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HSV-2 Immediate-Early Protein US1 Inhibits IFN-β Production by Suppressing Association of IRF-3 with IFN-β Promoter

Mudan Zhang,*† Yalan Liu,* Ping Wang,*† Xinneng Guan,*† Siyi He,*† Sukun Luo,*† Chang Li,*† Kai Hu,* Wei Jin,*† Tao Du,* Yan Yan,*† Zhenfeng Zhang,* Zhenhua Zheng,* Hanzhong Wang,* and Qinxue Hu*†

HSV-2 is the major cause of genital herpes, and its infection increases the risk of HIV-1 acquisition and transmission. After initial infection, HSV-2 can establish latency within the nervous system and thus maintains lifelong infection in humans. It has been suggested that HSV-2 can inhibit type I IFN signaling, but the underlying mechanism has yet to be determined. In this study, we demonstrate that productive HSV-2 infection suppresses Sendai virus (SeV) or polyinosinic-polycytidylic acid-induced IFN-β production. We further reveal that US1, an immediate-early protein of HSV-2, contributes to such suppression, showing that US1 inhibits IFN-β promoter activity and IFN-β production at both mRNA and protein levels, whereas US1 knockout significantly impairs such capability in the context of HSV-2 infection. US1 directly interacts with DNA binding domain of IRF-3, and such interaction suppresses the association of nuclear IRF-3 with the IFN-β responsive domain of IFN-β promoter, resulting in the suppression of IFN-β promoter activation. Additional studies demonstrate that the 217–414 aa domain of US1 is critical for the suppression of IFN-β production. Our results indicate that HSV-2 US1 downmodulates IFN-β production by suppressing the association of IRF-3 with the IFN-β responsive domain of IFN-β promoter. Our findings highlight the significance of HSV-2 US1 in inhibiting IFN-β production and provide insights into the molecular mechanism by which HSV-2 evades the host innate immunity, representing an unconventional strategy exploited by a dsDNA virus to interrupt type I IFN signaling pathway. The Journal of Immunology, 2015, 194: 3102–3115.

Innate immunity is the first line of host in response to viral infection (1–3). Type I IFN IFN-α/β, as a vital component of the innate immune system, plays a crucial role in eliminating the foreign pathogens at the early stage of infection (4). During viral infection, host pathogen recognition receptors recognize pathogen-associated molecular patterns, such as viral DNA, dsRNA, or proteins, to initiate the production of type I IFNs. The presence of dsRNA is usually recognized by endosomal TLR3 and two cytoplasmic sensors, retinoic acid inducible gene I (RIG-I) and melanoma-associated differentiation gene 5 (MDA-5) (5–9). After activation by cytoplasmic dsRNA, RIG-I and MDA-5 initiate a cascade of signals to induce type I IFN production.

IFN regulatory factor 3 (IRF-3) is a key transcription factor in the pathway of type I IFN production (10, 11). Inactive IRF-3 must go through sequential posttranslational modifications before associating with the IFN-β promoter (12, 13). Upon the signals triggered by viral infection, RIG-I/MDA-5 binds to dsRNA through the helicase domain and signals through caspase activation and recruitment domains to the adaptor molecule, mitochondrial antiviral signaling (MAVS) protein (also known as IPS-1, VISA, or Cardif). Activated MAVS leads to the activation of inhibitor of κB kinase ε (IKK-ε) and TANK-binding kinase 1 (TBK-1), which further leads to the phosphorylation and dimerization of IRF-3, resulting in the translocation of IRF-3 homodimer from the cytoplasm into the nucleus (14, 15). Subsequently, activated IRF-3 and other coactivators bind to IFN-α or -β promoter to boost IFN-α or -β transcription in a cooperative pattern (16).

Many viruses have evolved counteracting mechanisms by interfering with IRF-3 to escape the antiviral effect of type I IFNs. For example, viruses can bind directly to IRF-3 (17, 18) or interact with CBP/p300 coactivator (19–21) to impair the type I IFN production. In general, some viruses cause the degradation of activated IRF-3 (22, 23), whereas most viruses suppress the production of type I IFNs through inhibition of IRF-3 activation, including phosphorylation, dimerization, and nuclear translocation (24–30). Only a few viruses have been shown to interfere with the binding of activated IRF-3 to IFN-β promoter, resulting in the suppression of type I IFNs (31, 32).

HSV-2, a member of the α-herpesviridae subfamily, is a large enveloped dsDNA virus (33). HSV-2 mainly infects epithelial cells and causes genital herpes. It also infects neuronal cells and leu-
kocytes including dendritic cells (34–36), leading to encephalitis and disseminated diseases that affect other organ systems (37, 38). HSV-2 can establish a lifelong latent infection in sacral ganglia (39), resulting in up to 40% human adults living with HSV-2 latency (40). After initial infection, HSV-2 dsDNA, dsRNA, and proteins such as gB, gH/L, as pathogen-associated molecular patterns are most likely recognized by cellular sensors (1, 41–47), which causes the induction of host antiviral immune responses. Early investigation indicates that whole HSV-2 lysosomal tissues contain a large quantity of IFN-γ but surprisingly low levels of type I IFNs (48). Given that current understanding of HSV-2 immune evasion is limited, we investigated whether HSV-2 infection could modulate type I IFN production and investigated the underlying mechanisms.

We show that productive HSV-2 infection suppresses IFN-β production. We further demonstrate that US1, an immediate-early (IE) protein of HSV-2, inhibits the function of IRF-3 through suppressing the association of IRF-3 with IFN-β promoter and that a physical interaction between US1 and the DNA binding domain of IRF-3 contributes to the inhibitory activity of US1. In addition, we show that the 217–414 aa domain of US1 is crucial for inhibiting IFN-β production. Our study reveals a novel mechanism by which HSV-2 evade innate immunity, providing a basis for further understanding the complexity of HSV-2–host interactions.

Materials and Methods

Viruses, cell lines, and Abs

HSV-2 (G strain) was obtained from LGC standards and propagated in African green monkey kidney cells (Vero). Virus stock supplemented with 10% FBS (Life Technologies, 1099-141) was stored at -80˚C before use for infection. UV-irradiated HSV-2 was obtained by exposure to UV light for 15 min. HSV-2 titration was determined by plaque assay on Vero monolayers (49).

Sendai virus (SeV) was propagated in 12-d-old embryonated eggs and titrated by hemagglutination (HA) assay using chicken RBCs (50). Special pathogen-free embryonated eggs (Beijing Merial Vital Laboratory Animal Technology Corporation) were incubated at 37˚C for 12 d before inoculation with SeV. SeV was diluted to 100 hemagglutinating unit (HAU)/ml using FBS-free DMEM, and 300 μl of diluted SeV was injected into alantoic cavity of 12-d-old embryonated eggs, followed by incubation at 37˚C for 72 h. The embryonated eggs were stored at 4˚C overnight before the collection of allantoic fluids. Collected virus fluids were briefly centrifuged and stored at -80˚C before being used for infection. For HA assay, 2-fold serial dilutions of SeV were prepared and mixed with equal volumes of 1% (v/v) chicken RBCs, and then added to 96-well V-shaped bottom plates for 45 min at room temperature. The highest dilution of virus that formed diffuse lattice was defined as 1 HAU.

