Laboratory Breeding Base of Basic Science of Stomatology, Hubei Province and Key Laboratory of Oral Biomedicine, Ministry of Education (Hubei-MOST KLOS & KLOBME), School and Hospital of Stomatology, Wuhan University, Wuhan, 430079, China. 2State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan, 430007, China. 3Medical Research Institute, Collaborative Innovation Center for Viral Immunology, School of Medicine, Wuhan University, Wuhan, 430071, China. Correspondence and requests for materials should be addressed to Y.Q. (email: qinyue@whu.edu.cn)
In the present study, we found that SPI-2 and CrmA acted as inhibitors of both DNA- and RNA-virus-triggered induction of IFN-β. SPI-2 and CrmA functioned at the level of TBK1/IKKe to inhibit IRF3 but not NF-κB activation. SPI-2 and CrmA disrupted the MITA-TBK1/IKKe-IRF3 complex by interacting with TBK1 and IKKe. Our findings suggest that SPI-2 and CrmA antagonize the type I IFN pathway by targeting TBK1/IKKe, thus representing a newly identified mechanism of immune evasion of VACV and CPXV.

Results

Roles of SPI-2 orthologues in IFN-β induction. VACV strain Tian-Tan (TT), a widely-used smallpox vaccine strain in China, is less virulent than strain WR21,22. To evaluate the abilities of these VACV strains to stimulate type I IFN induction, THP-1 cells were infected with VACV strain WR and TT at an MOI of 1. Quantitative real-time PCR results indicated that both VACV strains triggered IFN-β induction in THP-1 cells. However, VACV strain TT activated IFN-β more than strain WR (Fig. 1A). In VACV strain WR and TT, several genes had polymorphic lengths, including A39R, B13R, C4L, C14L, and C16R23. Among them, C4L and C16R have been demonstrated to regulate type I IFN induction20,24. As type I IFN is critical in antiviral responses, we assumed that additional proteins target IFN induction signaling. SPI-2, encoded by the B13R gene in VACV strain WR, is split into two fragments (termed TB13R and TB14R) in VACV strain TT (Fig. 1B). As SPI-2 is highly homologous to CPXV CrmA, the well-known apoptosis inhibitor, we first constructed expression clones encoding VACV SPI-2, TB13R, and TB14R and identified their roles in IFN-β induction. Luciferase reporter assays were utilized to identify their abilities to regulate the activation of IFN-β promoter mediated by overexpression of cGAS and MITA in HEK293 cells. Ectopic expression of SPI-2 inhibited cGAS- and MITA-mediated activation of IFN-β promoter and ISRE reporter, but not NF-κB reporter, in a dose-dependent manner (Fig. 1C). However, ectopic expression of TB13R together with TB14R failed to inhibit cGAS- and MITA-mediated IFN-β promoter and ISRE reporter (Fig. 1D). Although the sequences of TB13R and TB14R are highly homologous to SPI-2, these two proteins do not retain the function of the full-length protein, which may be due to the conformational change resulting from splitting ORFs. These data indicated that full-length SPI-2 inhibits cGAS- and MITA-triggered IFN-β induction.

Next, we identified the roles of SPI-2 orthologues in IFN-β induction. CPXV CrmA and ECTV C7 proteins have 92% and 94% amino acid identity with SPI-2 protein, respectively (Fig. 1B). Ectopic expression of CrmA and C7 inhibited cGAS- and MITA-mediated activation of IFN-β promoter and ISRE reporter, but not NF-κB reporter, in a dose-dependent manner (Fig. 1E and F). Thus, the SPI-2 orthologues, CrmA and C7, inhibit cGAS- and MITA-triggered IFN-β induction.

Sendai virus (SeV) triggers induction of type I IFNs through RLR signaling. Consistent with their roles in regulation of cGAS- and MITA-mediated activation of IFN-β promoter, ectopic expression of SPI-2, CrmA, and C7, but not TB13R and TB14R, inhibited SeV-induced activation of IFN-β promoter and ISRE reporter but not NF-κB reporter (Supplementary Fig. S1A to D). They also had no marked effect on IFN-γ-induced activation of IRF1 promoter (Supplementary Fig. S1E to H). These data suggest that SPI-2 and its homologues, CrmA and C7, inhibit SeV-induced activation of IFN-β promoter.

SPI-2 and CrmA inhibit virus-triggered induction of endogenous IFN-β. To investigate the roles of SPI-2 and CrmA in virus-triggered induction of endogenous IFN-β, THP-1 cells were stably transfected with Flag-tagged SPI-2 or CrmA (Fig. 2A and B). Quantitative real-time PCR analysis indicated that ectopic expression of SPI-2 inhibited herpes simplex virus 1 (HSV-1)- and SeV-induced transcription of IFNB1 and its downstream genes RANTES and CXCL10, but not TNF, downstream of NF-κB signaling (Fig. 2C and D). Similarly, CrmA inhibited HSV-1- and SeV-induced transcription of IFNB1, RANTES, and CXCL10, but not TNF (Fig. 2E and F). These data suggest that SPI-2 and CrmA specifically inhibit virus-triggered induction of endogenous IFN-β.

Since SPI-2 and CrmA inhibited virus-triggered induction of IFN-β, we examined their roles in cellular antiviral responses. As shown in Supplementary Fig. S2, HSV-1 and Vesicular Stomatitis Virus (VSV) production was increased in THP-1 cells ectopically expressing SPI-2 or CrmA, consistent with the role of SPI-2 and CrmA in negative regulation of both DNA- and RNA-virus-triggered IFN-β induction. These results suggest that SPI-2 and CrmA negatively regulate cellular antiviral responses.

Knockdown of SPI-2 enhances VACV-triggered induction of IFN-β gene. The role of endogenous SPI-2 in innate antiviral responses was next examined. RNAi knockdown strategy used in our study has been successfully used in previous studies25-28. Two SPI-2-RNAi plasmids were constructed that could inhibit the mRNA level of B13R gene (Fig. 3A and B). In THP-1 cells stably transfected with the SPI-2-RNAi plasmids, the mRNA levels of B13R neighboring genes, B12R and B15R, were not dramatically decreased (Fig. 3B). Quantitative real-time PCR analysis indicated that knockdown of SPI-2 promoted VACV-induced transcription of IFNB1, RANTES, and CXCL10 (Fig. 3C), but it had no marked effect on HSV-1- or SeV-induced transcription of IFNB1 (Fig. 3D and E). These results suggest that knockdown of SPI-2 potentiates VACV-triggered induction of IFN-β and its downstream genes.

SPI-2 and CrmA inhibit IRF3 activation at the level of TBK1/IKKe. To determine which step of IFN-β induction was targeted by SPI-2/CrmA, luciferase assays was utilized in which SPI-2 or CrmA was co-expressed with the components of IFN-β induction signaling. Ectopic expression of SPI-2 inhibited the activation of IFN-β promoter and ISRE reporter mediated by overexpression of cGAS and MITA but not their downstream TBK1, IKKe, and IRF3 (Fig. 4A and B). IKKε and p65 act upstream of NF-κB activation. Consistent with the role of SPI-2 in NF-κB activation (Figs 1C, 2C and D), SPI-2 did not markedly affect IKK- or p65-mediated activation of IFN-β promoter (Fig. 4C). Similarly, CrmA inhibited cGAS-, MITA-, but not TBK1-, IKKe-, and IRF3-mediated activation of IFN-β promoter and ISRE reporter (Fig. 4D and E). Additionally, CrmA had no
marked effect on IKKβ- or p65-mediated activation of IFN-β promoter (Fig. 4F). SPI-2 and CrmA also inhibited the activation of IFN-β promoter and ISRE reporter mediated by the RLR adaptor VISA, which is upstream of TBK1/IKKε (Supplementary Fig. S3). These data indicate that SPI-2 and CrmA inhibit IRF3 activation at the level of TBK1/IKKε.