HEK 293T, Vero and human cervical epithelial cell line HeLa were cultured in DMEM (Life Technologies, 11665) supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin at 37˚C in a 5% CO2 incubator. Human cervical epithelial cell line ME180 was cultured in the same components as DMEM.

Abs against I-IFN and p-IFN-β were purchased from Cell Signaling Technology (4302S and 49475). Another Ab against IFN-β3 was purchased from Proteintech (116312-AF). Abs against HA tag were purchased from Santa Cruz Biotechnology (sc-7391) and Sigma-Aldrich (H6908). Ab against flag tag was from Sigma-Aldrich (F1804). Ab against β-actin was from Santa Cruz Biotechnology (sc-8432). Abs against proliferating cell nuclear Ag (PCNA) was from Proteintech (102052-AP). Cy3-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories. Ab against IRF-3 was purchased from Santa Cruz Biotechnology (sc-81178). Cy3-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories.

Construction of US1 deleted HSV-2

A bacterial artificial chromosome (BAC) plasmid carrying the complete genome of HSV-2 and GFP was provided by Dr. Yasushi Kawaguchi (University of Tokyo, Tokyo, Japan). The Escherichia coli strain GS1783 containing λ-Red recombineering system was provided by Dr. Klaus Osterrieder (Cornell University, New York, NY). The full-length HSV-2 BAC was electroplated into GS1783 and US1 deleted HSV-2 (referred to as US1 del HSV-2) was constructed via homologous recombination (51). GS1783 harboring HSV-2 BAC was grown at 32˚C in lysogenic broth (LB) medium with 30 μg/ml chloramphenicol to an A600 of 0.5–0.7. The culture was then transferred into a 42˚C water bath shaker for 15 min at 220 rpm to induce the expression of λ-Red recombineering system. Gel-purified PCR product was obtained using the kanamycin (kan) primers containing a 50-bp extension homologous to the us1 sequence (Supplemental Table I). Five microfilters of the purified PCR products were electroplated into 50 μl of competent GS1783 cells containing HSV-2 BAC, with the settings of 2.5 kV, 25 μF, and 200 Ω. Following the addition of 1 ml LB medium, the bacteria were then incubated with shaking at 32˚C for 2 h. Subsequently, 100 μl cultures were plated onto an agar plate containing 5% (v/v) glycerol, 50 μg/ml kanamycin, and 30 μg/ml chloramphenicol for 15 h, US1 del HSV-2 BAC DNA was extracted. US1 del HSV-2 construct was confirmed by PCR detection of kan gene and the 649–1242bp fragment of us1. US1 del HSV-2 BAC candidate was used as a template to amplify the sequence instead of us1 using detection primers (Supplemental Table I). Purified PCR products were verified by DNA sequencing (Sunny Biotechnology). BAC DNA was transfected into Vero cells using X-tremeGENE HP DNA transfection reagent (Roche, 06366236001) to produce viruses.

Plasmid construction

All primers used for plasmid construction are listed in Supplemental Table I. HSV-2 genomic DNA was extracted from the cells infected with HSV-2 for 48 h using QIAamp DNA Blood MIni Kit (Qiagen, 51104). The open reading frames encoding HSV-2 US1, US12, RS1, HU, US1-Flag, US1-HA, US1-Flank, RS1-Flag, RS1-HA, US1 (217–414 aa)-Flag and US1 (217–414 aa)-HA, respectively. All constructs were verified by DNA sequencing (Sunny Biotechnology).

IRF-3 and IRF-3/5D expression plasmids pIRES-hrGFP/IRF-3-Flag and pIRES-hrGFP/IRF-3/5D-Flag (constitutively active mutant of IRF-3) were provided by Dr. Yi-Ling Lin (Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan) (52). The reporter plasmid PRD (III-I)-Luc was provided by Dr. Stephan Ludwig (University of Muenster, Muenster, Germany) (53). pEF-Flag-RIG-IN (a carboxy-terminally truncated, constitutively active RIG-I mutant) expression plasmid was provided by Dr. Takashi Fujita (Kyoto University, Kyoto, Japan) (54). Expression plasmids pCDNA3-TBK-1-Flag and pCDNA3-IRF-3-Flag were gifts from Dr. Katherine Fitzgerald (University of Massachusetts Medical School, Worcester, MA) (14). Influenza virus RNA polymerase (PR/NS1, IRF-3 truncations (IRF-3 1–357 [residues 1–357], IRF-3 1–240 [residues 1–240], IRF-3 1–197 [residues 1–197], IRF-3 56–427 [residues 56–427], and IRF-3 112–427 [residues 112–427]) were provided by Dr. Stephan Ludwig (University of Muenster, Muenster, Germany) (53). pIRES-hrGFP/IRF-3/5D-Flag were provided by Dr. Yi-Ling Lin (Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan) (52). The reporter plasmid pIRES-hrGFP/IRF-3/5D-Flag were provided by Dr. Yi-Ling Lin (Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan) (52).

RNA isolation and quantitative PCR

Cells were collected and total RNA was extracted using TRIzol (Invitrogen, 15596-026) according to the manufacturer’s instructions. RNA-free DNase I (Fermentas, EN0521) was used to eliminate the contamination of genomic DNA. cDNA was synthesized by moloney murine leukemia virus transcriptase (Promega, M170B). The newly synthesized cDNA was used as the template for the amplification of IFN-β and GAPDH genes. The primer pairs for IFN-β and GAPDH were shown in Supplemental Table I. GAPDH was used as an internal control. Relative real-time quantitative PCR was performed on an ABI Prism 7700 apparatus using a SYBR Green Real-Time PCR Master Mix (Toyobo, QPK-201) according to the following conditions: 95˚C for 1 min, followed by 40 cycles of 95˚C for 15 s, 55˚C for 15 s, and 72˚C for 45 s. The expression difference was calculated on the basis of 2-ΔΔCt values.

Dual luciferase reporter assay

HEK 293T cells were seeded in 24-well plates overnight and cotransfected with empty vector or plasmid encoding US1, RS1, US12, UL54, US1 or transfected with US1. Renilla luciferase plasmid pCMV-Renilla plasmid p125-Luc or pCMV-Renilla plasmid p125-Luc or PRD(III-I)-Luc. Transfections were performed using Lipofectamine 2000 (Invitrogen, 11668-027) according to the manufacturer’s instructions. At 24 h posttransfection, cells were stimulated with 100 HAU of UV-inactivated HSV-2.