SPI-2 and CrmA disrupt the MITA-TBK1/IKKε-IRF3 complex by interacting with TBK1/IKKε. Since SPI-2 and CrmA function at the level of TBK1/IKKε, we examined whether SPI-2 and CrmA interacted with TBK1 and IKKε. Transient transfection and co-immunoprecipitation experiment indicated that SPI-2 and CrmA were associated with TBK1 and IKKε in HEK293 cells (Fig. 5A and B). To investigate the mechanism by which SPI-2 and CrmA regulated IRF3 activation, we examined whether SPI-2 and CrmA affected the interaction between components of the MITA-TBK1/IKKε-IRF3 complex as well as the stability of the components. Ectopic expression of SPI-2 and CrmA attenuated the interaction of TBK1/IKKε with MITA and IRF3, and SPI-2 and CrmA did not markedly affect the protein levels of MITA, TBK1, IKKε, and IRF3 (Fig. 5C and D). These results suggested that SPI-2 and CrmA disrupted the MITA-TBK1/IKKε-IRF3 complex through interacting with TBK1 and IKKε. Thus, SPI-2/CrmA inhibits IFN-β induction by targeting TBK1/IKKε.
Figure 2. Effects of SPI-2 and CrmA on virus-triggered induction of endogenous IFN-β. (A, B) Quantitative real-time PCR and immunoblot analysis of SPI-2 and CrmA expression in THP-1 cells stably transfected with SPI-2-Flag or CrmA-Flag plasmid. SPI-2-Flag (A) and CrmA-Flag (B) stable THP-1 cells were analyzed by quantitative real-time PCR and immunoblots. ND, not detected. (C, D) Effects of SPI-2 on HSV-1- and SeV-induced transcription of antiviral genes. SPI-2-Flag stable THP-1 cells were infected with HSV-1 (C) or SeV (D) for 12 hours before quantitative real-time PCR analysis. (E, F) Effects of CrmA on HSV-1- and SeV-induced transcription of antiviral genes. The experiments were performed similar to those in (C, D). All experiments were repeated at least three times with similar results. The bar graphs show the mean ± S.D. (n = 3) of a representative experiment performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001. The cropped blots from different gels are displayed, for full blots refer supplementary information.
Discussion

Previous studies have demonstrated that SPI-2 and CrmA have anti-apoptotic and anti-inflammatory functions\(^\text{15–20}\). Here, we identified SPI-2 and CrmA as negative regulators of virus-triggered induction of IFN-\(\beta\). Our findings reveal a novel mechanism of SPI-2/CrmA mediated OPXV immune evasion.
Figure 4. Effects of SPI-2 and CrmA on IFN-β promoter activation mediated by various components. (A,B) Effects of SPI-2 on IFN-β promoter (A) and ISRE reporter (B) activation mediated by cGAS, MITA, TBK1, IKKe, and IRF3. HEK293 cells were transfected with the IFN-β promoter or ISRE reporter and the indicated expression plasmids together with increasing amounts of SPI-2 for 20 hours before the luciferase reporter assays. (C) Effects of SPI-2 on IKKe- and p65-mediated activation of IFN-β promoter. HEK293 cells (2 × 10^5) were transfected with the IFN-β promoter (0.1 μg) and IKKe or p65 (0.1 μg each) together with increasing amounts of SPI-2 (25, 50, 100 ng) for 20 hours before the luciferase reporter assays. (D,E) Effects of CrmA on IFN-β promoter (D) and ISRE reporter (E) activation mediated by cGAS, MITA, TBK1, IKKe, and IRF3. The experiments were performed similarly to those in (C). All experiments were repeated at least three times with similar results. The bar graphs show the mean ± S.D. (n = 3) of a representative experiment performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001, relative to the controls transfected with the indicated components.
SPI-2 and its orthologue CrmA inhibited the induction of IFN-β triggered by both DNA and RNA viruses, and SPI-2 and CrmA inhibited the activation of IFN-β promoter triggered by cGAS and RLR signaling. SPI-2 and CrmA specifically inhibited virus-triggered induction of IFN-β by inhibiting of IRF3, but not NF-κB, activation. Luciferase reporter assays suggested that SPI-2 and CrmA inhibited the activation of IFN-β promoter and ISRE reporter at the level of TBK1 and IKKε, consistent with the roles of TBK1 and IKKε in the activation of IRF3 but not NF-κB. Further experiments suggested that SPI-2 and CrmA were associated with TBK1 and IKKε. Additionally, TBK1 and IKKε disrupted the MITA-TBK1/IKKε-IRF3 complex. Based on our findings, we developed a working model of how SPI-2 and CrmA negatively regulate virus-triggered type I IFN induction (Fig. 6).