Production of Plaque assay on Vero cells.
ml^{-1} SeV for 16 h. Cells were harvested and lysed, and the lysates were used for measuring firefly and Renilla luciferase activities using a Dual-Luciferase Reporter Assay System (Promega, E1980) according to the manufacturer’s instructions. For some experiments, HEK 293T cells were cotransfected with empty vector or US1 expression plasmid, reporter plasmids p125-Luc and phRL-TK, together with plasmid encoding IFN-β pathway inducer RIG-1, IPS-1, TBK-1, IKK-ε, or IRF-3/5/D. At 40 h posttransfection, the enzymatic activities of firefly and Renilla luciferase were measured. For ME180 cells, after cotransfection with reporter plasmids p125-Luc and phRL-TK for 4 h, cells were infected or mock infected with HSV-2, UV-inactivated HSV-2 or US1 del HSV-2 for 20 h. Cells were subsequently stimulated with or without 100 HAU ml^{-1} SeV for 16 h or transfected with or without 1 μg/ml poly-IC, and the activities of firefly and Renilla luciferase were measured.

**Immunofluorescence assay**

HeLa cells were seeded in 35-mm glass-bottom dishes and transfected with the indicated plasmids. At 24 h posttransfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 12 h, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. After three washes with PBS, cells were blocked in PBS containing 5% BSA at 4˚C overnight. Thereafter, cells were incubated with rabbit anti-human IRF-3 polyclonal Ab (pAb) and mouse anti-HA Ab or mouse anti-Flag mAb at a dilution of 1:100 at 37˚C for 1 h, respectively. After three washes with PBS, cells were then incubated with Cy3-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG at a dilution of 1:50 for 1 h at 37˚C. Cells were subsequently washed and incubated with Hoechst 33258 or DAPI for 10 min at 37˚C. Finally, cells were washed and subject to incubation with antifluorescence quenching reagent (Beyotime, P0126) and observed under a fluorescence microscope (Olympus IX51).

**Coimmunoprecipitation assay**

HEK 293T cells in six-well plates were cotransfected with IRF-3 expression plasmid or IRF-3 truncation mutant and US1-HA expression plasmid or empty vector. At 24 h posttransfection, cells were stimulated with or without 100 HAU ml^{-1} SeV for 16 h. Cells were harvested and lysed on ice for 10 min in 200 μl lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% NP40) containing protease inhibitor mixture (Roche, 11697498001). To eliminate the nonspecific binding of other proteins, the samples were pretreated with Dynabeads Protein G (Invitrogen, 10003D) for 2 h at room temperature followed by separation prior to coimmunoprecipitation assay. Meanwhile, 5 μg rabbit anti-HA Ab or control rabbit Ab was diluted in 200 μl PBS with 0.1% Tween-20 (PBST) and added to fresh Dynabeads protein G. After incubation with rotation for 1 h at room temperature, Dynabeads-Ab complexes were washed once with 200 μl PBST before mixed with the pretreated samples, followed by 30 min incubation with rotation at room temperature to allow the formation of Dynabeads-Ab-Ag complexes. The complexes were washed three times with PBST, and target Ags were subjected to Western blot analysis after elution by boiling.

**FIGURE 1.** HSV-2 infection inhibits the production of IFN-β. (A) HSV-2 infection suppresses the SeV-induced activation of IFN-β promoter. ME180 cells in 24-well plates were cotransfected with 250 ng p125-luc, 50 ng phRL-TK for 4 h followed by infection with HSV-2 at an MOI of 1, 2, or 3, or UV-inactivated HSV-2 at an MOI of 1 or 2. At 24 h posttransfection, cells were stimulated with or without SeV for 16 h. (B) HSV-2 infection inhibits the SeV-induced production of IFN-β. ME180 cells in six-well plates were infected with HSV-2 or UV-inactivated HSV-2 at an MOI of 1, or cultured alone for 20 h. Cells were stimulated with or without SeV for 16 h. (C) HSV-2 infection suppresses the poly-IC-induced activation of IFN-β promoter. ME180 cells in 24-well plates were cotransfected with 250 ng p125-luc, 50 ng phRL-TK for 4 h followed by infection with HSV-2 or UV-inactivated HSV-2 at an MOI of 1. At 24 h posttransfection, cells were stimulated with or without 1 μg/ml poly-IC for 16 h. (D) HSV-2 infection inhibits the poly-IC-induced IFN-β production. ME180 cells in six-well plates were infected with HSV-2 or UV-inactivated HSV-2 at an MOI of 1, or cultured alone for 20 h. Cells were stimulated with or without 1 μg/ml poly-IC for 16 h. Reporter activities were determined by DLR assay. The protein level of IFN-β in supernatants was measured with ELISA. Data shown are mean ± SD of three independent experiments with each condition performed in triplicate. MOI, multiplicity of infection; ND, not detected.
Western blot analysis

Western blot analysis was performed as described previously (55). Cytoplasmic and nuclear proteins were isolated using the Nucleus and Cytoplasm Protein Extraction Kit (Beyotime, P0028). Cell extracts were subjected to 10% or gradient 4–20% SDS-PAGE and transferred to PVDF membranes (0.45 μm; Millipore) followed by blocking with 5% nonfat milk in TBS-Tween (50 mM Tris-HCl [pH 7.5], 200 mM NaCl, 0.1% [v/v] Tween-20) and probed with appropriate primary Abs at room temperature for 2 h. After washing three times with TBS-Tween, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (BOSTER, BA1054) or goat anti-mouse IgG (BOSTER, BA1051) at room temperature for 1 h. Protein bands were visualized by exposure to FluorChem HD2 Imaging System (Alpha Innotech) after the addition of chemiluminescent substrate (Beyotime, P0018).

Chromatin immunoprecipitation (ChIP)

HEK 293T cells in six-well plates were co-transfected with plasmids expressing IRF-3 and US1-HA, US1 (1–216 aa)-HA, US1 (217–414 aa)-HA or empty vector. At 24 h posttransfection, cells were stimulated with or without 100 HAU mL⁻¹ SeV for 16 h. Chromatin immunoprecipitation (ChIP) assay was performed according to the manufacturer’s instructions.
Cells were fixed in 1% formaldehyde at room temperature for 10 min, followed by incubation in 1× glycine solution at room temperature for 5 min to quench unreacted formaldehyde. After three washes with cold PBS containing 1× Protease Inhibitor Cocktail II, cells were resuspended in nuclear lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing 1× Protease Inhibitor Cocktail II. After incubation in an ice bath for 10 min, DNA was sheared to small fragments by sonication, and supernatants were transferred into clean tubes. A small fraction of the supernatants was used for input sample detection. Magnetic protein G was added into the remaining supernatants followed by 2 h rotation at 4˚C to reduce the nonspecific binding of target protein or DNA to Protein G agarose. Thereafter, fresh magnetic protein G were added into the pretreated supernatants followed by incubation with mouse anti-Flag Ab, mouse anti-RNA polymerase II Ab (positive control) or normal mouse IgG (negative control) at 4˚C overnight. The next day, after several washes, the immunoprecipitated DNA/protein complexes were eluted by incubation at 65˚C for 4 h and 95˚C for 10 min. Final supernatants containing DNA were purified using spin columns.

**ELISA for IFN-β**

HEK 293T cells in six-well plates were transfected with empty vector, plasmid expressing US1, US1 (1–216 aa), US1 (217–414 aa), or NS1. At 24 h posttransfection, cells were stimulated with or without 100 HAU ml⁻¹ SeV for 16 h. In some cases, ME180 cells were infected with HSV-2 or UV-inactivated HSV-2 or infected with HSV-2 or US1 del HSV-2 for 20 h prior to SeV treatment. Cell culture supernatants were collected and centrifuged to remove cell debris. ELISA was used to quantify secreted IFN-β. Fifty microliters of supernatants was used for IFN-β detection using a human IFN-β ELISA kit (R&D PBL, 41410-1) according to the manufacturer’s instructions.

**Results**

**HSV-2 infection suppresses the production of IFN-β**

HSV-2 has been shown to evade mucosal innate immunity, but the underlying mechanisms remain to be elucidated (48). Considering the important role played by IFN-β in innate immunity (56) and that HSV-2 can inhibit type I IFN induced signaling (57), we examined the effect of HSV-2 infection on IFN-β production. HSV-2 infects cervical epithelial cells. We therefore conducted initial experiments using the human cervical epithelial cell line ME180 cells. ME180 cells were cotransfected with the reporter

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**Figure 3.** US1 interferes with the IRF-3–mediated signaling pathway. (A) US1 inhibits the activation of IRF-3–responsive IFN-β promoter. HEK 293T cells in 24-well plates were cotransfected with 500 ng US1 expression plasmid or empty vector together with 250 ng PRD(III-I)4–Luc and 50 ng phRL-TK. At 24 h posttransfection, cells were stimulated with or without SeV for 16 h. (B–F) US1 inhibits the activation of IRF-3 pathway. HEK 293T cells in 24-well plates were cotransfected with increasing quantities (0.5, 1.5, and 2.5 µg) of US1 expression plasmid or empty vector, 250 ng p125-Luc and 50 ng phRL-TK together with 50 ng RIG-IN (B), IPS-1 (C), TBK-1 (D), IKK-ε (E), or IRF-3/5D (F) expression plasmid. At 40 h posttransfection, cells were harvested and luciferase activities were measured. Reporter activities were determined with DLR assay. Values for the samples were normalized using Renilla luciferase values and expressed as a percentage of the value induced in cells transfected with empty vector. Protein expression was monitored with Western blot using anti-HA or anti-Flag Ab. β-actin was used as a loading control (lowest panel). Data shown are mean ± SD of three independent experiments with each condition performed in triplicate. **p < 0.001.
plasmid p125-Luc containing IFN-β promoter, which drives the expression of the firefly luciferase, and the internal control plasmid phRL-TK. At 4 h posttransfection, cells were infected with HSV-2 or UV-inactivated HSV-2 for 20 h. Cells were subsequently stimulated with SeV for 16 h to induce IFN synthesis. As shown in Fig. 1A, HSV-2 infection strongly suppressed the activation of IFN-β promoter. In contrast, treatment with UV-inactivated HSV-2 did not inhibit the activity of IFN-β promoter induced by SeV. To confirm the results generated from the IFN-β reporter assay, we performed ELISA to analyze IFN-β production at the protein level. ME180 cells were infected with HSV-2 or UV-inactivated HSV-2 for 20 h, followed by stimulation with SeV for 16 h. As shown in Fig. 1B, productive HSV-2 infection but not UV-inactivated HSV-2 inhibited IFN-β production. In addition to SeV stimulation, we also conducted experiments under the condition of IFN-β expression induced by poly-IC. ME180 cells were cotransfected with p125-Luc and phRL-TK. At 4 h post-transfection, cells were infected with HSV-2 for 20 h. Cells were subsequently transfected with or without poly-IC for 16 h to induce IFN expression. As shown in Fig. 1C, HSV-2 inhibited the activation of IFN-β promoter induced by poly-IC. We further assessed IFN-β production at protein level by ELISA. ME180 cells were infected or mock infected with HSV-2 for 20 h, followed by transfection with or without poly-IC for 16 h. As shown in Fig. 1D, HSV-2 significantly inhibited IFN-β production induced by poly-IC. These findings together indicate that HSV-2 can suppress the production of IFN-β and that productive HSV-2 infection is necessary for such suppression.

**HSV-2 US1 inhibits the production of IFN-β**

UV-inactivated HSV-2 did not affect IFN-β production, indicating that productive HSV-2 infection is required for the inhibition of IFN-β production. HSV-2 genome encodes at least 74 proteins in a well-ordered cascade fashion (58, 59). The firstly expressed IE proteins are...

**FIGURE 4.** US1 does not block IRF-3 activation. (A and B) US1 affects neither (A) the level of total IRF-3 nor (B) the phosphorylation of IRF-3. HEK 293T cells in six-well plates were transfected with 4 μg empty vector or US1 expression plasmid. At 24 h posttransfection, cells were stimulated with or without SeV for 16 h. Cytoplasmic and nuclear proteins were isolated. (C-F) US1 does not significantly inhibit IRF-3 nuclear translocation. HEK 293T cells in six-well plates were (E) transfected with 4 μg empty vector or US1 expression plasmid or (F) cotransfected with 2.5 μg IRF-3 and 2.5 μg US1 expression plasmids or empty vector for 24 h, followed by stimulation with SeV for 16 h. Cytoplasmic and nuclear proteins were isolated. p-IRF-3 and total IRF-3 levels were measured with anti-IRF-3 or anti-p-IRF-3 Ab. β-Actin and PCNA were used as loading controls for cytoplasmic and nuclear proteins, respectively. (C) The effect of US1 on IRF-3 nuclear translocation was determined by indirect immunofluorescence. HeLa cells in 35-mm dishes were transfected with 2.5 μg empty vector, US1, or NS1 expression plasmid and then stimulated with SeV for 12 h. Cells were stained with mouse anti-HA Ab and rabbit anti-IRF-3 pAb, followed by FITC-conjugated goat anti-mouse (green) and Cy3-conjugated goat anti-rabbit (red) as the secondary Abs. Total (D) The percentage of IRF-3 positive nuclei was quantified in a number of fields. (E) The images were obtained by fluorescence microscopy using a 60× objective. Densitometric analysis of p-IRF-3 level in nucleus was performed using Image J software. One representative experiment out of three is shown. C, cytoplasmic; N, nuclear.
proteins include ICP0 (RL2), ICP4 (RS1), ICP22 (US1), ICP27 (UL54), and ICP47 (US12), which peak between 3 and 5 h postinfection, followed by expression of early and late genes (60, 61). We therefore conducted experiments to address the potential roles of IE proteins in the inhibition of IFN-β induction. HEK 293T cells were cotransfected with reporter plasmids p125-luc, phRL-TK, and IE gene expression plasmid or empty vector for 24 h, followed by stimulation with or without SeV for 16 h, whereas a plasmid encoding NS1 of influenza virus was used as a positive control that is known to inhibit IFN-β induction (62). As shown in Fig. 2A, HSV-2 RL2 (ICP0) and UL54 (ICP27) inhibited the induction of IFN-β. In contrast, HSV-2 RS1 (ICP4) did not significantly inhibit IFN-β induction, whereas HSV-2 US12 (ICP47) seemed to increase the IFN-β activation. Of note, HSV-2 US1 significantly suppressed the activation of IFN-β promoter to a level similar to that of NS1, whereas HSV-1 US1 had no inhibitory effect on IFN-β induction (Fig. 2B). The expression of HSV-1 and HSV-2 US1 was confirmed as shown in Fig. 2C. HSV-2 US1 is a protein of 414 aa with a predicted molecular mass of 45 kDa migrating with an apparent size of 70 kDa, most likely because of posttranslational modifications such as phosphorylation and ubiquitination, which has been suggested in the study of HSV-1 US1 (63, 64).