The recognition of cytosolic viral DNA by PRRs triggers host antiviral responses. SPI-2 and CrmA inhibit the induction of type I IFNs by targeting TBK1 and IKKε. These findings have not only verified a novel immunomodulatory function of SPI-2/CrmA, but they also provide an example of how viruses escape host immune attack using distinct mechanisms mediated by one immunomodulator.

Consistent with the ability of SPI-2 to inhibit IFN-β induction, knockdown of SPI-2 by RNAi enhanced VACV-induced transcriptional activation of IFN-β gene in THP-1 cells. We observed that knockdown of B13R gene by SPI-2-RNAi plasmids slightly inhibited the mRNA levels of its neighboring genes, B12R and B15R, likely because the elevation of IFN-β production inhibited the replication of VACV in SPI-2 knockdown cells.

Multiple viral proteins may target a same cellular pathway to ensure successful immune evasion. VACV encodes several immunomodulatory proteins targeting type I IFNs induction signaling, including E3, A46, 32, 33,
C16, K7, C6, and N2. Among them, K7 and C6 have been reported to inhibit IRF3 activation by targeting the kinase complex containing TBK1 and IKKe. However, each protein targets distinct components of this complex. K7 inhibits IRF3 phosphorylation by targeting DDX31, and C6 interacts with TANK, NAP1, and SINTBAD, the scaffold proteins that associate with TBK1 and IKKe32. Our findings suggest that SPI-2 inhibits the activation of IRF3 in a different manner than K7 and C6. SPI-2 directly targets the kinases TBK1 and IKKe, which are responsible for the phosphorylation of both MITA and IRF3. SPI-2 may work in coordination with these immunomodulatory proteins to inhibit the activation of IRF3 triggered by VACV infection.

The mechanism study of immune evasion mediated by SPI-2 and CrmA not only reveals new insights into the virus-host interaction but also has important implications for rational vaccine design and antiviral drug development against OPXV infection.

**Materials and Methods**

**Reagents and antibodies.** Recombinant IFN-γ (R&D Systems) and mouse monoclonal antibodies against FLAG, Flag-HRP, β-actin (Sigma), Myc (CST), and HA (Covance) were purchased from the indicated manufacturers. HEK293 and THP-1 cells, SeV, HSV-1, and VSV have been previously described26, 34–36.

**Constructs.** IFN-β, ISRE, NF-κB and IRF1 luciferase reporter plasmids, mammalian expression plasmids for Flag- or HA-tagged cGAS, MITA, TBK1, IKKe, IRF3, and β-actin have been previously described26, 34. Mammalian expression plasmids for human Flag-, HA- or Myc-tagged SPI-2 and CrmA were constructed using standard molecular biology techniques.

**RNAi.** Double-stranded oligonucleotides corresponding to the target sequences were cloned into the pSuper.Retro-RNAi plasmid (Oligoengine). The following sequences were targeted for SPI-2 mRNA: #1 5′-GGAGAACATGGATAAGGTT-3′, #2 5′-GCGATATTCTGCCGTGTTT-3′. The sequence targeted by the control RNAi plasmid is 5′-GGAAGATGTATGGAGACATGG-3′.

**Transfection and reporter assays.** HEK293 cells were seeded and transfected the following day using the standard calcium phosphate precipitation method or FuGENE (Roche), according to the procedures recommended by the manufacturer. Empty control plasmids were added to ensure that each transfection received the same amount of total DNA. To normalize for transfection efficiency, pRL-TK Renilla luciferase reporter plasmids were added to each transfection. Luciferase assays were performed using a dual-specific luciferase assay kit (Promega). Firefly luciferase activities were normalized based on Renilla luciferase activities.

**Quantitative real-time PCR.** Total RNA was isolated from THP-1 cells (1 × 10⁶) using TRIzol reagent (Takara) according to the manufacturer’s instructions. cDNA was synthesized from 2 μg of purified total RNA using reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using Ssoadvanced Universal SYBR Green Supermix (Bio-Rad) to measure the expression of mRNA. The related fold difference in expression...
The target gene between the cells were analyzed with 2^ΔΔCt method. Data represent the relative abundance of the indicated mRNA normalized to that of GAPDH. Gene-specific primer sequences were as described or as follows: B13R: (forward, 5′-CTCCGCACGGAATGGTAGAT-3′; reverse, 5′-TGCCGAATGGATCTCCCTTACA-3′). DNA-A: (forward, 5′-GTGTGCAGGACTATCAGCAC-3′; reverse, 5′-GATGAAAGGAGTATCCTGAC-3′). B12R: (forward, 5′-ACAACCTGACACGGGAACG-3′; reverse, 5′-CTCTTTGGGGCAATACCTT-3′). B13R: (forward, 5′-TAAACCGGCTGAATGTATCG-3′; reverse, 5′-CAGTAAGTTCTGTCTGGAATG-3′).

RNAi-transduced stable THP-1 cells. HEK293 cells were transfected with two packaging plasmids (pGAG-Pol and pVSV-G) together with control or SPI-2-RNAi retroviral plasmids respectively by calcium phosphate precipitation. Twenty-four hours after transfection, the cells were incubated with new medium without antibiotics for another twenty-four hours. The recombinant virus-containing medium was filtered with a 0.22-μm filter (Millex) and then added to cultured THP-1 cells in the presence of polyeylene (4μg/mL). The infected cells were selected with puromycin (0.5μg/mL) for at least seven days before performing additional experiments.

Coimmunoprecipitation and immunoblot analysis. HEK293 cells (5 × 10^6) were lysed in 1 mL IP lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM phenylmethylsulphonyl fluoride) on wet ice. Coimmunoprecipitation and immunoblot analysis were performed as previously described.