Given that RL2 (ICP0) (13, 23, 65, 66) and UL54 (ICP27) (67, 68) of HSV-1 have previously been reported to inhibit IFN-β induction and that HSV-1 US1 did not have such inhibitory effect in our study (Fig. 2A, 2B), we subsequently focused our study on HSV-2 US1 to address the mechanisms as to how HSV-2 US1 inhibits IFN-β induction. We first assessed whether HSV-2 US1 blocked the production of IFN-β mRNA. HEK 293T cells were transfected with empty vector or construct expressing HSV-2 US1 for 24 h, followed by stimulation with or without SeV for 16 h. US12, another IE protein of HSV-2 and the influenza NS1 were used as controls. Total RNA was extracted and IFN-β mRNA was analyzed by relative real-time quantitative PCR. As shown in Fig. 2D, HSV-2 US1 significantly inhibited IFN-β mRNA production. The expression of IE proteins and NS1 was confirmed (Fig. 2E). We next examined whether US1 could indeed inhibit IFN-β production. HEK 293T cells were transfected with empty vector or US1, NS1 expression plasmid for 24 h followed by stimulation with or without SeV for 16 h. The protein level of IFN-β in supernatants was measured by ELISA. In agreement with the data from IFN-β promoter assay, US1 downregulated the production of IFN-β induced by SeV at protein level (Fig. 2F).

We further confirmed the impact of US1 on IFN-β induction in the context of HSV-2 infection using a newly constructed US1 del HSV-2 (Supplemental Fig. 1A). US1 del HSV-2 BAC was transfected into Vero cells to produce infectious progeny US1 del HSV-2 (Supplemental Fig. 1B, 1C). As shown in Supplemental Fig. 1D and 1E, US1 knockout did not significantly affect HSV-2 replication and release compared with HSV-2. In the context of HSV-2 infection, ME180 cells were infected or mock infected with HSV-2 or US1 del HSV-2 for 20 h, or cotransfected with p125-Luc, and phRL-TK for 4 h and subsequently infected or mock infected with HSV-2 or US1 del HSV-2 for 20 h, followed by stimulation with or without SeV for 16 h. As shown in Fig. 2G and 2H, US1 knockout significantly impaired the capability of HSV-2 in inhibiting IFN-β induction at both promoter activation and protein expression levels. The remaining inhibitory activity of US1 del HSV-2 on IFN-β production was likely due to other viral proteins, including RL2 (ICP0) and UL54 (ICP27) as demonstrated in Fig. 2A, which have previously been reported to inhibit IFN-β induction in several HSV-1 studies (13, 23, 65–68).

**FIGURE 5.** US1 interacts with the DNA binding domain of IRF-3. (A) US1 interacts with IRF-3. (B) Schematic representation of IRF-3 and IRF-3 deletion mutants. Numbers indicate amino acid positions of IRF-3. (C) US1 interacts with the DNA binding domain of IRF-3. HEK 293T cells in six-well plates were cotransfected with 2.5 μg plasmid expressing (A) flag-tagged IRF-3 or (C) IRF-3 truncations and 2.5 μg HA-tagged US1 expression plasmid or empty vector for 24 h, followed by stimulation with SeV for 16 h. Cells were lysed and subjected to coimmunoprecipitation (IP) using rabbit anti-HA Ab. Rabbit normal IgG was used as a negative control. IP products and 5% input samples were examined using mouse anti-HA and mouse anti-Flag Abs with Western blot. One representative experiment out of three is shown. IB, immunoblotting.
Taken together, our data collectively demonstrate that HSV-2 US1 plays a crucial role in the inhibition of IFN-β production.

**US1 interferes with IFN-3-mediated signaling pathway**

Several viral proteins from different viruses have been shown to inhibit IFN production by interfering with IFN-3 signaling pathway (17–30, 69, 70). We next determined the effects of US1 on IFN-3-dependent, SeV-induced activation of the IFN-β promoter. HEK 293T cells were cotransfected with PRD(III-I)4–Luc containing four repeats of the IFN-3 responsive domain of IFN-β promoter, phRL-TK and US1-expressing plasmid or empty vector. Influenza virus NS1 was used as a positive control for suppressing the activation of IFN-3 responsive promoter. As shown in Fig. 3A, SeV induced the activation of IFN-3-responsive promoter in cells transfected with the empty vector, whereas US1 blocked the activation of IFN-3-responsive promoter to the background level, indicating that US1 inhibits IFN-β production likely through IFN-3–dependent signaling pathway.

In response to foreign Ags, RIG-I binds to dsRNA and signals through the adaptor IPS-1. Engagement of RIG-I initiates signaling through two protein kinase complex, TBK-1/IKK-ε, leading to the phosphorylation and further activation of IFN-3. To identify the potential mechanism by which US1 inhibits IFN-β production, plasmid expressing RIG-IN, IPS-1, TBK-1, IKK-ε, or IFN-3/5D, which are inducers of IFN-β in the IFN-3 signaling pathway, was transfected into HEK 293T cells together with p125-Luc, phRL-TK, US1 expression plasmid, or empty vector. As shown in Fig. 3A, SeV induced the activation of IFN-3-responsive promoter in cells transfected with the empty vector, whereas US1 blocked the activation of IFN-3-responsive promoter to the background level, indicating that US1 inhibits IFN-β production likely through IFN-3–dependent signaling pathway.

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**US1 does not significantly block IFN-3 activation**

IFR-3 is a key transcription activator in IRF3-mediated signaling pathway. Many viruses have evolved mechanisms to evade the host innate immunity by interacting with IRF-3, such as HSV-1 ICP0 (71), human papillomavirus 16 E6 (17), HHV-2 vIRF-1 (72), and hepatitis C virus serine protease (29). Upon phosphorylation, IRF-3 dimerizes and translocates from the cytoplasm to the nucleus, where it acts as a transcription factor to activate IFN-β promoter (14, 15). Because US1 inhibited IRF-3 pathway, we investigated whether it affected SeV-induced IRF-3 phosphorylation and nuclear translocation. Phosphorylation of IRF-3 was analyzed in cells transfected with empty vector or plasmid expressing US1 followed by stimulation with SeV to activate IRF-3. NS1 was used as a positive control for the inhibition of IRF-3 phosphorylation. In agreement with previous study (73), SeV stimulation rapidly induced an apparent increase of phosphorylated IRF-3 as detected by a specific anti-p-IRF-3 Ab. Of note, US1 affected neither the level of total IRF-3 (Fig. 4A) nor the phosphorylation (Fig. 4B) of IRF-3 induced by SeV.