Data Availability. All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

References
1. Smith, G. L. Rapid spreading and immune evasion by vaccinia virus. *Advances in experimental medicine and biology* **808**, 65–76, doi:10.1007/978-81-322-1774-9_6 (2014).
2. Alshbanova, D. & Fruh, K. Modulation of the host immune response by cowpox virus. *Microbes and infection / Institut Pasteur* **12**, 900–909, doi:10.1016/j.micinf.2010.07.007 (2010).
3. Smith, G. L. et al. Vaccinia virus immune evasion mechanisms: virulence and immunogenicity. *The Journal of general virology* **94**, 2367–2392, doi:10.1099/vir.0.055921-0 (2013).
4. Budgood, S. R. & Mercer, J. Cloak and Dagger: Alternative Immune Evasion and Modulation Strategies of Poxviruses. *Cell host & microbe* **18**, 480–485, doi:10.1016/j.chom.2017.01.001 (2017).
5. Sett, R. B., Sun, L., Ea, C. K. & Chen, Z. J. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein. *Immunological reviews* **243**, 61–73, doi:10.1111/1600-065X.2011.01048.x (2011).
6. Takeuchi, O. & Akira, S. Pattern recognition receptors and inflammation. *Cell* **140**, 805–820, doi:10.1016/j.cell.2010.01.022 (2010).
7. Kawasaki, T., Kawai, T. & Akira, S. Recognition of nucleic acids by pattern-recognition receptors and its relevance in autoimmunity. *Immunological reviews* **243**, 61–73, doi:10.1111/1600-065X.2011.01048.x (2011).
8. Sun, L., Wu, J., Du, F., Chen, X. & Chen, Z. J. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* **339**, 786–791, doi:10.1126/science.1232458 (2013).
9. Seth, R. B., Sun, L., Ea, C. K. & Chen, Z. J. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* **122**, 669–682, doi:10.1016/j.cell.2005.08.012 (2005).
10. Xu, L. G. et al. VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Molecular cell* **19**, 727–740, doi:10.1016/j.molcel.2005.08.014 (2005).
11. Ishikawa, H. & Barber, G. N. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* **455**, 674–678, doi:10.1038/nature07317 (2008).
12. Zhong, B. et al. The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity* **29**, 538–550, doi:10.1016/j.immuni.2008.09.003 (2008).
13. Ishikawa, H., Ma, Z. & Barber, G. N. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* **461**, 788–792, doi:10.1038/nature08476 (2009).
14. Liu, S. et al. Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation. *Science* **347**, aa2630, doi:10.1126/science.aac2630 (2015).
15. Kettle, S., Blake, N. W., Law, K. M. & Smith, G. L. Vaccinia virus serpins B13R and B22R encode M(r) 38.5 and 40K, intracellular polypeptides that do not affect virus virulence in a murine intranasal model. *Journal of virology* **87**, 136–147 (1993).
16. Dobbelstein, M. & Shenk, T. Protection against apoptosis by the vaccinia virus SPI-2 (B13R) gene product. *Journal of virology* **70**, 6479–6485 (1996).
17. Kettle, S. et al. Vaccinia virus serpin B13R (SPI-2) inhibits interleukin-1beta-converting enzyme and protects virus-infected cells from TNF- and Fas-mediated apoptosis, but does not prevent IL-1beta-induced fever. *The Journal of general virology* **78**(Pt 3), 777–785, doi:10.1099/0022-1317-78-3-677 (1997).
18. Ray, C. A. et al. Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1 beta converting enzyme. *Cell* **69**, 597–604 (1992).
19. Komiyama, T. et al. Inhibition of interleukin-1 beta converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. *The Journal of biological chemistry* **269**, 19331–19337 (1994).
20. Nathaniel, R., MacNeill, A. L., Wang, Y. X., Turner, P. C. & Moyer, R. W. Cowpox virus CrmA, Myxoma virus SERP2 and baculovirus P35 are not functionally interchangeable caspase inhibitors in poxvirus infections. *The Journal of general virology* **85**, 1267–1278, doi:10.1099/vir.0.79905-0 (2004).
21. Fang, Q. et al. Host range, growth property, and virulence of the smallpox vaccine: vaccinia virus Tian Tan strain. *Virology* **353**, 242–251, doi:10.1016/j.virology.2005.02.014 (2005).
22. Zhang, Q. et al. Genomic sequence and virulence of clonal isolates of vaccinia virus Tianan, the Chinese smallpox vaccine strain. *PloS one* **8**, e60557, doi:10.1371/journal.pone.0060557 (2013).
23. Ember, S. W., Ren, H., Ferguson, B. J. & Smith, G. L. Vaccinia virus protein C4 inhibits NF-kappaB activation and promotes virus virulence. *The Journal of general virology* **93**, 2098–2108, doi:10.1099/vir.0.045070-0 (2012).
24. Peters, N. E. et al. A mechanism for the inhibition of DNA-PK-mediated DNA sensing by a virus. *PLoS pathogens* **9**, e1003649, doi:10.1371/journal.ppat.1003649 (2013).
25. Lau, L., Gray, E. E., Brunette, R. L. & Stetson, D. B. DNA tumor virus oncogenes antagonize the cGAS-STING DNA-sensing pathway. *Science* **350**, 568–571, doi:10.1126/science.aab3291 (2015).
26. Fu, Y. Z. et al. Human Cytomegalovirus Tegument Protein UL82 Inhibits STING-Mediated Signaling to Evade Antiviral Immunity. *Cell host & microbe* , doi:10.1016/j.chom.2017.01.001 (2017).
27. Zhang, P. & Samuel, C. E. Induction of protein kinase PKR-dependent activation of interferon regulatory factor 3 by vaccinia virus occurs through adapter IPS-1 signaling. *The Journal of biological chemistry* **283**, 34580–34587, doi:10.1074/jbc.M807029200 (2008).