We next examined whether US1 affects IRF-3 translocation. Nuclear translocation of IRF-3 was analyzed in cells transfected with empty vector or plasmid expressing US1 followed by stimulation with SeV to induce IRF-3 translocation from the cytoplasm to the nucleus. NS1 was used as a positive control for the blockade of IRF-3 nuclear translocation. Cells were examined by indirect immuno-fluorescence (Fig. 4C) and the percentage of IRF-3 positive nuclei in a number of fields was quantified (Fig. 4D). Cytoplasmic and nuclear proteins were subsequently isolated from HEK 293T cells, followed by Western blot (Fig. 4E) to determine the distribution of IRF-3. As shown in Fig. 4C and 4E, after stimulation with SeV, almost all the IRF-3 proteins in cells transfected with empty vector were translocated from the cytoplasm into the nucleus, and such IRF-3 translocation was not significantly inhibited when cells were transfected with US1 expression plasmid. In contrast, influenza virus NS1 significantly blocked IRF-3 translocation.

Given that IRF-3 translocation was not significantly blocked by US1, we performed the experiment to examine the level of p-IRF-3 in nuclei under the condition of IRF-3 overexpression. HEK 293T cells were cotransfected with plasmid expressing IRF-3 together with US1 expression plasmid or empty vector. At 24 h posttransfection, cells were stimulated with SeV for 16 h. Cytoplasmic and nuclear proteins were subsequently isolated, followed by Western blot. As shown in Fig. 4F, US1 did not block the nuclear translocation of IRF-3 when IRF-3 was over-expressed. These data collectively indicate that US1 does not inhibit IRF-3 activation.

**FIGURE 6.** US1 suppresses the association of IRF-3 with IFN-β promoter. (A) Schematic representation of the four domains within IFN-β promoter. (B) US1 inhibits the association of IRF-3 with IFN-β promoter. HEK 293T cells in six-well plates were cotransfected with 2.5 μg flag-tagged IRF-3 and 2.5 μg HA-tagged US1 expression plasmids or empty vector for 24 h, followed by stimulation with SeV for 16 h. Cells were lysed and subjected to ChIP assay using mouse anti-Flag mAb, mouse anti-RNA polymerase II mAb (positive control), or mouse normal IgG (negative control) for immunoprecipitation. (C) The expression of IRF-3 and US1 was stained using anti-Flag or HA Ab. One representative experiment out of three is shown.
US1 interacts with DNA binding domain of IRF-3

Given that US1 was mainly localized in the nucleus (Fig. 4C), we hypothesized that US1 interferes with the function of IRF-3 in the nucleus. To test this hypothesis, coimmunoprecipitation was performed to determine the interaction between US1 and IRF-3. HEK 293T cells were cotransfected with plasmids expressing IRF-3 and US1-HA or empty vector. At 24 h posttransfection, cells were stimulated with SeV for 16 h. Precleared cell lysates from the transfected cells were incubated with anti-HA Ab against US1-HA. The precipitates were analyzed by Western blot using anti-Flag Ab against flag-tagged IRF-3. As shown in Fig. 5A, the anti-HA Ab was able to specifically precipitate the immune complex that contained US1 and IRF-3, indicating a physical association of US1 with IRF-3. IRF-3 consists of 427 aa, including a DNA binding domain (aa 1–112) responsible for DNA binding and an IRF association domain (IAD; aa 197–394) responsible for IRF-3 phosphorylation, dimerization and interaction with CBP/p300 co-activators (32). To map the region in IRF-3 responsible for US1 binding, a series of IRF-3 truncated mutants (Fig. 5B) were used in coimmunoprecipitation assay. HEK 293T cells were cotransfected with plasmid expressing US1-HA or empty vector and full-length IRF-3 or truncated IRF-3 expression plasmid. At 24 h posttransfection, cells were stimulated with SeV for 16 h. As shown in Fig. 5C, deletion of the entire DNA binding domain (IRF-3 aa 112–427) abolished the interaction between IRF-3 and US1. In contrast, US1 still bound to IRF-3 mutants with partial or entire IAD deletion, indicating that the DNA binding domain of IRF-3 is essential for US1 binding.

US1 suppresses the association of IRF-3 with IFN-β promoter

Given that DNA binding domain of IRF-3 plays a critical role in mediating the interaction between IRF-3 and IFN-β promoter (Fig. 6A), its engagement by US1 likely consequently suppresses the association of IRF-3 with its responsive DNA promoter. ChIP was used to test this hypothesis. HEK 293T cells were cotransfected with plasmids expressing IRF-3 and US1 or empty vector for 24 h followed by stimulation with SeV for 16 h. Chromosomes were fragmented by ultrasonication followed by ChIP assay. As seen in Fig. 6B, IRF-3 bound poorly to IFN-β promoter when cotransfection with US1 expression plasmid, suggesting that US1 inhibits the binding of IRF-3 to IFN-β promoter after IRF-3 nuclear translocation. The expression of US1 and IRF-3 was confirmed as shown in Fig. 6C. These data indicate that US1 inhibits the association of IRF-3 with IFN-β promoter.

The 217–414 aa domain of US1 is essential for the inhibition of IFN-β production

To map the functional region of US1 involved in the inhibition of IFN-β production, we constructed two truncation mutants US1 (1–216 aa) and US1 (217–414 aa). HEK 293T cells were cotransfected with reporter plasmids p125-luc, phRL-TK and the plasmid expressing US1, truncated US1, or empty vector for 24 h followed by stimulation with or without SeV for 16 h, whereas NS1 was used as a positive control. Reporter activities were determined by DLR assay. As shown in Fig. 7A, deletion of the 217–414 aa domain (US1 [1–216 aa]) barely suppressed the activation of IFN-β promoter induced by SeV, whereas deletion of the 1–216 aa domain (US1 [217–414 aa]) significantly inhibited the activation of IFN-β promoter. To confirm our findings further at the protein level, HEK 293T cells were transfected with plasmid expressing US1, US1 (1–216 aa), US1 (217–414 aa), or empty vector for 24 h followed by stimulation with or without SeV for 16 h. The protein level of IFN-β in supernatants was measured with ELISA. In agreement with the data from IFN-β promoter assay, US1 (217–414 aa) downregulated the production of IFN-β induced by SeV (Fig. 7B). The expression of US1, truncated US1 and NS1 was confirmed as shown in Fig. 7C. These results together indicate that the 217–414 aa domain of HSV-2 US1 is essential for the inhibition of IFN-β production.