28. Myśliwiec, C., Arsenio, J., van Bruggen, R., Deschambault, Y. & Cao, J. Vaccinia virus E3 suppresses expression of diverse cytokines through inhibition of the PKR, NF-kappaB, and IRF3 pathways. *Journal of virology* **83**, 6757–6768, doi:10.1128/JVI.02570-08 (2009).

29. Lysakova-Devine, T. *et al.* Viral inhibitory peptide of TLR4, a peptide derived from vaccinia protein A46, specifically inhibits TLR4 by directly targeting MyD88 adaptor-like and TRIF-related adaptor molecule. *Journal of immunology* **185**, 4261–4271, doi:10.4049/jimmunol.1002013 (2010).

30. Stack, J. *et al.* Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. *The Journal of experimental medicine* **201**, 1007–1018, doi:10.1084/jem.20041442 (2005).

31. Schroder, M., Baran, M. & Bowie, A. G. Viral targeting of DEAD box protein 3 reveals its role in TBK1/IKKepsilon-mediated IRF activation. *The EMBO journal* **27**, 2147–2157, doi:10.1038/emboj.2008.143 (2008).

32. Unterholzner, L. *et al.* TRIM38 Negatively Regulates TLR3/4-Mediated Innate Immune and Inflammatory Responses by Two Sequential and Distinct Mechanisms. *Journal of immunology* **195**, 4415–4425, doi:10.4049/jimmunol.1500859 (2015).

Acknowledgements

We thank Yu-Zhi Fu, Dan Li, Tian-Tian Liu, Tian Xia, and Ze-Min Song for technical assistance and stimulating discussions; we thank Xue-Wu Zhang, Cao-Qi Lei, and Yu Zhang for critically reading the manuscript; and we thank Yan-Yi Wang (Wuhan Institute of Virology, Chinese Academy of Sciences), Zhi-Gao Bu and Xi-Jun Wang (Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences) for reagents. This work was supported by the National Natural Science Foundation of China (31600696, 81470727, and 81501750), the National Key Research and Development Program (2016YFC1000505), the Fundamental Research Funds for the Central Universities (413000006), and the Open Research Fund Program of Hubei-MOST KLOS & KLOBME (201501).

Author Contributions

Conceived and designed the experiments: Y.Q., M.L., H.B.S. Performed the experiments: Y.Q., M.L. Analyzed the data: Y.Q., S.L.Z., W.Y., Z.B., H.B.S. Wrote the paper: Y.Q.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-11016-3

Competing Interests: The authors declare that they have no competing interests.

Accession Number: UniProtKB/Swiss-Prot accession numbers (parentheses) are indicated for the proteins mentioned in text: SPI-2 (P15059), CrmA (P07385), TBK1 (Q9UHD2), IKKε (Q14164), MITA (Q86WV6), IRF3 (Q14653).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2017