The 217–414 aa domain of US1 inhibits IFN-β production in the same manner as full-length US1

Because US1 downmodulates IFN-β production by impeding the association of IRF-3 with IFN-β promoter, it is likely that the 217–414 aa domain of US1 interferes with IFN-β production in the same manner as full-length US1. Subsequent experiments were conducted to examined the roles of US1 (217–414 aa). In indirect immunofluorescence assay, HeLa cells were transfected with empty vector, US1 or US1 mutant expression plasmid for 24 h followed by stimulation with SeV for 12 h. As shown in Fig. 8A, SeV-induced IRF-3 translocation was not significantly blocked when cells were transfected with US1, US1 (1–216 aa), or US1

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**FIGURE 7.** The 217–414 aa domain of US1 is essential for the inhibition of IFN-β production. (A) The 217–414 aa domain of US1 inhibits the activation of IFN-β promoter. HEK 293T cells in 24-well plates were cotransfected with 500 ng US1 or truncated US1 expression plasmid or empty vector together with 250 ng p125-Luc and 50 ng pHRL-TK. At 24 h posttransfection, cells were stimulated with or without SeV for 16 h. (B) US1 (217–414 aa) inhibits the production of IFN-β. HEK 293T cells in six-well plates were transfected with 4 μg empty vector, US1, US1 (1–216 aa), or US1 (217–414 aa) expression plasmid for 24 h followed by stimulation with or without SeV for 16 h. The protein level of IFN-β in supernatants was measured by ELISA. *p < 0.05. (C) The expression of US1, US1 (1–216 aa), US1 (217–414 aa), and NS1 was stained using anti-Flag or anti-HA Ab. One representative experiment out of three is shown. IB, immunoblotting; ND, not detected.
(217–414 aa) expression plasmid. The percentage of IRF-3 positive nuclei was quantified in a number of fields (Fig. 8B). In Western blot assay, HEK 293T cells were transfected with empty vector, US1, US1 (1–216 aa) or US1 (217–414 aa) expression plasmid, or cotransfected with IRF-3 expression plasmid and empty vector or plasmid expressing US1, US1 (1–216 aa), or US1 (217–414 aa) for 24 h followed by stimulation with SeV for 16 h. Cytoplasmic and nuclear proteins were subsequently isolated, followed by Western blot (Fig. 8C, 8D) to determine the distribution of IRF-3. As shown in Fig. 8C, SeV-induced IRF-3 translocation was not significantly blocked when cells were transfected with US1 or US1 mutant expression plasmid. As shown in Fig. 8D, US1 (217–414 aa) did not inhibit IRF-3 translocation when IRF-3 was overexpressed. In ChIP assay, HEK 293T cells were cotransfected with IRF-3 expression plasmid and empty vector or plasmid expressing US1, US1 (1–216 aa) or US1 (217–414 aa) for 24 h followed by stimulation with SeV for 16 h. As shown in Fig. 8E, the interaction between IRF-3 and IFN-β promoter was significantly inhibited by US1 and US1 (217–414 aa), but US1 (1–216 aa) had no such effect on the interaction between IRF-3 and IFN-β promoter. The expression of US1, US1 mutants and IRF-3 was confirmed as shown in Fig. 8F. These results collectively indicate the 217–414 aa domain of US1 inhibits IFN-β production in the same manner as full-length US1 by impeding the association of IRF-3 with IFN-β promoter.

**Discussion**

HSV-2 is one of the most common sexually transmitted infections worldwide and the primary cause of genital and neonatal herpes and genital ulcer disease (74). HSV-2 and HSV-1 are closely re-

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**FIGURE 8.** The 217–414 aa domain of US1 inhibits IFN-β production in the same manner as full-length US1. (A) US1 (217–414 aa) does not significantly block the IRF-3 nuclear translocation. HeLa cells in 35-mm dishes were transfected with 2.5 μg empty vector, US1, or US1 mutant expression plasmid and then stimulated with or without SeV for 12 h. Cells were stained with mouse anti-Flag mAb and rabbit anti-IRF-3 pAb, followed by FITC conjugated goat anti-mouse (green) and Cy3-conjugated goat anti-rabbit (red) as the secondary Abs. Cell nuclei (blue) were stained with DAPI. The images were obtained by fluorescence microscopy using 60× objective. (B) The percentage of IRF-3 positive nuclei was quantified in a number of fields. (C and D) The effect of US1 (217–414 aa) on IRF-3 nuclear translocation was determined with Western blot. HEK 293T cells in six-well plates were (C) transfected with 4 μg empty vector, US1, or US1 mutant expression plasmid, or (D) cotransfected with 2.5 μg IRF-3 and 2.5 μg US1 mutant expression plasmids or 2.5 μg empty vector. At 24 h posttransfection, cells were stimulated with or without SeV for 16 h. NS1 was used as a positive control for the blockade of nuclear translocation of IRF-3. Cytoplasmic and nuclear proteins were isolated. PCNA was used as a loading control for nuclear proteins. (E) US1 (217–414 aa) inhibits the association of IRF-3 with IFN-β promoter. HEK 293T cells in six-well plates were cotransfected with 2.5 μg flag-tagged IRF-3 and 2.5 μg HA-tagged US1 or US1 mutant expression plasmid or empty vector for 24 h, followed by stimulation with or without SeV for 16 h. Cells were lysed and subjected to ChIP assay using mouse anti-Flag mAb, mouse anti-RNA polymerase II mAb (positive control), or mouse normal IgG (negative control) for immunoprecipitation. (F) The expression of IRF-3, US1, and US1 mutants was stained using anti-Flag or anti-HA Ab. Densitometric analysis of p-IRF-3 level in nucleus was performed using Image J software. One representative experiment out of three is shown. N, nuclear.
lated, and both can establish latency in sensory ganglia and re-
currently reactivate to cause diseases, but they exhibit substantial
differences in latency and reactivation patterns (75). A large
number of studies have demonstrated that HSV-1 evolved multiple
countermeasures to subvert the production of type I IFNs (67, 71,
76–80). However, apart from the report that HSV-2 virion host
shutoff (vhs) protein can suppress IFN-β expression (81), current
understanding of HSV-2 immune evasion against the production
of type I IFNs is limited.

In the current study, we demonstrated that HSV-2 infection
inhibited the activity of IFN-β promoter and downregulated the
production of IFN-β at protein level. In addition, we found that
UV-inactivated HSV-2 was unable to inhibit IFN-β production,
suggesting that such inhibition requires productive HSV-2 infec-
tion. To identify HSV-2 components that contribute to the sup-
pression of IFN-β production, we initially focused on the firstly
expressed IE proteins. We found that HSV-2 US1 markedly sup-
pressed IFN-β induction, whereas HSV-1 US1, which shares
~60% of amino acid sequence with HSV-2 US1, did not have such
inhibitory effect. To our knowledge, our findings demonstrate for
the first time a countermeasure exploited by HSV-2 US1 to subvert
the production of IFN-β. We found that, similar to productive
HSV-2 infection, HSV-2 US1 inhibited the activity of IFN-β
promoter and suppressed the production of IFN-β at both mRNA
and protein levels. Moreover, US1 knockout significantly impaired
the inhibitory activity of HSV-2 on IFN-β production. Given the
complexity of HSV-2 genome containing over 70 genes, we
cannot rule out the involvement of other HSV-2 components that
might contribute to the suppression of IFN-β production. Indeed,
we observed that US1 del HSV-2 did not completely lose the in-
hibitory activity on IFN-β induction and that the IE proteins RL2
and UL54 also inhibited the activation of IFN-β promoter to
certain degrees. Similar findings were previously observed on RL2
(ICP0) and UL54 (ICP27) of HSV-1 (13, 23, 65–68). Moreover,
HSV-2 vhs has also been reported to play a role in inhibiting IFN-β expression via suppressing IRF-3 activation (82). We demonstrate in this study that HSV-2 US1 does not inhibit IRF-3 activation, but instead antagonizes the production of IFN-β by suppressing the association of IRF-3 with IFN-β promoter, which may be one of the strategies used by HSV-2 to counteract the host innate immune responses.

To date, most of the studies on US1 have focused on HSV-1. Little is known about the functions of HSV-2 US1. HSV-1 US1 is an IE protein and a multifunctional viral regulator. For example, HSV-1 US1 interacts with RNA polymerase II (83–89) and P-TEFb (90) to regulate viral replication (91). HSV-1 US1 is glycosylated, adenylated, and phosphorylated, and at least part of these post-translational modifications involve the viral protein kinases encoded by UL13 and US3 and casein kinase II (92–95). Despite intensive investigation of HSV-1 US1, there is no suggestion of its involvement in evading type I IFN signaling pathway. Indeed, we found that HSV-1 US1 had no inhibitory effect on IFN-β induction. Unlike HSV-2, HSV-1 appears to use other viral components, including vhs, ICP0, ICP27, ICP34.5, VP16, US3, and US11, to counteract host innate antiviral responses (68, 71, 76–82, 96, 97).

In the current study, we have demonstrated a novel function of HSV-2 US1, showing that HSV-2 US1 inhibits the production of IFN-β. Although other functions of HSV-2 US1 are beyond the scope of this study, future work is warranted to explore them. IRF-3 plays a key role in type I IFN-mediated antiviral immune response (98, 99). Both DNA and RNA viruses have evolved strategies to evade innate immune responses by interfering with IRF-3. For example, HCV suppresses host antiviral response by inhibiting the dimerization and phosphorylation of IRF-3 (100). Varicella zoster virus IE protein ORF61 abrogates IRF-3-mediated innate immune response through degradation of activated IRF-3 (22). Ebola virus VP35 protein inhibits the induction of antiviral genes by blocking the activation of IRF-3 (24). HSV-1 ICP0, ICP27, and US3 also block IRF-3-mediated antiviral response via interacting with IRF-3 (71, 78, 80). We hypothesized that HSV-2 US1 might inhibit the IRF-3-mediated signaling pathway, leading to the suppression of IFN-β production. Indeed, we found that HSV-2 US1 inhibited PRD(III-I)-responsive promoter activity, and blocked RIG-IN–, IPS-1–, TBK-1–, IKK-ε–, or IRF-3/5D-induced activation of IFN-β promoter activity in a dose-dependent manner, indicating that US1 inhibits IFN-β production probably depending on the downstream of IRF-3 activation.

Activation of IRF-3 during IFN-β production includes several key steps: phosphorylation, dimerization, and cytoplasm-to-nucleus translocation. Of interest, we found that, unlike the mechanisms used by other viruses, HSV-2 US1 did not significantly suppress the phosphorylation and nuclear translocation of IRF-3, but it appeared to function after IRF-3 translocation from the cytoplasm into the nucleus. Indeed, our data collectively indicated that HSV-2 US1 suppresses IFN-β production via a physical association of US1 with IRF-3. In agreement, we observed that HSV-2 US1 was mainly localized in the nucleus as evidenced by immunofluorescence assay. IRF-3 consists of 427 aa, including a DNA binding domain (aa 1–112) responsible for DNA binding and an IAD (aa 197–394) responsible for IRF-3 phosphorylation, dimerization, and interaction with CBP/p300 coactivators (32). By analyzing a series of truncated IRF-3 mutants, we demonstrated that HSV-2 US1 inhibited the association of IRF-3 with IFN-β promoter by interacting with the DNA binding domain of IRF-3, which is crucial for the activation of IFN-β promoter. After assessing US1 mutants, we further revealed that the 217-414 domain of US1 inhibited IFN-β production in the same manner as full-length US1. To date, only a few viral components, including human bocavirus nucleoprotein NP-1 and Kaposi sarcoma–associated herpesvirus latency-associated nuclear Ag, have been reported to interfere with the binding of activated IRF-3 to IFN-β promoter (31, 32). We demonstrate in this study that HSV-2 US1 blocks the production of IFN-β by directly suppressing the association of IRF-3 with IFN-β promoter.

Although other mechanisms likely exist, we proposed one molecular model based on the RIG-I signaling pathway as described in Fig. 9. HSV-2 infection yields a number of by-products such as viral dsRNA, which can be recognized by RIG-I receptor. RIG-I is a cytoplasmic helicase containing two N-terminal caspase activation and recruitment domains and a C-terminal DExD/H-Box RNA helicase domain. It binds to dsRNA through the helicase domain and signals through caspase activation and recruitment domains to the adaptor IPS-1. Engagement of RIG-I initiates signaling through two protein kinase complex, TBK-1 and IKK-ε, leading to the phosphorylation and dimerization of IRF-3 (101, 102). IRF-3 dimers translocate from the cytoplasm into the nucleus to bind to the IFN-β promoter and to boost IFN-β transcription (16). Our data indicate that HSV-2 US1 downmodulates IFN-β production by suppressing the association of nuclear IRF-3 with the IRF-3–responsive domain of the IFN-β promoter. Ultimately, the production of IFN-β is suppressed, which likely contributes to HSV-2 immune evasion. Findings in our study provide one molecular explanation for the suppression of type I IFN production by HSV-2.

In conclusion, based on our observation that productive HSV-2 infection suppresses IFN-β production, we demonstrate that HSV-2 US1 inhibits the production of IFN-β by suppressing the activation of IFN-β promoter by interfering with the association of nuclear IRF-3 with IRF-3–responsive domain of the IFN-β promoter. We also reveal that the 217–414 aa domain of US1 is the functional region of US1 to block IFN-β production. Our findings highlight the significance of HSV-2 US1 in inhibiting IFN-β production, providing a novel mechanism for the evasion of host innate immunity by HSV-2.

Disclosures
The authors have no financial conflicts of interest.

References
3114 HSV-2 US1 INHIBITS IFN-β PRODUCTION


This page contains multiple references to various studies and articles, mostly related to herpes simplex virus (HSV). The text includes references to the infection, spread, and mechanisms of HSV-1, including its immediate-early ICP0 protein and its impact on the immune system. The text also references studies on the expression of HSV-1 tegument protein VP16 and its role in blocking interferon regulatory factor-3 (IRF-3) and IRF-7 in type I interferon-mediated control of murine microglial digestion. The text further discusses the role of HSV-1 in the accumulation of alpha and gamma mRNAs and the impact of HSV-1 on cellular mechanisms such as the secretion of a type I interferon-antagonizing protein and the inhibition of transcription of the beta interferon gene by the human herpesvirus 6 immediate-early 1 protein. The text also references studies on the potential role in blocking IFN-beta induction and the spread of HSV-1 to the spinal cord.

The following references are cited in the text: