THE INTERPLAY BETWEEN INNATE IMMUNITY AND HERPESVIRUSES

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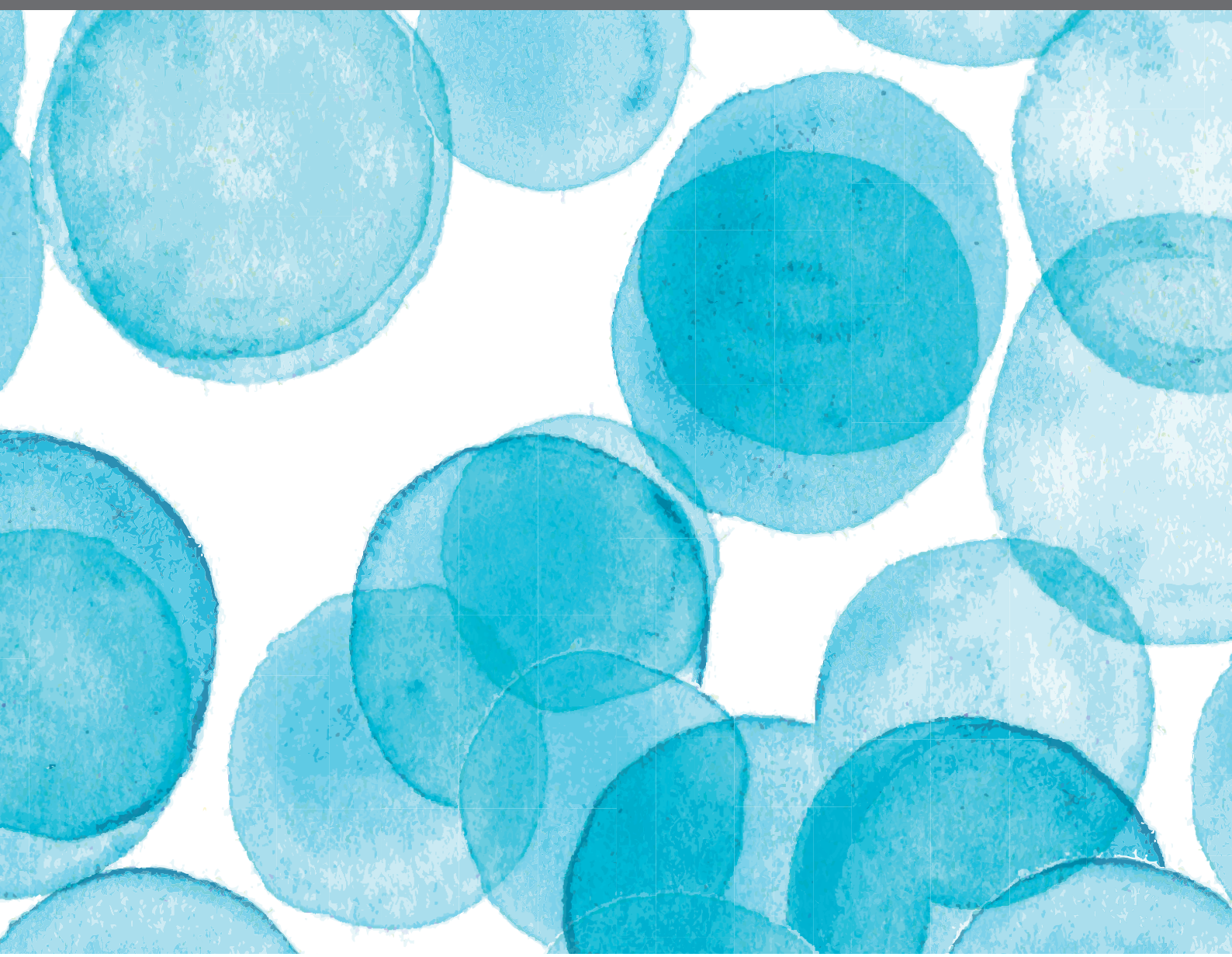

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THE INTERPLAY BETWEEN INNATE IMMUNITY AND HERPESVIRUSES

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Extracellular Vesicles From KSHV-Infected Cells Stimulate Antiviral Immune Response Through Mitochondrial DNA

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Kaposi's Sarcoma-associated herpesvirus (KSHV) is the etiologic agent of Kaposi's sarcoma, which is the most common cancer in acquired immune deficiency syndrome patients. KSHV contains a variety of immunoregulatory proteins. There have been many studies on the modulation of antiviral response by these immunoregulatory proteins of KSHV. However, the antiviral effects of extracellular vesicles (EVs) during *de novo* KSHV infection have not been investigated to our best knowledge. In this study, we showed that KSHV-infected cells induce interferon-stimulated genes (ISGs) response but not type I interferon in uninfected bystander cells using EVs. mRNA microarray analysis showed that ISGs and IRF-activating genes were prominently activated in EVs from KSHV-infected cells (KSHV EVs)-treated human endothelial cells, which were validated by RT-qPCR and western blot analysis. We also found that this response was not associated with cell death or apoptosis by virus infection. Mechanistically, the cGAS-STING pathway was linked with these KSHV EVs-mediated ISGs expressions, and mitochondrial DNA on the surface of KSHV EVs was one of the causative factors. Besides, KSHV EVs-treated cells showed lower infectivity for KSHV and viral replication activity than mock EVs-treated cells. Our results indicate that EVs from KSHV-infected cells could be an initiating factor for the innate immune response against viral infection, which may be critical to understanding the microenvironment of virus-infected cells.

Keywords: extracellular vesicles, interferon-stimulated gens, innate immunity, KSHV, virus, antiviral response

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INTRODUCTION

Cells release vesicles of varying sizes both via the endosomal pathway and by budding from the plasma membrane. These vesicles are referred to by various names, such as exosomes, microvesicles, microparticles, and apoptotic bodies, collectively termed extracellular vesicles (EVs) (1). EVs are a heterogeneous collection of membrane-bound carriers with complex cargoes including proteins, lipids, and nucleic acids, which work as crucial players in intercellular communication (2).

In many aspects, EVs resemble viruses, especially an enveloped virus (3). Their size and structure share similar features. Both are surrounded by a lipid membrane that also contains cell membrane proteins. EVs carry genetic material, which can change the functions of the recipient cells. Apparently, unlike viruses, EVs do not cause infection and replication. However, increasing

evidence indicates that EVs from virus-infected cells affect immune response during viral infection. Dreux et al. reported that EVs released from Hepatitis C virus-infected cells can induce interferon (IFN)-α release from uninfected plasmacytoid dendritic cells due to the viral RNA present within the EVs (4).

Type I IFN and interferon-stimulated genes (ISGs) are indispensable for vertebrates to control viral infection (5, 6). Induction of type I IFN gene expression is tightly regulated. Generally, primary *de novo* viral infection and reactivation from latency elicit a host antiviral immune response. However, Kaposi's sarcoma-associated herpesvirus (KSHV), the etiologic agent of Kaposi's sarcoma, has multiple mechanisms to block type I IFN response (7–9). Especially, various tegument proteins in the virion work on antagonizing type I IFN response from the viral entry stage. Indeed, a previous study showed that KSHV induced little or very weak antiviral response during *de novo* infection (10). However, the antiviral effect in bystander cells during *de novo* KSHV infection has not been investigated so far. In this study, we demonstrated that EVs from KSHV-infected cells (KSHV EVs) can induce ISGs but not type I IFNs in human endothelial cells through the cGAS-STING pathway. EVs were isolated prior to virion production from KSHV-infected cells, and cell death or apoptosis was not observed at this time. We showed that mitochondrial DNA on EVs was one of the associated-factors inducing ISG expression. These results are important to understand the microenvironment of virus-infected cells because currently, little is known regarding the fact that virus-infected cells induce antiviral responses in bystander cells using independent mechanisms from type I IFN. Furthermore, we found that *de novo* infection of KSHV and human herpes simplex virus type 1 are partially blocked in KSHV EVs-pretreated cells.

MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Allendale, NJ) and cultured in endothelial cell growth medium-2 (EGM-2; Lonza) bullet kit. Vero cells and lenti-X-293T cells were obtained from Korean Cell Line Bank (Seoul, South Korea) and Takara (Otsu, Japan), respectively. They were cultured in Dulbecco's modified Eagle's medium (DMEM; GE Healthcare, Little Chalfont, UK) supplemented with 10% fetal bovine serum (FBS; Wellgene, Seoul, South Korea) and 1% antibiotics (Lonza). The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C according to the manufacturer's instructions. An absence of contamination of mycoplasma in all cultured cells was tested by mycoplasma detecting PCR every month as described previously (11).

Virus Isolation and Infection

iSLK BAC16 cells harboring recombinant KSHV BAC16 were used as the source of the virus, as described previously (12). Infectious KSHV BAC16 virions from iSLK BAC16 cells were induced by treatment with doxycycline and sodium butyrate for 3 days. The culture supernatant was collected, filtered through a 0.22 μm filter, and centrifuged at 100,000 × g for 1 h. The pellet was resuspended in phosphate-buffered saline (PBS) and stored at −70°C as infectious viral particles. HUVECs were infected with KSHV according to methods used in a previous study (13).

Affymetrix Whole Transcript Expression Array Analysis

The Affymetrix whole transcript expression array process was executed according to the manufacturer's protocol (GeneChip Whole Transcript PLUS reagent Kit, Thermo Scientific, Waltham, MA). cDNA was synthesized using the GeneChip WT (Whole Transcript) Amplification kit as described by the manufacturer. The sense cDNA was then fragmented and biotin-labeled with TdT (terminal deoxynucleotidyl transferase) using the GeneChip WT Terminal Labeling kit. Approximately 5.5 μg of labeled DNA target was hybridized to the Affymetrix GeneChip Human 2.0 ST Array at 45°C for 16 h. Hybridized arrays were washed and stained on a GeneChip Fluidics Station 450 and scanned on a GCS3000 Scanner (Affymetrix, Santa Clara, CA). Signal values were computed using the Affymetrix® GeneChip™ Command Console software. Six most significant biofunctions were identified using Ingenuity Pathways Analysis (Ingenuity Systems; www. Ingenuity.com). Data are based on transcripts differentially expressed in mock- or KSHV-infected cells-derived EV-treated HUVECs. The *P*-value indicates the likelihood that an association of the specific set of transcripts with the indicated process or pathway is the result of random chance. B-H *P*-value indicates *P*-values after Benjamini-Hochberg correction for multiple comparisons.

Isolation of EVs by Differential Centrifugation

For EVs isolation, HUVECs were incubated in EGM-2 for 24 h. Cell supernatants were collected and centrifuged at 2,000 × g for 10 min to remove cells, followed by filtration through a 0.22-μm pore filter (Sartorius, Göttingen, Germany) to remove cell debris. The collected supernatant was then ultracentrifuged at 100,000 × g for 60 min, and the precipitate was resuspended with PBS.

Nanoparticle Tracking Analysis

The size distribution and concentration of EVs were determined by NTA, using a ZetaView (Particle Metrix GmbH, Meerbusch, Germany). Preparations of EVs were diluted in PBS and passed through 0.22 μm filters before the analysis. The analysis parameters were as follows: max size 200, min size 20, brightness 20, sensitivity 75, and temperature 25°C.

Quantitative Real-Time Reverse Transcription PCR (RT-qPCR)

Total RNA from cells was isolated by NucleoSpin RNA II as recommended by the manufacturer (MACHEREY-NAGEL Inc., Bethlehem, PA). Total RNA was reverse-transcribed to obtain the first-strand cDNA using the ReverTra Ace qPCR RT kit (TOYOBO CO, Osaka, Japan). Real-time PCR was performed using the SYBR® FAST qPCR mix (Takara). The cycling conditions were as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 10 s. The specificity of the amplified products was confirmed by analyzing the melting curves. All

samples were tested in triplicates and normalized by β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers were synthesized by Genotech (Daejeon, South Korea) and their sequences are described in **Table 1**.

Western Blotting

Western blotting was performed as previously described (14) with minor modifications. Cellular proteins were isolated using 1 × RIPA buffer containing a protease inhibitor and a phosphatase inhibitor. The proteins were resolved by electrophoresis in a 10–15% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (GE Healthcare). The membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20. Rabbit monoclonal anti-STING (Cell Signaling Technology, Beverly, MA), Rabbit monoclonal anti-cGAS (Cell Signaling Technology), rabbit polyclonal anti-Rab27b (Bioss Antibodies Inc., Woburn. MA), mouse monoclonal anti-KSHV ORF65 (14), rabbit polyclonal anti-GAPDH (Cusabio, Houston, TX), rabbit polyclonal anti-calnexin (Bioss Antibodies Inc.), mouse monoclonal anti-HDAC1 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-mtTFA (Santa Cruz Biotechnology), rabbit polyclonal anti-MX1 (Bioss Antibodies Inc.), rabbit polyclonal anti-IFIT1 (Bioss Antibodies Inc.), rabbit polyclonal anti-IFIT44L (Bioss Antibodies Inc.) and mouse monoclonal anti-β-actin antibodies (Sigma, St. Louis, MO) were used as primary antibodies. HRP-conjugated anti-rabbit or anti-mouse antibodies (Bethyl Laboratories Inc., Montgomery, TX) were used as secondary antibodies. The results were visualized using an ECL detection reagent (Bio-Rad, Hercules, CA).

ELISA for Type I Interferon

Mock EVs or KSHV EVs added to HUVECs and incubate for 24 h, followed by isolating the culture supernatant. Type I interferon in the culture supernatant was analyzed by human interferon α and β ELISA kit (Cusabio) according to the manufacturer's instructions.

Immunofluorescence Assay (IFA)

IFA was performed as previously described (14). A mouse monoclonal antibody to ORF65 was used for tracking of KSHV particles. Infection of KSHV was analyzed by detection of LANA using a rat monoclonal antibody to KSHV ORF73 (Abcam, Cambridge, MA).

Tracking of EVs by Fluorescent Labeling

For fluorescent labeling of the EV membrane, Exo-Glow (System Bioscience, Palo Alto, CA), acridine orange nucleic acid-selective fluorescent dye was added to the purified EV according to the manufacturer's instructions. After EV membranes were fluorescently labeled, ultracentrifugation was performed at 100,000 × g for 60 min to remove the unlabeled dye. The labeled EVs were then added to HUVECs. After 4–8 h of incubation, the cells were gently washed with 1 × PBS and analyzed by flow cytometry or fluorescence microscopy.

Flow Cytometry

Flow cytometry experiments were performed to assess the infectivity of KSHV, apoptosis, and tracking of labeled EVs. Cells suspended in 1% FBS/PBS were analyzed using a Guava easyCyte Flow Cytometer and the InCyte 3.1 software (Merck Millipore, Bedford, MA). For apoptosis assay, FITC Annexin V apoptosis detection kit (BD Bioscience, San Jose, CA) was used as recommended by the manufacturer's instruction.

LDH Release Assay

Media from mock- or KSHV-infected HUVEC cells at 8 h of postinfection was isolated and centrifuged at 300 × g for 3 min. Cytotoxicity detection kit plus LDH (Roche, Mannheim, Germany) was used to measure lactate dehydrogenase (LDH) released from dead cells. The prepared culture media was added to the same volume of LDH reagent and incubated for 30 min in the dark. The absorbance was measured at 490 and 650 nm by a microplate reader (Molecular Devices, Silicon Valley, CA).

Lentivirus Infections

Plasmids containing shRNAs for human Rab27b (TRCN0000293978 and TRCN0000294016, Sigma), STING (TRCN0000163029, TRCN0000163296, Sigma), cGAS (TRCN0000428336, TRCN0000128706, Sigma), or a scramble shRNA (#1864, Addgene, Cambridge, MA) were co-transfected with pPACKF1 packaging plasmid mix (System Bioscience) into Lenti-X-293T cells (Takara) using Lipofectamine 3000 transfection reagent (Thermo Scientific) as per the manufacturer's recommendations. HUVECs were infected with viral supernatants from 293T cells along with polybrene (5 μg/mL) for 24 h. After 10 days of selection with puromycin (0.5 μg/mL), the efficiency of knockdown was evaluated by western blotting.

Analysis of Virion DNA of KSHV and Mitochondrial DNA (mtDNA)

The supernatants of KSHV-infected HUVECs were collected and centrifuged at 100,000 × g for 1 h. For detect virion DNA, the pellet was resuspended in 1 × DNase buffer and treated by RQ1 RNase-free DNase I (Promega, Madison, WI) at 37°C for 1 h. DNA was extracted from DNAase-treated virion or EVs using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Real-time PCR analysis was carried out using the SYBR® FAST qPCR mix (Takara) with primers in **Table 1**. KSHV ORF26 and NADH sub1/5 was amplified to analyze virion DNA and mtDNA, respectively. The cycling conditions were as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 10 s.

Cell Viability Assay

Cell viability was measured by the WST-1 cell proliferation reagent (Roche) according to the manufacturer's protocol. Briefly, WST-1 reagent was added into cells on 96-well culture plate (1:10) and incubated for 90 min in a humidified atmosphere of 5% CO_2 at 37°C. Absorbance at 450 nm was measured with the reference wavelength set at 650 nm.

TABLE 1 | List of primers used for PCR.

Gene	Sense primer	Antisense primer	Amplicon
IFI44L	ATC TCT GCC ATT TAT GTT GT	GTA GAA TGC TCA GGT GTA AT	153 bp
IFIT1	AAT AGA CTG TGA GGA AGG A	ATA GGC AGA GAT CGC ATA	139 bp
MX1	CAG GAC TAC GAG ATT GAG AT	GTT ATG CCA GGA AGG TCT A	170 bp
GAPDH	GGT ATC GTG GAA GGA CTC	GTA GAS GCA GGG ATG ATG	91 bp
β-actin	AGA GCT ACG AGC TGC CTG AC	AGC ACT GTG TTG GCG TAC AG	164 bp
IFN-α	AAT GCG GAC TCC ATC TTG	GGG CTG TAT TTC TTC TCT GT	130 bp
IFN-β	CAT TAC CTG AAG GCC AAG GA	CAG CAT CTG CTG GTT GAA GA	147 bp
tRNA-LEU(UUR)	CAC CCA AGA ACA GGG TTT GT	TGG CCA TGG GTA TGT TGT TA	107 bp
β2-microglobulin	TGC TGT CTC CAT GTT TGA TGT ATC T	TCT CTG CTC CCC ACC TCT AAG T	86 bp
IFIT1	TAG AAC AGG CAT CAT TAA CAA G	CTC CAG GGC TTC ATT CAT A	152 bp
IFIT3	GAC TGA ATC CTC TGA ATG C	CCT TAT TGA ATG GTG TCT GAT	78 bp
OAS1	TCA GTC AGC AGA AGA GAT AA	CAA TGA ACT TGT CCA GAG ATT	118 bp
cGAS	CCT GCT GTA ACA CTT CTT AT	TAG TCG TAG TTG CTT CCT AA	147 bp
NADH sub1	TTC TAA TCG CAA TGG CAT TCC T	AAG GGT TGT AGT AGC CCG TAG	146 bp
NADH sub5	TTC ATC CCT GTA GCA TTG TTC G	GTT GGA ATA GGT TGT TAG CGG TA	184 bp
IFI44	CGG TAA CAT TCG TGA TAG ATA	TCT GAG AGG AGA AGT ATT GA	152 bp
ISG15	GCA GAT CAC CCA GAA GAT	CCT TGT TAT TCC TCA CCA G	182 bp
KSHV ORF26	GGA GAT TGC CAC CGT TTA	ACT GCA TAA TTT GGA TGT AGT C	93 bp

In vitro Antiviral Assay and Plaque Formation Assay

HUVECs were pretreated with or without 2-fold serial dilution of IFN-α starting from 1,000 to 1.8 U/mL for 24 h. HSV-1 at a multiplicity of infection (MOI) of 0.1–64 was added to the medium containing the cells using opti-MEM (Thermo Scientific) for 1 h at 37°C. Viral supernatant was then removed, and the cells were refreshed with complete medium. The medium was removed 48 h of post infection and cells were fixed with 10% formaldehyde solution for 20 min at room temperature. After fixation, cells were visualized with 0.4% crystal violet. The excessive dye was then removed by immersing the plate in PBS. Each treatment was performed in duplicate. For plaque formation assay to measure the MOI of HSV-1, different dilutions of supernatant from virus-infected cells were used to infect Vero cells in opti-MEM for 1 h, followed by overlaying 2% FBS in DMEM containing 1% agarose (Bio-Rad) to immobilize the virus. After 24 h, cells were fixed and visualized with crystal violet, and the plaques were enumerated.

Statistical Analysis

Results are shown as the mean ± standard deviation. The two-tailed Student's *t*-test was used to assess the statistically significant difference between groups. Statistical significance at $P < 0.05$ and < 0.01 is indicated by * and **, respectively.

RESULTS

EVs From KSHV-Infected Cells Stimulate the Expression of ISG-Related Transcripts

In the previous study, we isolated EVs from KSHV-infected human endothelial cells at 24 h of postinfection and characterized them (15). A schematic isolation process for EVs is presented as **Figure 1A**. Previously, EVs were analyzed by western blotting, nanoparticle tracking analysis, and electron microscopy. EVs-related proteins including CD81, CD63, and HSP70 were detected in EVs by western blot. We confirmed that these EVs were not contaminated with KSHV virions. In this study, we tried to investigate the influence of KSHV EVs on uninfected bystander cells. The isolated EVs from mock-infected (mock EVs) or KSHV-infected cells (KSHV EVs) were treated with naïve HUVECs for 24 h. Using a microarray, the differential expression of transcripts was analyzed with two sets of RNA samples independently prepared from EV-treated cells (**Figure 1B**). Gene expression profiling of KSHV EV-treated HUVECs revealed an enrichment of ISGs and antiviral signaling factors (**Figures 1C,D**). We observed increased expression of ISGs with direct antiviral activity (IFIT1, IFIT3, IFITM1, MX1, and OAS1) and positive regulators (cGAS, IRF4, IRF9, Stat1, and Stat2) reinforcing the antiviral response.

To validate the microarray results, the mRNA expression of ISGs in KSHV EVs-treated HUVECs were analyzed by RT-qPCR analysis (**Figure 2A**). Although there were some variations in differences between mock- and KSHV EVs-treated cells, the eight ISGs that were analyzed showed significant differences. We also validated the protein expression of IFIT1, MX1, IFI44L, and cGAS (**Figure 2B**). As ISGs have known to be induced by type I interferons (IFNs), we analyzed the expression of type I IFNs by KSHV EVs. After HUVECs were treated with mock EVs or KSHV EVs for 24 h, IFN-α and IFN-β in their supernatant were analyzed by ELISA (**Figure 2C**). A significant increase of type I IFNs was not observed in KSHV EVs-treated HUVECs compared to mock EVs-treated cells. A previous study showed that *de novo* KSHV infection suppressed the type I IFNs response by tegument proteins, ORF45, in KSHV (10). Interestingly,

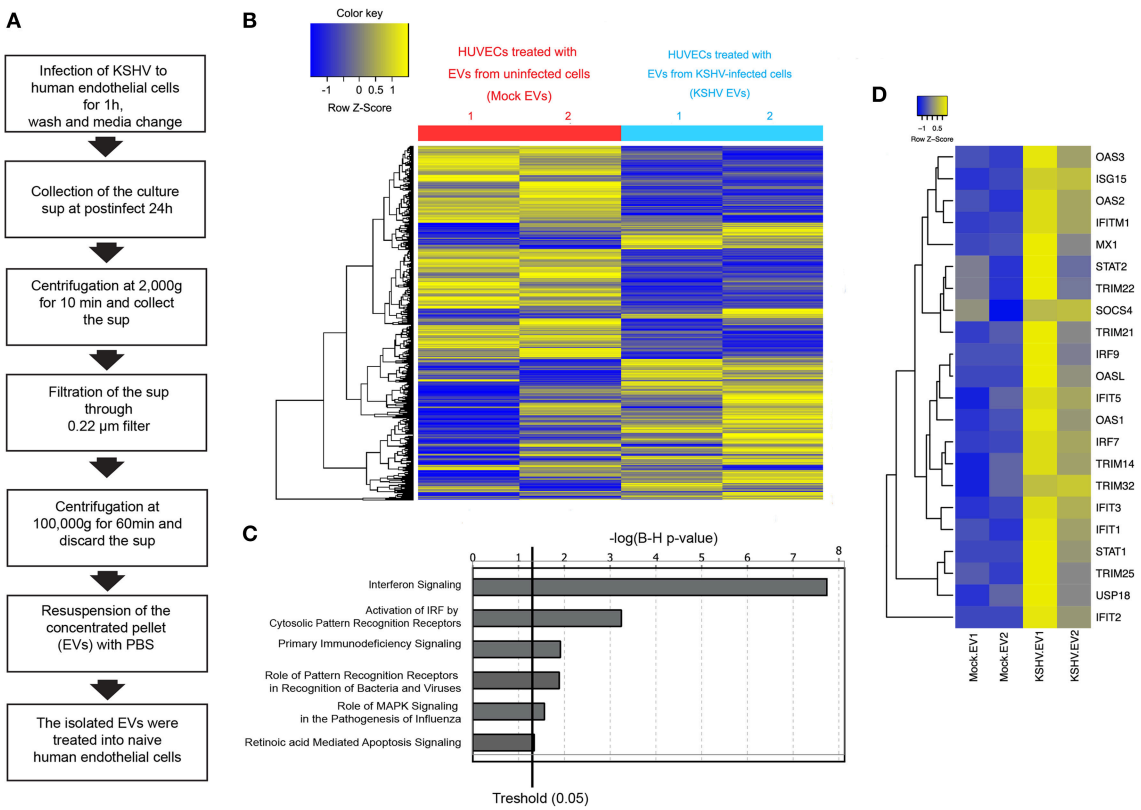

FIGURE 1 | Microarray analysis for mRNA expression in human endothelial cells treated with EVs from mock- and KSHV-infected cells. **(A)** Schematic experimental processes of extracellular vesicle (EV) isolation from Kaposi's sarcoma-associated herpesvirus (KSHV)-infected human umbilical cord vein endothelial cells (HUVECs). Isolated EVs were treated to uninfected HUVECs, followed by analyzing for mRNA expression by microarray. **(B)** Hierarchical clustering analysis of mRNA levels in HUVECs treated with mock- vs. KSHV-infected cells-derived EVs (mock EVs vs. KSHV EVs). **(C)** Altered cell function and signal pathways in KSHV EVs-treated HUVECs as assessed by microarray analysis. **(D)** Heatmap for Interferon Stimulating Genes (ISGs) based on transcripts differentially expressed in mock- or KSHV-infected cells-derived EVs-treated HUVECs.

ISGs were highly upregulated in KSHV-infected cells at 24 h of postinfection (**Supplementary Figure 1**) in our results. These results suggest that KSHV EVs- or KSHV-infection-mediated ISG response might have an independent mechanism from type I IFN response of human endothelial cells.

Stimulation of ISG Expression by KSHV EVs Is Not Associated With a Virus or a Product From Cell Death

In our initial study design, we isolated KSHV EVs at 24 h of postinfection and treated them with HUVECs. To determine the time taken to release an effective EV for ISGs after KSHV infection, KSHV EVs were isolated at various time points after KSHV infection (**Figures 3A,B**). Interestingly, 4 h after KSHV infection was enough for the isolated KSHV EVs to induce ISG expression, confirming that the induction of ISGs would not be associated with KSHV because KSHV is generally produced 48 h of postinfection (16). To confirm the presence of viral nucleic acids and proteins, KSHV ORF26 was amplified from KSHV EVs by PCR, and KSHV envelope protein, ORF65, was analyzed by western blotting (**Supplementary Figure 2A**). We could not detect KSHV DNA or viral protein in KSHV EVs. Furthermore, viral particles or viral gene expressions in KSHV-infected or KSHV EVs-treated cells were also investigated. As expected, viral particles or viral gene expressions were not detected in KSHV EVs-treated HUVECs (**Supplementary Figures 2B–E**). These results showed that KSHV EVs did not contain KSHV virion, suggesting KSHV EVs alone can cause ISG response without the virus. Some previous studies showed that EVs from apoptotic cells could induce inflammation by their harboring proteins or nucleic acids. For example, apoptotic bodies from endothelial cells contained IL-1α (17) and EVs from apoptotic T cell blasts triggered the secretion of IFN-α in plasmacytoid dendritic cells (18). Since ISGs might be stimulated by apoptosis or cell death, apoptosis and cell death in KSHV-infected HUVECs was analyzed at 8 h of postinfection, which was the highest time point of ISG expression. We could not find significantly increased apoptosis or cell death by KSHV infection in Annexin V/PI staining (**Figures 3C,D**). LDH release in the culture supernatant was also analyzed at the same time point (**Figure 3E**). In KSHV-infected cells, LDH release was not increased at all compared

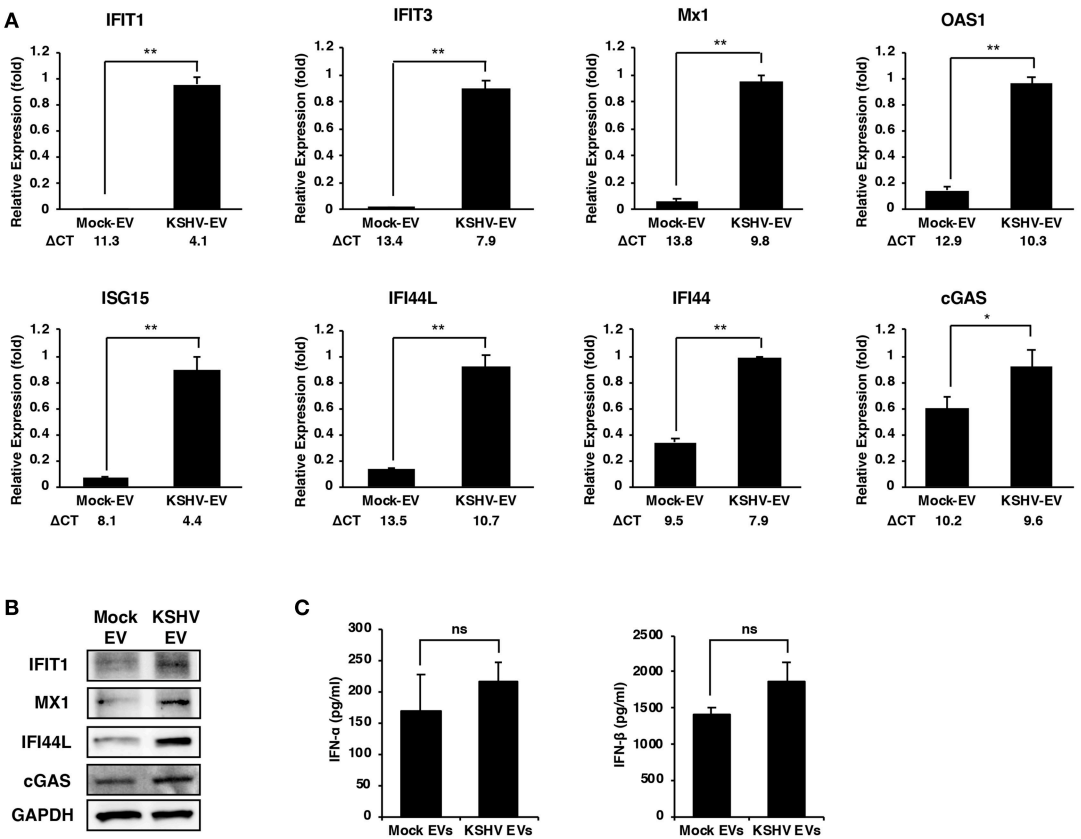

FIGURE 2 | Increased expression of ISGs in human endothelial cells by EVs from KSHV-infected cells. **(A,B)** mRNA and protein expression of the indicated ISGs in mock- or KSHV EVs-treated HUVECs were analyzed by RT-qPCR **(A)** and western blotting **(B)**, respectively. ΔCT indicated the normalized CT value of ISGs with reference gene, β-actin. The grouping of blots cropped from different gels and full-length blots are included in a **Supplementary Figure 4**. Data are shown as the mean ± SD, $n = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$. **(C)** Analysis for IFN-α and IFN-β in the supernatant from mock- or KSHV EVs-treated HUVECs by ELISA. Data are shown as the mean ± SD, $n = 4$, ns: not significant.

to mock-infected cells (**Figure 3E**). Taken together, our results indicated that the induction of ISGs by KSHV EVs would not be caused by a product from apoptosis or cell death during KSHV infection.

Entry of KSHV EVs Is More Prominent Than Mock EVs

To investigate whether KSHV EVs were taken up by HUVECs, EVs were stained with a fluorescence dye, Exoglow. Then, the labeled EVs were treated to HUVECs, followed by analyzing their entry by flow cytometry and fluorescent microscopy (**Figures 4A,B**). Compared to mock EVs, KSHV EVs-treated cells showed higher fluorescence intensity in flow cytometry (**Figure 4A**). More particles of EVs were also detected in the microscopic analysis (**Figure 4B**). In nanoparticle tracking analysis, an overall 10-fold higher number of particles was detected in EVs from KSHV-infected cells than those from mock-infected cells (**Figure 4C**). Therefore, increased entry of EVs in KSHV EV-treated cells may be caused by the larger quantity of EVs.

Induction of ISGs Is Specifically Mediated by KSHV EVs

To confirm whether KSHV EVs-mediated ISGs response is not stimulated by cytokines or small proteins from KSHV-infected cells, the conditioned medium containing KSHV-infected cells was separated into high molecular weight (HMW) proteins and low molecular weight (LMW) proteins using centrifugal filter device, Amicon ultra-2 100 kDa. Each of them added to uninfected HUVECs, and the mRNA expression of IFIT1, a representative ISG, was analyzed (**Figure 5A**). While KSHV HMW proteins-treated cells showed highly upregulated IFIT1, KSHV LMW proteins did not induce its expression. These results are consistent with the previous results with EVs isolated by differential centrifugation (**Figures 1**, **2**), suggesting that KSHV EVs-mediated ISGs expression would not be mediated by small-sized proteins including cytokines but by EVs or large-sized proteins. To investigate whether KSHV EV-mediated induction of ISGs depended on the amount of EVs, serially diluted EVs were treated with HUVECs. A dose-dependent decrease of IFIT1 expression was observed in KSHV EV-treated cells (**Figure 5B**), indicating that our

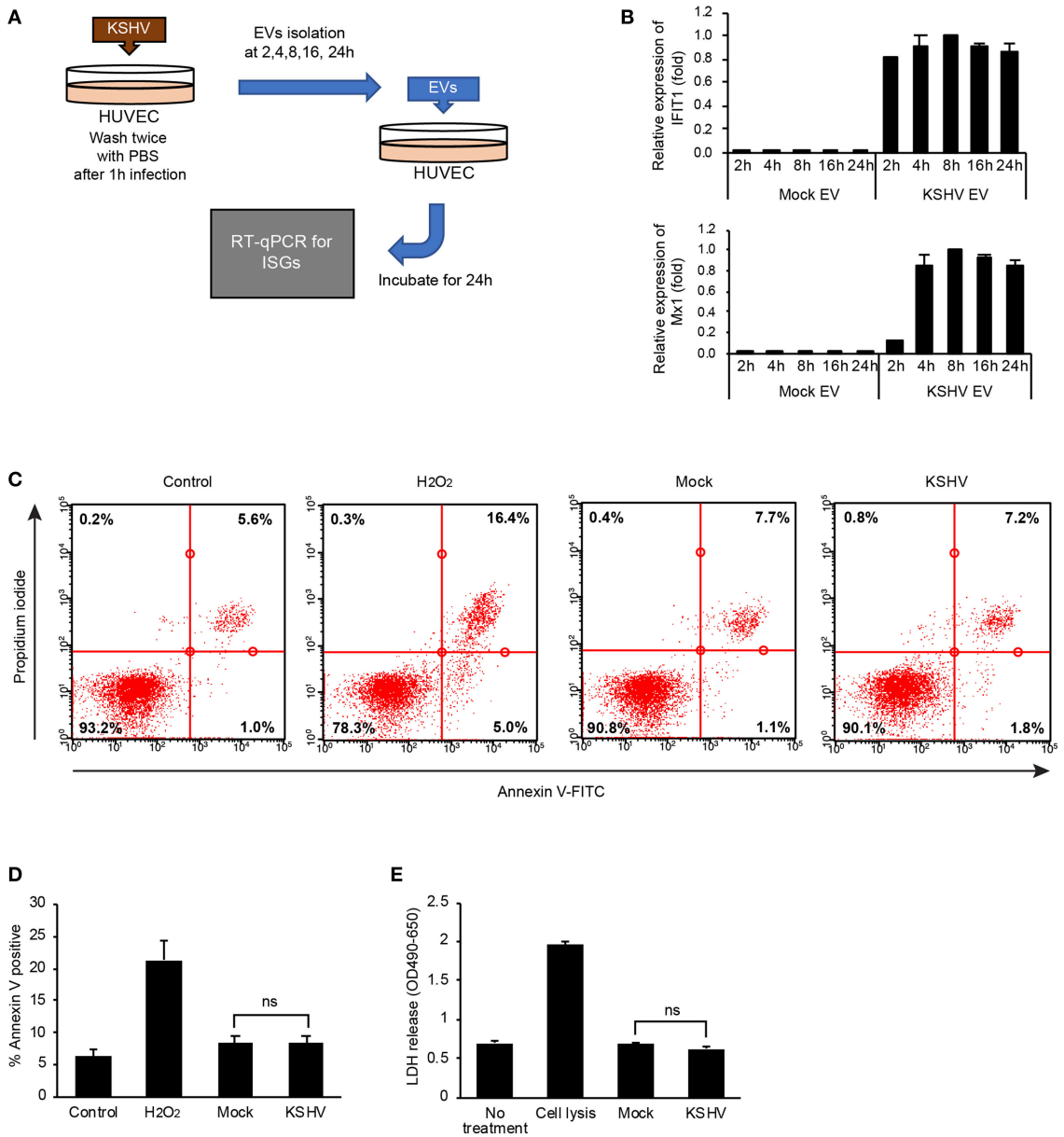

FIGURE 3 | Stimulation of ISG expression by KSHV EVs is not associated with a production of virus or cell death. **(A)** Schematic summary of the experimental process. KSHV infected to HUVECs for indicated periods and EVs were isolated from the supernatant of KSHV-infected cells. Then, each isolated EVs was treated with HUVECs for 24 h, and mRNA expressions for ISGs were analyzed. **(B)** mRNA expression of mock EVs vs. KSHV EVs-treated HUVECs. Each time point represents the time that EVs were isolated after KSHV infection. Data are shown as the mean ± SD, $n = 6$. **(C,D)** Apoptosis and cell death in KSHV-infected HUVECs at 8 h of postinfection. Mock- or KSHV-infected HUVECs were detached from culture plate and stained with FITC-conjugated Annexin V and propidium iodide. Representative and average values from three independent experiments are shown in **(C)** and **(D)**, respectively. PBS and H_2O_2 were used as negative and positive control, respectively. Data are shown as the mean ± SD, $n = 3$. ns, not significant. **(E)** LDH assay for mock- or KSHV-infected HUVECs at 8 h of postinfection. Data are shown as the mean ± SD, $n = 6$. ns, not significant.

results meet the requirements of dose-response studies of EVs recommended in MISEV2018 (19). Furthermore, 16-fold diluted KSHV EVs induced higher expression of IFIT1 in HUVECs compared to 1-fold diluted mock EVs, suggesting a similar number of KSHV EVs still induce the expression of ISGs compared to mock EVs. Next, to confirm if induction of ISGs was specifically mediated by EVs, an essential protein for biogenesis of EVs, Rab27b, was suppressed by shRNA in HUVECs (**Figure 5C**). After 2 weeks of incubation with a selection marker, puromycin, for the shRNA-transduced cells, the expression of Rab27b decreased in knockdown cells. The prepared cells were infected with KSHV, and EVs were isolated

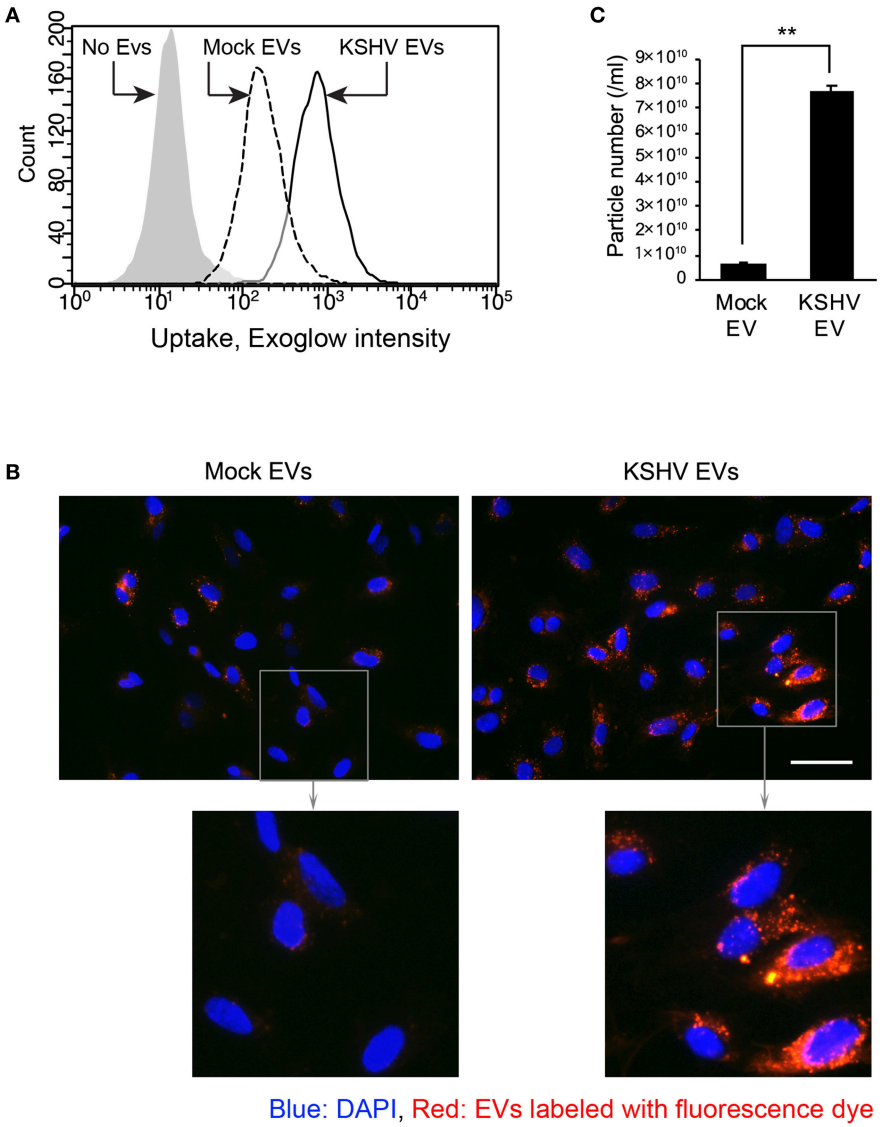

FIGURE 4 | Entry of KSHV EVs into human endothelial cells. Mock EVs or KSHV EVs were isolated from the same amount of conditioned media, and each EV was labeled with fluorescence dye. The labeled EVs were treated with HUVECs, and their entry was analyzed by flow cytometry **(A)** and fluorescence microscopy **(B)**. Scale bar: 50 μm. **(C)** Particle number of the EVs of experiments-applied. Data are shown as the mean ± SD, $n = 3$, $^{**}p < 0.01$.

from each culture supernatant. These isolated EVs were treated to uninfected HUVECs, followed by analysis of mRNA expression of MX1 and IFIT1. mRNA expressions of both ISGs were significantly suppressed in HUVECs treated with KSHV EVs from Rab27b knockdown cells, indicating biogenesis of EVs as a critical factor for KSHV EVs to induce ISGs in human endothelial cells.

Mitochondrial DNA on KSHV EVs Originated From the Cytosol of KSHV-Infected Cells Is a Stimulant for ISGs

Nucleic acids are recognized by the innate immune system, which provides key signals to initiate antiviral responses, including ISGs (20, 21). To determine whether the DNA or RNA on EVs is associated with the induction of ISGs, the isolated EVs were treated with DNase I or RNase, followed by addition to HUVECs. Interestingly, only DNase I treatment of EVs significantly suppressed the expression of ISGs of HUVECs, as observed from the mRNA expression data (**Figure 6A**). These results indicate that the external DNA on EVs might be one of the causative factors for the induction of ISGs, which is consistent with recent studies showing that the external dsDNA on EVs could be an inducing agent for inflammation (22, 23). A previous study showed that mitochondrial DNA (mtDNA) stress primed the antiviral innate immune response (24, 25). Moreover, Sun et al. indicated that infection of dengue virus activates innate immune response via the release

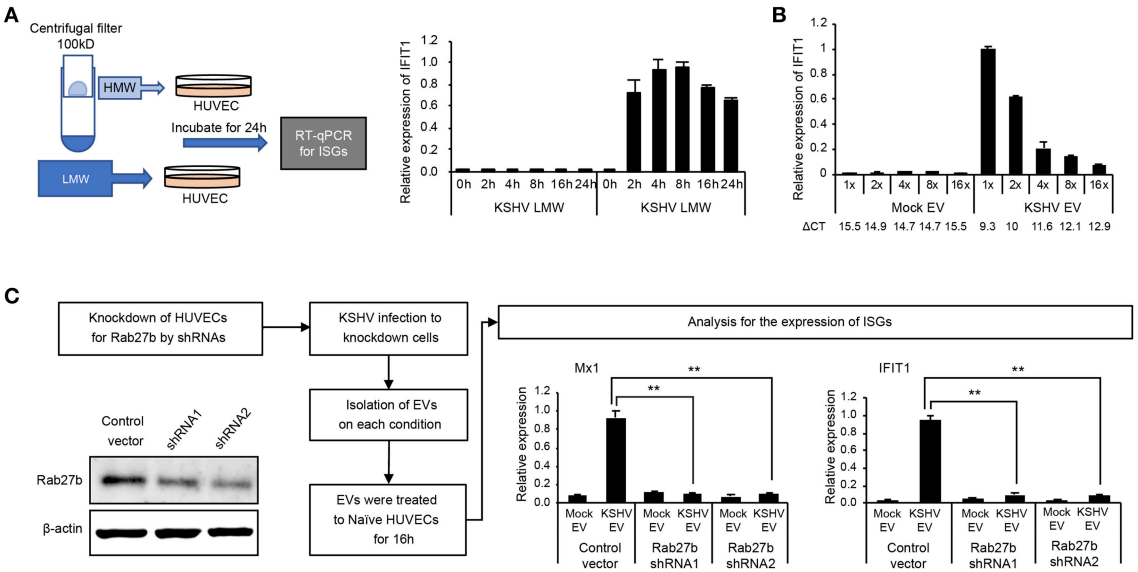

FIGURE 5 | EV was an essential factor in the induction of ISGs by the supernatant from KSHV-infected cells. **(A)** KSHV EVs isolated by centrifugal filtration induced IFIT1 expression. The supernatant from KSHV-infected cells was separated by centrifugal filter device with a cut-off of 100 kDa. High molecular weight (HMW) proteins (the retained materials by a filter) and low molecular weight (LMW) proteins (the flow-through) was applied to HUVECs, followed by analyzing mRNA expression by RT-qPCR. **(B)** Induction of ISGs was correlated with the amount of EVs. The same volume of EVs was isolated from the same amount of the supernatant from mock- or KSHV-infected cells. Then, each EV was applied to HUVECs, followed by analyzing mRNA expression by RT-qPCR. ΔCT indicated the normalized CT value of IFIT1 with reference gene, β-actin. **(C)** Knockdown of Rab27b suppressed the induction of ISGs by KSHV EVs. The expression of Rab27b was suppressed by shRNA in HUVECs. After KSHV infection, mock EVs or KSHV EVs were isolated from the Rab27b-suppressed HUVECs. Each prepared EV was applied to uninfected HUVECs, and mRNA expressions for ISGs were analyzed. The grouping of blots cropped from different gels and full-length blots are included in a **Supplementary Figure 5**. Data are shown as the mean ± SD, $n = 6$, $^{**}p < 0.01$.

of mtDNA (26). Therefore, we analyzed mtDNA in EVs from mock and KSHV-infected cells (**Figure 6B**). Interestingly, a larger quantity of mtDNA was detected in KSHV EVs than in mock EVs, which is consistent with DNase I-treated experiments (**Figure 6A**). We also found that the quantity of mtDNA of EVs was increased in the time course of KSHV infection (**Supplementary Figure 3**). For genomic DNA in the same samples, we could not find an amplification of GAPDH and β-actin (data not shown). To determine whether KSHV-infected HUVECs release mtDNA into the cytosol, we extracted the cytosolic fraction from KSHV-infected cells without any contamination of the nucleus or other cellular organelles (**Figure 6C**). Genomic and mitochondrial DNA were analyzed in the cytosolic fraction derived from mock- or KSHV-infected cells (**Figure 6D**). The cytosolic fraction from KSHV-infected cells contained a larger amount of mtDNA than that from mock-infected cells, which might be the origin of mtDNA of KSHV EVs. We next examined the involvement of the cytosolic DNA sensor cGAS in mtDNA stress signaling, as it mediates ISG expression in response to exogenous and endogenous immunostimulatory DNA species. Knockdown of cGAS in KSHV EVs-treated HUVECs significantly suppressed IFIT1 expression (**Figure 6E**). Besides, IFIT1 mRNA in KSHV EVs-treated HUVECs were also reduced upon STING knockdown (**Figure 6F**), indicating that cGAS-STING signaling would be a driver of KSHV EVs-induced ISG expression. STING signals via the TBK1-IRF3/7 axis to trigger antiviral gene expression. In the microarray analysis (**Figures 1C,D**), TBK1 was analyzed as the top regulator of effect network in KSHV EVs-treated HUVECs, which supports the association of cGAS-STING pathway in KSHV EVs-treated cells. Taken together, these results indicate that mtDNA from KSHV EVs facilitates cGAS-dependent sensing of cytoplasmic mtDNA, resulting in STING-TBK-IRF3 signaling to trigger ISG expression.

Antiviral Effect of KSHV EVs in Human Endothelial Cells

To establish a functional significance of KSHV EVs-induced antiviral priming, KSHV was challenged with the KSHV EVs-pretreated HUVECs. In contrast to mock EVs-treated HUVECs, KSHV EVs-treated cells showed significantly less infectivity for KSHV (**Figures 7A,B**). From those cells, genomic DNA was isolated and KSHV ORF26 DNA was quantified by real-time PCR (**Figure 7C**). KSHV EVs-treated cells showed significantly lesser KSHV DNA than mock EVs-treated cells, which is consistent with KSHV infectivity results. To evaluate the antiviral effect against another virus, human herpes simplex virus type 1 (HSV-1) was used to infect EVs-pretreated HUVECs. For HSV-1 infection, more live cells were observed in KSHV EVs-treated cells than in mock EVs-treated cells (**Figures 7D–F**), indicating that KSHV EVs provide higher resistance to HSV-1 infection in HUVECs than mock EVs.

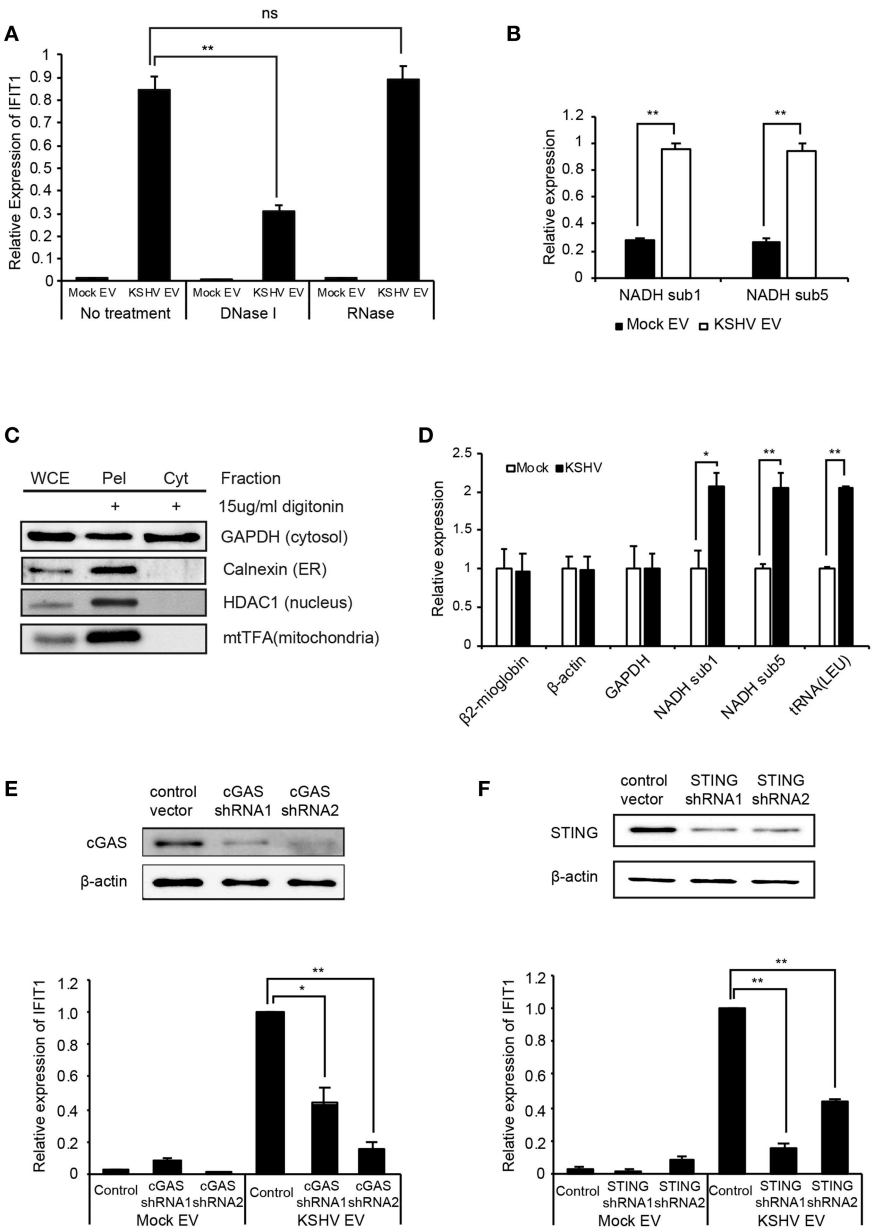

FIGURE 6 | Induction of ISGs by KSHV EVs is associated with mtDNA. **(A)** mRNA expression of IFIT1 in HUVECs treated with DNase I or RNase-treated EVs. **(B)** Quantification of mtDNA in mock EVs vs. KSHV EVs. Genomic DNA was isolated from the same number of EVs, and mtDNA-related genes were analyzed by qPCR. **(C)** Western blotting for the cytoplasmic fraction from KSHV-infected HUVECs. Cytoplasmic fraction was extracted by digitonin, and its purity was analyzed by western blot analysis. WCE: whole cell extract, Pel: pellet after extraction of the cytoplasmic fraction, Cyt: cytoplasmic fraction. **(D)** Quantification for genomic and mitochondrial DNA in the cytoplasmic fraction from mock- vs. KSHV-infected HUVECs. **(E,F)** Induction of IFIT1 in cGAS or STING-suppressed HUVECs by KSHV EVs. The expression of cGAS **(E)** or STING **(F)** was suppressed by shRNAs. Mock EVs or KSHV EVs were treated with each indicated knockdown cell, and the induction in IFIT1 expression was analyzed by RT-qPCR. Data are shown as the mean ± SD, $n = 6$, ns, not significant, $^{*}p < 0.05$, and $^{**}p < 0.01$.The grouping of blots cropped from different gels and full-length blots are included in a **Supplementary Figures 6–8**.

DISCUSSION

To protect multicellular organisms against viruses, it is vital that infected cells trigger antiviral defense responses that can be rapidly transmitted to non-infected cells. The spread of innate immune responses is generally attributed to the production of cytokines, including type I IFNs, which have broad antiviral activities through the induction of ISGs (6).

Increasing evidence suggests that EVs from some virus-infected cells modulate cellular processes including immune responses (27–29). Studying EVs in viral infections poses a limitation: separation of EVs from viral particles is

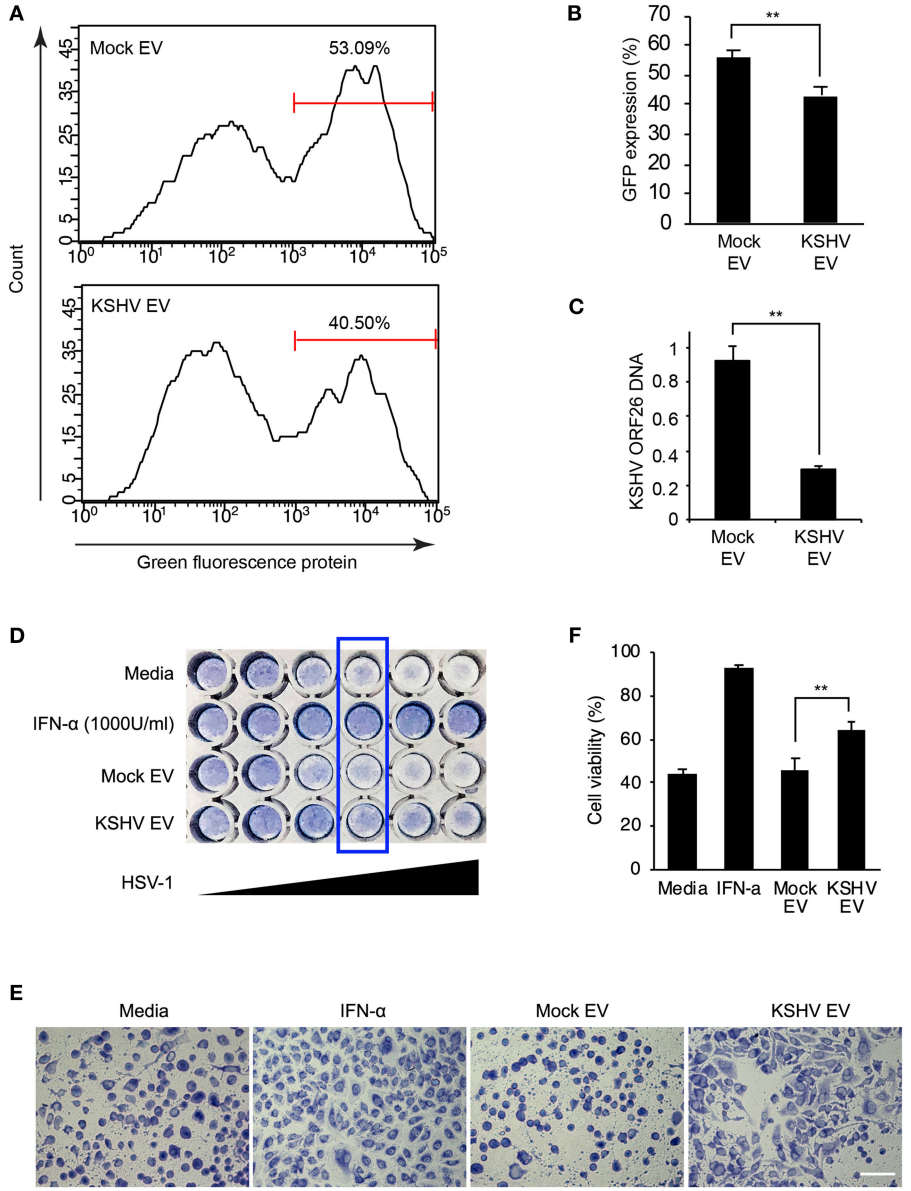

FIGURE 7 | Antiviral effect of KSHV EV in human endothelial cells. **(A–C)** KSHV infectivity was decreased by KSHV EVs. Mock EVs and KSHV EVs were pretreated to human endothelial cells for 24 h, and KSHV infected into the prepared cells. KSHV infectivity was analyzed by flow cytometry through GFP expression **(A,B)**. After KSHV infection, KSHV DNA was compared between mock EVs vs. KSHV EVs-treated cells **(C)**. Data are shown as the mean ± SD, $n = 3$, $^{**}p < 0.01$. **(D–F)** KSHV EVs inhibit the infection of HSV-1. HSV-1 (MOI = 64) was serially diluted into mock EVs or KSHV EVs-treated cells. After 24 h of incubation, the cytopathic effect was analyzed by staining (D). Cellular morphology in the wells of boxed area from **(D)** was visualized by microscopy **(E)**. Scale bar: 100 μm. **(F)** The ratio of cell viability was measured by the WST-1 assay (MOI of HSV-1 = 4). Data are shown as the mean ± SD, $n = 4$, $^{**}p < 0.01$.

challenging. In our previous study, EVs were successfully isolated from KSHV-infected cells in the early phase of infection (15). Using these EVs from KSHV-infected cells, we demonstrated that EVs from KSHV-infected cells trigger an antiviral response by inducing ISGs in human endothelial cells. There have been a few studies on EVs from KSHV-infected cells (30, 31). However, mostly viral microRNA in EVs have been highlighted so far. In this study, we showed that KSHV EVs stimulate ISGs in bystander cells using host mtDNA, demonstrating that virus-infected cells can mediate early antiviral defenses by modulating the production and content of EVs. An EVs-mediated antiviral effect may provide the basis for therapeutic strategies to control viral infection.

In hepatitis B and C viral infections, an antiviral effect could be transferred from cell to cell through exosomes (4, 32). These studies showed that EVs could deliver not only viral components but also molecules with antiviral activity. To our best knowledge, this is the first study that demonstrates EVs with mtDNA from virus-infected cells to be a triggering factor

for an antiviral response. Previous studies showed that mtDNA activates innate immune responses through cGAS or TLR9 (24, 33, 34). Additionally, cGAS-mediated antiviral signaling was spread from dengue virus-infected cells to neighboring cells via gap junctions using mtDNA (26). Considering all of these observations, the antiviral response by EVs containing mtDNA seems to be a reasonable response to viral infection. EVs mediate intercellular communication and regulate immune signaling. Previous studies indicated that double-stranded genomic DNA is located in circulating EVs and a large proportion of human blood plasma cell-free DNA is localized in EVs (35, 36), suggesting that blood circulating DNA or anti-DNA antibodies in autoimmune diseases might be associated with DNA-containing EVs.

In this study, we demonstrated that KSHV-infected cells release approximately 10-folds of EVs particles compared to uninfected cells, which might be associated with extruding the increased mtDNA in the cytosol of KSHV-infected cells to favor cell survival. Cytosolic mtDNA accumulates have known to trigger cell injury (37). In patients with non-alcoholic steatohepatitis, hepatocytes have shown to release mtDNA through microparticles (38). Therefore, the secretion of mtDNA through EVs might be a mechanism for cellular homeostasis. Although the exact functions and mechanisms remain to be elucidated, some virus-infected cells showed increased production of EVs (15, 39, 40). As the small Rab GTPase are well-known to control the secretion of EVs (41, 42), some Rab proteins appear to be factors to regulate the release of EVs in virus-infected cells. Infection of CMV increased the level of Rab27a, which was related to CMV production (43). HSV-1 also exploits Rab27a for its intracellular transport and exocytosis (44, 45). Interaction of virus and Rab GTPase might modulate not only the production of the virus but also the release of EVs. Another pathway that might lead to EVs production in virus-infected cells is the tetraspanin-dependent pathways (46). A recent paper showed that HSV-1 triggered the release of CD63 positive EVs but not alter the exocytosis of TSG101 or Alix, suggesting the infection triggers ESCRT-independent pathways for the release of EVs (47). Understanding and manipulation of EVs biogenesis during virus infection may reveal potential targets for antiviral therapy.

While we suggest mtDNA is a causative factor for the stimulation of ISGs by KSHV EVs, the mechanisms of KSHV EVs-mediated ISGs response is not entirely resolved here, and other factors and pathways may be associated with them. We could not extensively investigate the effect of DNA inside the EVs because DNase treatment removed only surface DNA and permeabilizing agent disrupted a functional structure of EVs. More research should be required to elucidate the exact mechanisms of EVs-mediated antiviral response and their biological significance *in vivo*. Nevertheless, we provide clear evidence that EVs from KSHV-infected HUVECs restricted infection of KSHV and HSV-1, suggesting that DNA-carrying EVs might be important mediators for antiviral response. Taken together, our findings would contribute to the current understanding of the antiviral immune response of EVs from virus-infected cells.

AUTHOR CONTRIBUTIONS

M-SL designed the study. HJ, JL, SL, S-KK, SP, S-MY, and M-SL performed the experiments and analyzed data. HJ and M-SL wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00876/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

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Innate Immune Evasion of Alphaherpesvirus Tegument Proteins

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Alphaherpesviruses are a large family of highly successful human and animal DNA viruses that can establish lifelong latent infection in neurons. All alphaherpesviruses have a protein-rich layer called the tegument that, connects the DNA-containing capsid to the envelope. Tegument proteins have a variety of functions, playing roles in viral entry, secondary envelopment, viral capsid nuclear transportation during infection, and immune evasion. Recently, many studies have made substantial breakthroughs in characterizing the innate immune evasion of tegument proteins. A wide range of antiviral tegument protein factors that control incoming infectious pathogens are induced by the type I interferon (IFN) signaling pathway and other innate immune responses. In this review, we discuss the immune evasion of tegument proteins with a focus on herpes simplex virus type I.

Keywords: alphaherpesvirus, immune evasion, tegument protein, IFN, signaling pathway

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INTRODUCTION

Herpesviruses are divided into three subfamilies, alpha-, beta- and gammaherpesviruses, all of which share a common viral morphology and approximately 40 conserved genes that are important for virus production. The alphaherpesvirus subfamily has a wide range of host (1). Herpes simplex virus 1 (HSV)-1, HSV-2, and varicella-zoster virus (VZV) belong to the human alphaherpesvirus subfamily, while veterinary alphaherpesviruses include bovine herpesvirus (BHV), pseudorabies virus (PRV), and waterfowl duck enteritis virus (DEV) (2).

Herpesviruses undergo two forms of replication, lytic replication, and latent infection. In the lytic replication cycle, the virus first enters a cell, and the viral DNA begins to replicate after the capsid DNA is released into the nucleus. Subsequently, after assembly and genome packaging, the capsid leaves the nucleus (3). The viral particles then undergo primary envelopment and de-envelopment at the nuclear envelope, with tegumentation and secondary envelopment occurring in the cytoplasm. Finally, the virions leave the host by exocytosis (**Figure 2**) (4–6). After some alphaherpesviruses replicate at the infection site, the nervous system is invaded by the fusion of some alphaherpesviruses with the neuronal membrane at the end of an axon. When alphaherpesvirus DNA enters ganglion cell nuclei, some viral particles immediately assemble into a chromatin structure, forming heterochromatin, and resulting in latent infection (7). Not all neuronal infections lead to chromatinization, and in some cases, neuronal infection leads to lytic replication. The occurrence of lytic replication may depend on both viral and cellular factors

that are differentially expressed in distinct types of neurons. Epithelial cells are the primary sites for alphaherpesvirus infection and are typically asymptomatic. Infected humans or animals become carriers without symptoms, with the infection becoming detectable only when progeny viral particles intermittently leave the host cells through germination, exocytosis or induction of apoptosis, making herpesviruses difficult to monitor and control (3, 8).

During latent infection, the viral genome remains in the nucleus, wherein new viral particles accumulate due to periodic reactivation of the lytic replication cycle and are transported along axons into epithelial cells, resulting in symptomatic or asymptomatic shedding (1, 9–12). Lytic replication releases infectious particles that elicit a strong immune response, whereas in latent infection, viruses use various strategies to weaken the presentation of antigens and prolong the lifespans of host cells (3, 13). This approach is highly beneficial to the survival of viruses and the establishment of latent infections.

Alphaherpesviruses encode ∼8 capsid proteins, 23 tegument proteins (**Table 1**), and 14 envelope proteins (14, 15). The tegument is located between the capsid and the envelope (**Figure 1**). The alphaherpesvirus tegument is a self-supporting structure consisting of thousands of densely packaged protein molecules. A proteomic analysis of extracellular HSV-1 by mass spectrometry identified 23 types of virus-encoded tegument proteins as well as some host cell enzymes, chaperones and structural proteins, some of which may be incorporated into the tegument (14). The density of the tegument at the icosahedral vertices of the HSV-1 capsid has been observed by cryo-electron microscopy (cryo-EM), which revealed C-capsid-specific and capsid-apex-specific components (16). More generally, in different subfamilies of herpesviruses, the components are referred to the capsid-associated tegument complex (CATC) (17). Tegument proteins are typically designated as internal or external tegument components depending on whether they preferentially bind to the capsid or viral membrane during entry and exit or on their fractionation behavior after virus decomposition with non-ionic detergents. Although the outer tegument appears to be amorphous, the inner layer has a partial icosahedral order because of its close relationship with the capsid (18). Tegument proteins promote viral replication by regulating genes transcription, halting cell protein synthesis, and destroying host innate immune responses. They can also provide scaffolds for viral particles assembly and create interaction networks to link viral capsids and envelope proteins (1, 19). In addition to the important role of some tegument proteins in viral particles for immune evasion, some other viral proteins are also important for their survival, as they prompte viral replication and participate in the viral immune process. For example, HSV-1 UL24 (20) has key roles in modulating innate immunity. However, this review focuses on the innate immune escape of tegument proteins. The mechanism by which tegument proteins facilitate innate immune evasion remains unclear.

TABLE 1 | Alternative alphaherpesvirus tegument genes and their homologs.

HSV-1/2	VZV	PRV
Tegument proteins involved in innate immune evasion		
UL13 (VP18.8)	ORF47	UL13 (VP18.8)
UL36 (VP1-2)	ORF22 (p22)	UL36
UL37	ORF21	UL37
UL41 (VHS)	ORF17	UL41
UL48 (VP16)	ORF10	UL48
UL49 (VP22)	ORF9	UL49
UL50 (dUTPase)	ORF8	UL50
US3	ORF66	US3
US10	ORF64/69	/
US11	/	/
RL1 (ICP34.5)	/	/
RL2 (ICP0)	ORF61	EP0 (ICP0)
RS1 (ICP4)	ORF62/71 (IE62)	IE180 (ICP4)
UL54 (ICP27)	IE63	UL54 (ICP27)
Other tegument proteins		
UL7	ORF53	UL7
UL11	ORF49	UL11
UL14	ORF46	UL14
UL16	ORF44	UL16
UL21	ORF38	UL21
UL23	ORF36	TK
UL47 (VP13-14)	ORF11	UL47
UL51	ORF7	UL51
UL55	ORF3	/
US2	/	/

Pattern recognition receptors (PRRs) are recognition molecules that are primarily expressed on the surface and in the intracellular compartments of innate immune cells. PRRs can recognize one or more pathogen-associated molecular patterns (PAMPs). The type I interferon (IFN) signaling pathway plays an important role in the innate immune response and is the first line of host defense against viruses (21). Among PRRs, Toll-like receptors (TLRs) were the first PAMP-detecting receptors to be discovered. Nucleic acids are detected by TLR3, TLR7, TLR8, and TLR9, which locate on the endosomal membrane (22, 23). TLRs detect PAMPs and subsequently recruit downstream binding proteins, such as bone marrow differentiation primary response protein 88 (MyD88), MyD88 binding protein-like protein (Mal), Toll/interleukin (IL)-1 receptor domain-containing adapter protein (TIRAP), and Toll/interleukin, which play important roles in the immune processes of HSV-1 infection. TLR3 can be activated by recognizing short double-stranded (dsRNA) and then further recruits and activates the adapter protein Toll/IL-1 receptor (TIR) domain-containing adaptor TRIF. Stimulation of the TLR3-TRIF signaling pathway activates

Abbreviations: HSV-1, Herpes simplex virus 1; BHV, Bovine herpesvirus; PRV, Pseudorabies virus; DEV, Duck enteritis virus; PRRs, Pattern recognition receptors; PAMPs, Pathogen-associated molecular patterns; IFN, Interferon; TLRs, Toll-like receptors; VZV, Varicella zoster virus; RIG-I, Retinoic acid-inducible gene I; MDA5, Melanoma differentiation-associated gene 5; TBK1, Tank-binding kinase 1; cGAS, cyclic GMP-AMP synthase; DDR, DNA damage response; ISGs, IFN-stimulated genes; NF-κB, Nuclear factor kappa B; TNF-α, Tumor necrosis factor alpha; ZAP, Zinc finger antiviral protein; SVV, Simian varicella virus; SOCS, Suppressor of cytokine signaling; ATM, Ataxia telangiectasia mutated.

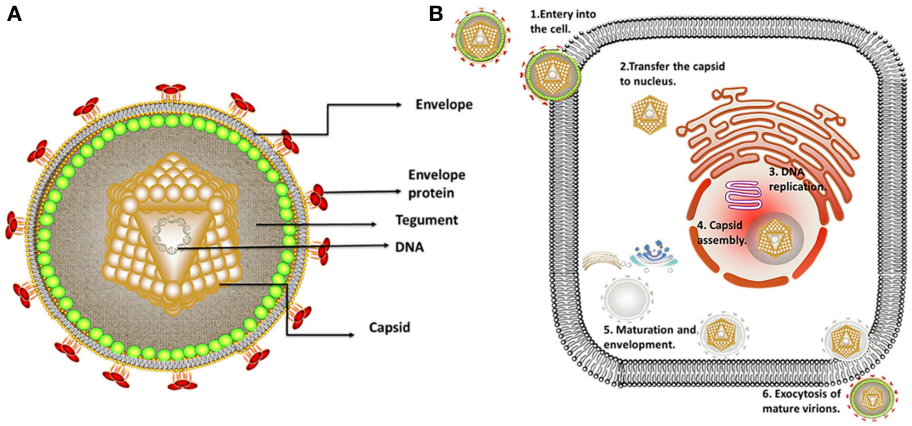

FIGURE 1 | Structure and replication process of herpes virus. **(A)** Structure of alphaherpesviruses. The viral particle structure of alphaherpesviruses includes the genome, tegument, envelope, and capsid. **(B)** The viral replication process of alphaherpesviruses. The viral replication process of alphaherpesviruses includes adsorption, replication, and assembly, secondary envelopment and exocytosis. Some inspiration for this figure was obtained from previous articles (3).

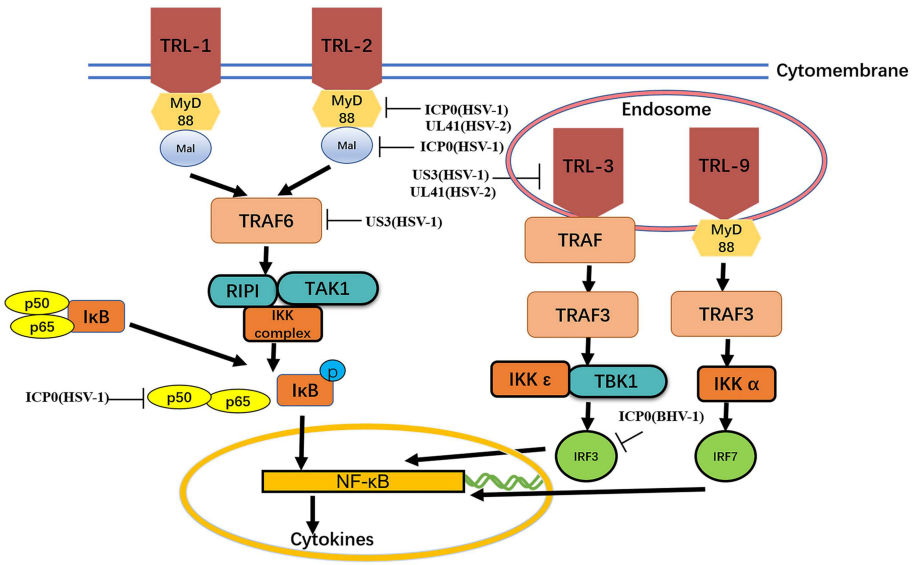

FIGURE 2 | MyD88, Mal, TIRAP,TIRAP-induced IFN-β and TRAM. The formation of protein complexes of unique TBK1 and IKK inhibitors leads to activation of the transcription factors IRF3 and IRF7 and induction of IFN-β expression. Viral proteins can degrade TLRs and interfere with TLR recognition. The ubiquitination activity of viral proteins can inhibit MyD88, Mal, and TRAF6. A series of strategies is used for virus immune evasion. Some inspiration for this figure was obtained from previous articles (4).

the transcription factors NF-κB and IFN regulator factor 3/7 (IRF3/7), resulting in the translocation of NF-κB and IRF3/7 into the nucleus and the production of various cytokines, such as type I IFN (24). According to previous studies, HSV-1 can be detected by TLR2, TLR3, TLR4, and TLR9 (25). The cellular recognition of dsRNA or 5′-triphosphate dsRNA activates the expression of the retinoic acid-induced gene I (RIG-I) and melanoma differentiation-associated gene (MDA-5), resulting in homo-oligomerization of the mitochondrial antiviral signaling (MAVS) protein and activation of tank-binding kinase 1 (TBK1). In recent years, DNA sensors capable of detecting cytoplasmic DNA have been identified, including cyclic GMP-AMP (CGAMP) synthase (cGAS), IFN-γ inducible protein 16 (IFI16), DEAD-box polypeptide 41 (DDX41), DNA-dependent activator of IRF (DAI), and several proteins involved in the DNA damage response (DDR) (21, 26). Bacterial DNA, viral DNA, synthetic double-stranded DNA (dsDNA) and even dsDNA isolated from mammalian cells can be sensed in the cytosol if their lengths exceed 40–50 bp. The key DNA sensor cGAS, which binds to dsDNA and catalyzes the production of the second messenger 2′3′- cGAMP. cGAMP then binds to the binding protein stimulator of the IFN gene (STING), causing

a conformational change in the dimerization of STING. TBK1 phosphorylates the serine at position 366 of STING and then recruits IRF3 (27). In addition to the DNA sensor cGAS, RNA polymerase III (POL III) also functions as a DNA sensor, and cytosolic POL III acts as an innate PRR that recognizes abundant foreign DNA in the cytosol. POL III transcribes the exogenous AT-rich DNA into 5′-ppp RNA, which is recognized by the cytoplasmic RNA sensor RIG-I, thereby allowing downstream signaling via the adaptor MAVS to activate NF-κB and IRF3. The activation of these proteins finally initiates host innate immune responses, including IFNs and proinflammatory cytokines (28–30). The binding of secretory IFNs to the homologous dimer receptors type I IFN receptor (IFNAR1) and type II IFN receptor (IFNAR2) induces the downstream Janus kinase (JAK)-signal transducer and activator of transcription factor (STAT) signaling pathway and antiviral IFN-stimulating gene (ISG) transcription (24).

Human and mouse genetic studies have found that type I IFN responses play an important role in controlling host innate immune responses to alphaherpesvirus infection. Human with mutations in STAT1, TLR3, or UNC-93B, whose gene products are involved in the production or responses of type I IFNs, are susceptible to HSV-induced encephalitis (31–34). Mouse models have demonstrated that type I IFNs are important for controlling acute alphaherpesvirus infection, and many gene products encoded by HSV can antagonize host type I IFN antiviral activity (35). Additionally, ISGs, such as ISG15 and 2′-5′-oligonucleotide synthase (OAS1), have been shown to be important for controlling acute alphaherpesvirus infection in mice (36, 37). Defects in TLR3 increases susceptibility to HSV encephalitis, while impairment of POL III induces predisposition to VZV encephalitis. This specificity may due to the important role of TLR3 in recognizing HSV in the central nervous system, while POL III appears to be an important sensor for the AT-rich VZV genome (38). Carter-Timofte et al. identified mutations in the POL III gene, located in the subunits POLR3A and POLR3E, in two of eight patients by whole-exome sequencing. Functional analysis demonstrated impaired expression of antiviral and inflammatory cytokines in response to the POL III agonist Poly (dA: dT) and increased viral replication in patient cells compared to these features in controls (39).

In addition to the type I IFN signaling pathway, some other innate immune pathways are also involved (40, 41). Chromosome breaks at specific sites caused by HSV-1 infection interact with cellular pathways that identify and repair DNA damage, also known as the DDR. Studies have shown that the DDR plays an active role in antiviral activity (42, 43). Autophagy functions in regulating the activity of specific signals utilized by cells and can remove the threat of intracellular pathogens and prevent the damage or accumulation of long-lived and aggregation-prone proteins (44). Therefore, autophagy is an important aspect of innate immunity. Moreover, because viral infection also induces the formation of antiviral cytoplasmic granules known as stress granules (SGs), this process is closely associated with SG formation and type I IFN production (45).

TABLE 2 | Tegument proteins that inhibit the TLR pathway.

Protein	Virus	Function	References
RL2 (ICP0)	HSV-1	Reduces the inflammatory response triggered by TLR2	(48)
		Decreases MyD88 and Mal	(49)
US3	HSV-1	Reduces the levels of TLR3 and type I IFNs	(50)
		Inhibits TLR2 signaling by reducing TRAF6 polyubiquitination	(51)
UL41	HSV-2	Reduces the expression of TLR2 and TLR3	(52)

IFN Induction and IFN-Dependent Signaling Pathways

Tegument Proteins Inhibit the TLR Signaling Pathway

TLRs are type I transmembrane protein that recognize microorganisms invading the body and activate immune responses (46, 47) and are thus believed to play a key role in the innate immune system. Downstream binding proteins of the TLR signaling pathway include MyD88, Mal, TIRAP, TRIF, and TRAM. TBK1 is ubiquitinated and autophosphorylated, leading to activation of the transcription factors IRF3 and IRF7 and induction of IFN-β expression (23) (**Figure 2** and **Table 2**).

TLR2

The TLR2-dependent induction of type I IFNs occurs only in response to viral ligands. TLR2 can directly or indirectly promotes the synthesis and release of proinflammatory factors and enhances antiviral activities. Studies have also shown that infected cell protein 0 (ICP0) reduces the inflammatory response triggered by TLR2 during HSV-1 infection (48, 53, 54). van Lint and colleagues elucidated a process in which ICP0 promoted the degradation of TLR adapter molecules and inhibited inflammatory responses. ICP0 reduced the TLR-2-mediated inflammatory response to HSV-1 infection, and ICP0 expression alone is sufficient to block the expression of TLR-2 in MyD88 adapter complexes through the E3 ligase function of ICP0 (55–57). Yao and Rosenthal found that the expression of TLR2 in VK2 epithelial cells transfected with the HSV-2 virion host shutoff (VHS) protein was reduced, consistent with the findings in HEK 293 cells (52).

TLR3

TLR2 has been reported to linked to the recognition of several DNA viruses, while dsRNA is a particularly potent nucleic acid intermediate that activates TLR3 (58). TLR3 is capable of inducing the expression of type I IFNs and inflammatory cytokines after detecting the dsRNA. TLR3-deficient fibroblasts produced much less type I IFN during HSV-1 infection than the control group, and impaired TLR3 signaling also resulted in high level of viral replication (59). Cellular proteasomal activity is required for this inhibitory activity. Peri and colleagues observed that pUS3 interferes with TLR3 recognition and MxA induction following inhibition of type I IFN mRNA in HSV-1

infected cells (50). Similarly, Yao and Rosenthal found that the expression of TLR3 in VK2 epithelial cells transfected with the VHS protein was reduced, consistent with the findings in HEK 293 cells (52).

MyD88 and mal

MyD88 is an essential adapter molecule associated with inflammatory cytokines upon activation of all TLRs. The cascade pathway activates the transcription factor NF-κB and promotes the production of the proinflammatory cytokines IL-1β, IL-6, IL-8, IL-12, and monocyte chemotactic peptide 1 (MCP-1). Another MyD88-like protein, Mal, activates NF-kB, Jun amino-terminal kinase (JNK) and extracellular signal-regulated kinase-1 and−2. Mal can form homo- and heterodimers with MyD88 (60). van Lint showed that ICP0 can also decrease the level of MyD88 and Mal through its E3 ligase function (49).

TRAF6

The E3 ubiquitin ligase TNF receptor-associated factor 6 (TRAF6) interacts with TGF-β-activated kinase 1 (TAK1), subsequently activatingTAK1 (61). This interaction leads to activation of the IKK complex, which then phosphorylates the inhibitor of κB, causing κB ubiquitination and degradation (62). The HSV-1 kinase pUS3 can inhibit the TLR-2 signaling pathway by reducing TRAF6 polyubiquitination, which depends on its kinase activity before or at the stage of TRAF6 ubiquitination (51, 63).

Tegument Proteins Inhibit the RIG-I Signaling Pathway

RIG-I and MDA-5 are members of the RIG-I-like receptor (RLR) family (64, 65) and can identify RNA viruses in cells and induce production of type I IFNs and immune factors (66). RIG-I activates NF-κB and IRFs through MAVS (67). Kato's gene knockout experiments showed that loss of RIG-I or MAVS could severely inhibit the innate immune response of mice, resulting in highly increased viral replication (68) (**Figure 3** and **Table 3**).

RIG-I and MDA-5

RIG-I and MDA-5 act as two cytoplasmic dsRNA sensors. RIG-I primarily recognizes RNA containing 5′-triphosphate, while MDA-5 typically recognizes dsRNAs >2,000 bp in length. RIG-I and MDA-5 recruit MAVS to deliver signals to the kinase TBK1 and induce IκB kinase (IKKi), which phosphorylates

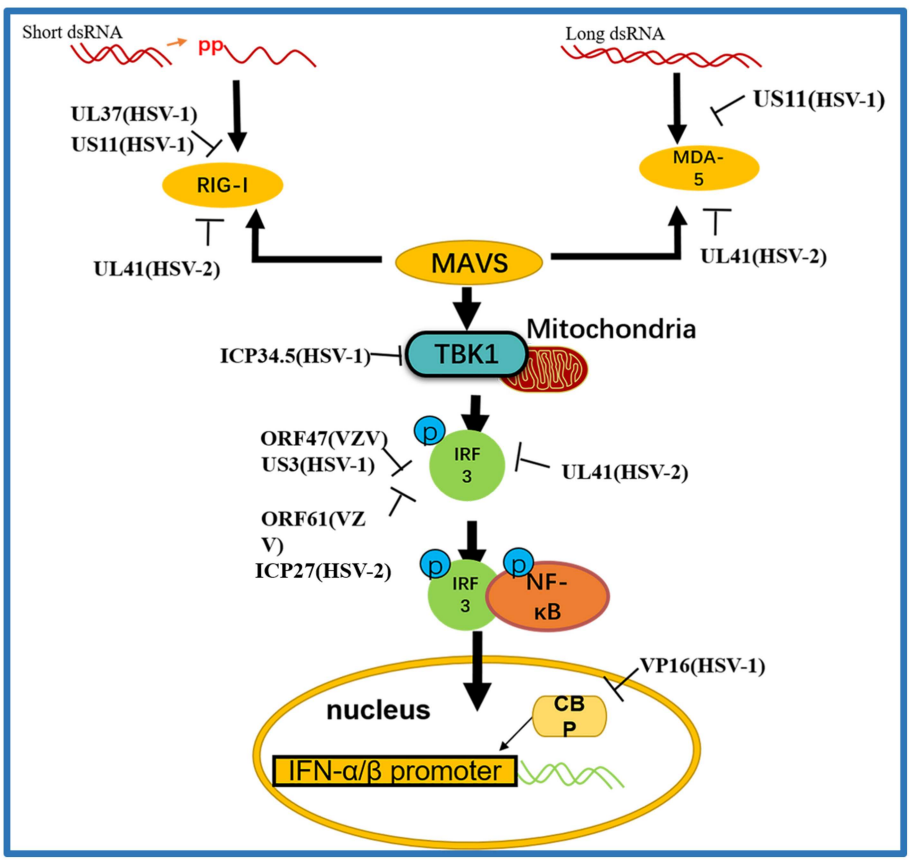

FIGURE 3 | A schematic diagram of the pathogen-derived molecules used to escape intracellular RNA sensing pathways. The sensors in the pathway include RIG-I and MDA-5, which can detect different RNA species, primarily those containing 5′ diphosphate or triphosphate or long dsRNA, respectively. Pathogen-derived degradative or inhibitory helper proteins inhibit RIG-I activation through direct binding to block the interaction between RIG-I and MAVS and prevent RIG-I from entering mitochondria.

TABLE 3 | Tegument proteins that inhibit the RIG-I pathway.

Protein	Virus	Function	References
US3	HSV-1	Interacts with and hyperphosphorylates IRF3 to prevent IRF3 activation	(69)
US11	HSV-1	Binds to RIG-I and MDA5 inhibits their downstream signaling pathway	(70)
UL36 (VP1-2)	HSV-1	Deubiquitinates TRAF3 to prevent the recruitment of TBK1	(71)
UL37	HSV-1	Blocks RNA-induced activation by targeting RIG-I	(67)
RL1 (ICP34.5)	HSV-1	Binds to and sequesters TBK1	(72)
		Controls IRF3 activation by reversing translational shutoff and sustaining the expression of other IFN inhibitors	(73)
UL46	HSV-1	Blocks the interaction of TBK1 and IRF3 and inhibits the dimerization of TBK1	(74)
UL48 (VP16)	HSV-1	Blocks MAVS-Pex-mediated early ISG production	(75)
ORF61	VZV	Degrades activated IRF3	(76)
ORF47	VZV	Prevents IRF3 homodimerization and subsequent induction of IFN-β and ISG15	(77)
UL41	HSV-2	Inhibits RIG-I and MDA-5 as well as IRF3 dimerization and translocation	(52)
ORF62(IE62)	VZV	Blocks the phosphorylation of serine residues 396, 398, and 402 in IRF3	(78)
UL54(ICP27)	HSV-2	Inhibit IRF3 phosphorylation and nuclear translocation	(79)

both IRF3 and IκB kinase beta (IKKβ) and then activates the NF-κB signaling pathway (80). Once activated, IRF3 transfers to the nucleus and binds to positive regulatory domains I and III of the IFNβ promoter to induce IFNβ expression. Through coimmunoprecipitation analyses, Xing and colleagues demonstrated that in HSV-1 infected cells, the pUS11 C-terminus interacts with endogenous RIG-I and MDA-5 through an RNA binding domain. HSV-1 pUS11 can block IFN-β production and inhibit downstream signaling pathway activation by binding to RIG-I and MDA-5 (70). Zhao and colleagues observed that HSV-1 pUL37 is a deaminase protein that blocks RNA-induced activation by targeting RIG-I. Upon interacting with pUL37, RIG-I activation was inhibited (67). Yao and his colleague found that HSV-2 pUL41/VHS can inhibit the expression of RIG-I and MDA-5, thereby facilitating virus to evade host innate immune responses (52).

MAVS

Activated RIG-I and MDA-5 induce downstream signal transduction by binding to MAVS. The N-terminus of MAVS contains a CARD-like domain that binds to RIG-I and MDA-5, and through a CARD-CARD interaction to activate NF-κB and IRFs. MAVS is located in the outer mitochondrial membrane and interacts with RIG-I and MDA-5 to self-oligomerize (81). Peroxisome MAVS (MAVS-Pex) signaling has been reported to trigger the rapid production of IFN-dependent ISG in response to invasive pathogens (82). For example, pUL48/VP16, a tegument protein encoded by HSV-1, blocks the early production of ISG mediated by MAVS-Pex and inhibits the early innate immune signaling of peroxisomes (75).

TRAF3

TRAF3 is an important molecule in the RLR signaling pathway. The downstream kinases TBK1 and IκB kinase ε of RIG-I are recruited by the K63-mediated multiubiquitination of TRAF3, which results in IRF3 phosphorylation and the subsequent production of type I IFNs (83). pUL36 ubiquitin-specific protease has been shown to deubiquitinate TRAF3 and block the recruitment of the downstream adaptor TBK1 to decrease the production of IFN-γ during HSV-1 infection (71).

TBK1

As an IκB kinase-related kinase, TBK1 can phosphorylate a variety of substrates that are involved in various cellular processes (84). After DNA and RNA sensors detecting nucleic acids, TBK1 is activated. TBK1 triggers the phosphorylation of IRF3, the activation of NF-κB and the expression of type I IFNs. HSV-1 ICP34.5 is a neurotoxic factor with multiple functions and plays a crucial role in viral pathogenesis (85). Previous studies have reported that HSV-1 ICP34.5 can regulate IFN production by binding to and isolating TBK1 (72) and suppressing the induction of the ISG56 promoter by TBK1. Recently, study found that HSV-1 pUL46 interacted with TBK1 and reduced TBK1 activation and its downstream signaling. The results showed that pUL46 impaired the interaction between TBK1 and IRF3 and downregulated the activation of IRF3 by inhibiting the dimerization of TBK1 to reduce the production of type I IFN and immunostimulatory DNA (74).

IRF3

Activated IRF3 is essential for the effective transcription of type I IFNs, and IRF3 plays an important role in RLR-independent signal transduction. Activated IRF3 dimerizes and migrates to the nucleus, wherein it identifies specific sequence-based IFN stimulus response elements in the regulatory regions of target genes (86). Studies have shown that US3 protein expression can significantly inhibit the activation of IFN-γ, IFN stimulatory response element (ISRE) promoters and transcription of IFN, ISG54, and ISG56 via the neurovirus Sendai virus (SEV) (87). In addition, the SEV-induced dimerization and nuclear translocation of IRF3 have been shown to be blocked by pUS3. pUS3 can interact with and hyperphosphorylate IRF3 at serine 175, thus blocking IRF3 activation (69). Manivanh provided evidence that ICP34.5 controlled IRF3 activation via its ability to regulate translational shutoff reversal and by maintaining the expression of other IFN inhibitors encoded by viruses (73). The VZV immediate-early protein ORF61, a protein homologous to HSV-1 ICP0, attenuates the IRF3-mediated innate immune response through degradation of activated

IRF3 (76). Vandevenne observed that during VZV infection, the VZV kinase ORF47, a protein homologous to UL13, can atypically inhibit the phosphorylation of IRF3, which blocks the homodimerization and induction of target genes such as IFN-β and ISG15 (77). VZV ORF62/IE62 is a protein homologous to HSV ICP4. Sen and colleagues found that the inhibition mediated by VZV IE62 may be the three serine residues (396, 398, and 402) on IRF3 were inhibited, thus blocking the downstream signal transduction mediated by IRF3 (78). Additional studies revealed that HSV-2 ICP27 directly associates with IRF3 and inhibits its phosphorylation and nuclear translocation, resulting in the inhibition of IFN-β induction (79) (**Table 2**).

Tegument Proteins Inhibit the NF-κB Signaling Pathway

The NF-κB signaling pathway is an important factor in antiviral immunity (88, 89) that promotes the expression of proteins contributing to viral replication and induces specific and adaptive immune responses (90). PPRs, TLRs, and RLRs can all lead to the induction of the NF-κB signaling pathway.

During HSV-1 infection, pUL48 can block IFN-β production by inhibiting NF-κB activation and interfering with the IRF3 recruitment of its coactivator CBP (91). The ORF61 protein of VZV and simian varicella virus (SVV) is involved in immune evasion and can prevent IκB-α from ubiquitination. Travis further demonstrated that SVV ORF61 can interact with β-TrCP, a subunit of the SCF ubiquitin ligase complex, to mediate the degradation of IκB-α (92). Sloan and colleagues observed that VZV ORF61 could inhibit the activity of the NF-κB reporter induced by tumor necrosis factor alpha (TNF-α). In addition, ORF61 mutation experiments revealed that the E3 ubiquitin ligase domain was necessary to inhibit the NF-κB pathway (93). During HSV-1 infection, ICP0 can interact with p65 and p50 and degrade the proteasomal protein p50 to block the nuclear translocation of p65 and reduce NF-κB-dependent genes expression (94). In contrast, another study showed that ICP0 can also ubiquitinate IκB-α and activate the transcription of NF-κB target genes (95). In addition, the replication of HSV-1 can be directly enhanced by stimulation of NF-κB, with recruitment of the ICP0 promoter by NF-κB activating the transcription and replication of ICP0 (96). The HSV-1 protein kinase US3 hyperphosphorylated p65 at serine 75 and blocked its nuclear translocation, significantly inhibiting NF-κB activation and decreasing the expression of the inflammatory chemokine IL-8 (97, 98) (**Figure 2** and **Table 4**).

TABLE 4 | Tegument proteins that inhibit the NF-κB pathway.

Protein	Virus	Function	References
UL48 (VP16)	HSV-1	Inhibits NF-κB activation and interferes with the IRF-3-mediated recruitment of its coactivator CBP	(91)
ORF61	SVV	Prevents IκBα ubiquitination and interacts with β-TrCP	(92)
	VZV	Inhibits TNF-α-induced NF-κB reporter activity	(93)
RL2 (ICP0)	HSV-1	Interacts with p50 and p60 and degrades the proteasomal protein p50	(94)
US3	HSV-1	Ubiquitinates IκBα	(95)
		Hyperphosphorylates p65 at serine 75 and blocks p65 nuclear translocation	(97)

Tegument Proteins Inhibit the DNA Sensor Signaling Pathway

In recent years, substantial advances have been made in research on DNA sensors. Several important cytoplasmic DNA sensors have been identified and characterized, providing insights into the mechanisms of sensor signaling pathways (21) (**Figure 4** and **Table 5**).

cGAS

Among the DNA sensors, cGAS, a nucleotidyltransferase, is responsible for identifying various DNA ligands present in certain cell types. cGAS is activated by binding to cytosolic dsDNA and uses ATP and GTP to produce cGAMP through its enzymatic activity (107). Huang and colleagues showed that VP22 encoded by the HSV-1 UL49 gene is a tegument protein. VP22 participates in the innate immune antiviral process by inhibiting the enzymatic activity of cGAS and thus antagonizing the DNA-mediated innate immune signaling pathway (103). Zhang et al. discovered the innate immune evasion mechanism of the HSV-1 pUL37 deaminase protein and revealed that the human and mouse cGAS proteins (but not the nonhuman primate cGAS proteins) are targets for UL37 deamidation, promoting the lytic replication of HSV-1 (105).

STING

When STING is activated, it recruits TBK1, activates IRF3 and induces the production of IFN-β (103). As a broad antimicrobial factor, the DNA sensor STING antagonizes HSV by activating type I IFNs and proinflammatory responses upon sensing foreign DNA or non-canonical cyclic dinucleotides, the latter of which are synthesized by cGAS (108). Previous data suggested that ICP0 blocks the STING pathway (100). The transcription factor IRF3, a primary component of the STING pathway, is known to be blocked by ICP0 (101, 102), although the associated mechanism is unclear. Deschamps and colleagues showed that STING was degraded in cells expressing HSV-1 pUL46, which blocked the accumulation of STING transcripts (100). ICP27 interacted with TBK1 and STING in a manner that was dependent on TBK1 activity and the RGG motif in ICP27. Thus, HSV-1 inhibits the expression of type I IFNs in human macrophages through ICP27-dependent targeting of the TBK1-activated STING signalsome (106).

IFI16

IFI16, a member of the PYHIN protein family, was originally reported to be a cytosolic DNA sensor and has been implicated in the type I IFN response to HSV-1 (109–111). IFI16 localizes in the nuclei of many types of cells, making it a potential

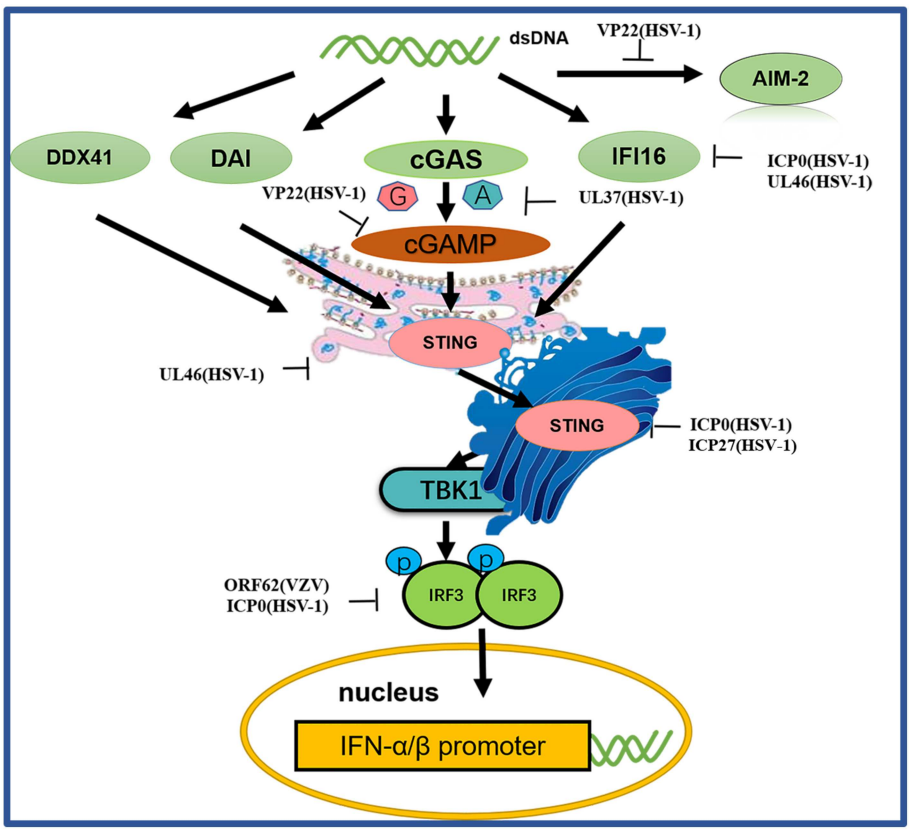

FIGURE 4 | A schematic diagram of pathogen-derived molecules used to escape intracellular DNA sensing pathways. The primary sensor of cytoplasmic DNA is cGAS, which is responsible for activating the binding protein STING. Pathogen-mediated degradation targets cGAS to prevent it from binding to DNA or to inhibit its catalytic activity. At the same time, pathogen invaders also degrade cGAMP and bacterial circulating dinucleotides. IFI16 positively affects the activation of the cGAS-STING pathway. Other DNA sensors, such as DAI and AIM2, are also viral factors that block DNA binding and downstream pathway activation. Viral proteolytic enzymes can decompose and degrade these factors; blocking their translocation and preventing their interaction from the downstream signaling protein TBK1, thus hindering STING function.

candidate for sensing HSV-1 DNA in the nucleus (112). Studies have shown that HSV ICP0 triggers IFI16 degradation, thereby inhibiting additional signaling and IRF3 activation (99, 113). Deschamps and colleagues also showed that IFI16 is degraded in cells constitutively expressing HSV-1 pUL46, which blocks the accumulation of IFI16 transcripts (104).

Other DNA sensors

Before the study of cGAS, proteins such as DAI, DDX41, DNA-dependent protein kinase (DNA-pk) and AIM2 were identified as cytosolic DNA sensor candidates (114). Although these proteins were reported to inhibit viral replication, further studies have shown that they are not necessarily involved in the DNA-induced responses in many human cells, suggesting that they may play a redundant or cell- type-specific role. HSV-1 AIM2-dependent inflammatory activation has been shown to be inhibited by the HSV-1 tegument protein VP22. VP22 can interact with AIM2 and prevent AIM2 oligomerization, which is the first step in AIM2 inflammasome activation (115).

Tegument Proteins Inhibit IFN-Stimulated Genes

Type I IFNs triggers numerous ISGs, such as viperin, zinc finger antiviral protein (ZAP), tetherin, dsRNA-dependent protein kinase (PKR), and OAS. Different combinations of ISGs can enhance the signaling transduction of type I IFNs and the antiviral activity of host to inhibit viral replication (21, 116) (**Figure 5** and **Table 6**).

Viperin

Viperin was first to be identified as a highly conserved protein that can induce IFN-γ protein production and is comprised of 361 amino acids. A number of studies have shown that viperin is directly induced by human cytomegalovirus and exhibits low expression (124). The viperin gene (also known as CIG5 or RASD2) can also be classified as an antiviral ISG that restricts the replication of DNA and RNA viruses (125). However, it is unclear whether viperin plays a role in HSV-1 infection. HSV-1 pUL41 can degrade host mRNA by cutting it at a preferential site, and UL41 promotes the replication of HSV-1 by degrading viperin mRNA (117).

ZAP

In addition to viperin, ZAP is an antiretroviral factor that was originally identified in rats. Viruses that contain ZAP response elements (ZREs) in their RNA are sensitive to ZAP. Studies have shown that human ZAP (hZAP) has no inhibitory effect on the replication of HSV-1, and as an antagonist of hZAP, HSV-1 pUL41/VHS can degrade hZAP mRNA (118).

Tetherin

Tetherin (BST-2 or CD317) is a membrane glycoprotein that can induce the production of IFNs and effectively exert antiviral activity by inhibiting the release of many envelope viruses (126). Helen showed that overexpression of tetherin can inhibit the replication of HSV-1 and that HSV-1 pUL41/VHS can deplete tetherin mRNA via its mRNA degradation function (120).

PKR

Binding of dsRNA activates PKR, which then phosphorylates the α subunit of eIF2α, resulting in translational inhibition (127). HSV-1 pUS11, a late-stage gene, inhibits PKR activation by binding to both dsRNA and PKR to prevent them from binding to each other (123), and then inhibits PKR phosphorylation. Other studies have shown that during early infection, the HSV-1 pUL41 VHS RNase protein degrades RNAs that activate PKR. The VHS RNase protein and mitogen-activated protein kinase act cooperatively to block the activation of PKR (121).

TABLE 5 | Tegument proteins that inhibit the DNA sensor signaling pathway.

Protein	Virus	Function	References
RL2 (ICP0)	HSV-1	Targets IFI16 degradation, inhibiting additional signaling and IRF-3 activation	(99)
		Blocks STING and the transcription factor IRF3	(100–102)
UL49 (VP22)	HSV-1	Inhibits the enzymatic activity of cGAS	(103)
UL46 (VP11-12)	HSV-1	Blocks STING and IFI16 transcript accumulation	(104)
UL37	HSV-1	Deamidates cGAS proteins	(105)
UL54 (ICP27)	HSV-1	Targets the TBK1-activated STING signalsome	(106)

TABLE 6 | Immune evasion of tegument proteins through ISGs.

Protein	Virus	Function	References
UL41	HSV-1	Degrades viperin mRNA	(117)
		Degrades hZAP mRNA	(118)
		Reduces the accumulation of IFIT3 mRNA	(119)
		Depletes tetherin mRNA	(120)
		Block the activation of PKR	(121)
US11	HSV-1	Inhibits OAS	(122)
		Inhibits PKR	(123)

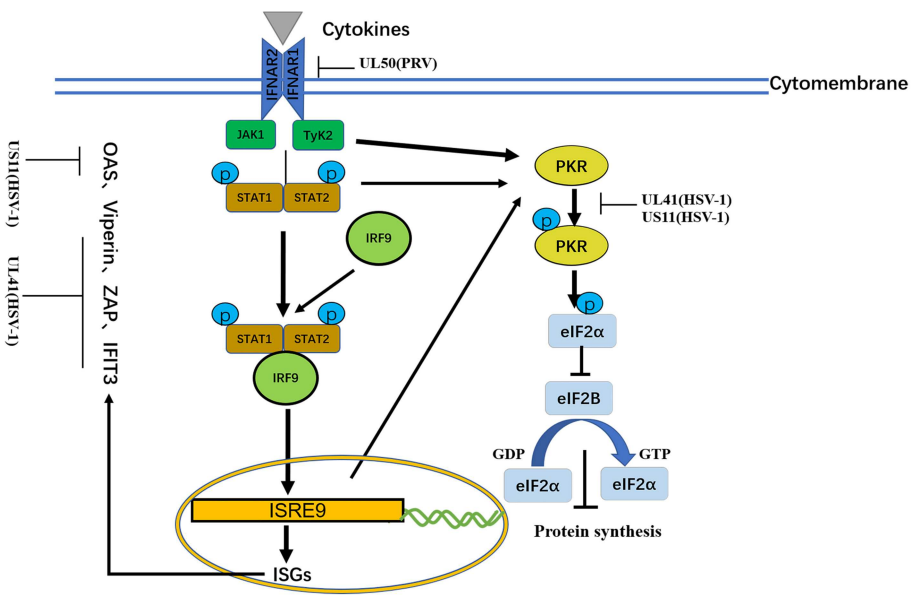

FIGURE 5 | A schematic diagram of the pathogen-derived molecules used to escape cytokine-sensing pathways in cells. In the basic transmission process of the JAK/STAT signaling pathway, the binding of cytokines to their receptors causes dimerization of the receptor molecules. The close proximity, of JAKs to the receptors enables their activation through interactive tyrosine phosphorylation. The immune evasion effect of the virus can be achieved through the degradation of IFNAR1 and ISG mRNA.

OAS

Similar to PKR, OAS recognizes dsRNA. The three primary forms of OAS recognize dsRNA through positively charged channels in the molecule. Conformational changes in OAS after binding to dsRNA lead to the synthesis of 2′,5′-oligoadenylates (2–5As), which then activate latent RNase L, leading to the degradation of viruses and endogenous RNA and the inhibition of viral replication. OAS is essential for host defense and can be inhibited by pUS11 via its dsRNA binding domain, with pUS11 sequestering any available dsRNA produced during infection as the potential underlying mechanism (122).

IFIT3

The IFN-induced protein with tetratricopeptide repeats (IFIT) family includes IFIT1 (ISG56), IFIT2 (ISG54), IFIT3 (ISG60), and IFIT5 (ISG58), which are distributed on human chromosome. Recent studies have shown that IFIT proteins restrict viral replication by altering protein synthesis, binding viral RNA or interacting with structural or non-structural viral proteins to exert antiviral effects (128). Jiang showed for the first time that IFIT3 has little effect on the replication of HSV-1, because pUL41/VHS reduces the accumulation of IFIT3 mRNA and disrupts its antiviral activity (119).

JAK/STAT Signaling Pathway

During viral infection, IFNs exert their antiviral function by inducing antiviral proteins via the JAK/STAT pathway (129). Four JAKs and seven STATs have been identified in mammals. JAKs are tyrosine kinases of the Janus family, include JAK-1, JAK-2, JAK-3, and Tyk-2. The STATs include STAT-1, STAT-2, STAT-3, STAT-4, STAT-5a, STAT-5b, and STAT6. JAK-1, JAK-2, Tyk-2, STAT-1, STAT-2, STAT-4, and STAT-5 are directly involved in IFN-mediated signaling transduction pathways. The JAK/STAT signaling pathway is a common pathway that includes many cytokine signaling molecules and plays extensive roles in cell proliferation, differentiation, apoptosis and inflammation. This pathway exerts its function by interacting with negative regulators in other signaling pathways and STAT-mediated covalent modifications. In the basic transmission process of the JAK/STAT signaling pathway, the binding of cytokines with their receptors induces receptor molecule dimerization. The proximity of JAKs to receptors then enables JAK activation through interactive tyrosine phosphorylation. Activated JAKs catalyze the tyrosine phosphorylation of receptors and form corresponding STAT docking sites, which enable STATs to bind to receptors through the SH2 domain and cooperate with JAKs. After phosphorylation, STATs form homodimers that divert into the nucleus, wherein they bind to the promoters of target genes to activate their transcription and expression (**Figure 5** and **Table 7**).

IFNAR1

Because lacking intrinsic protein kinase domains, IFNAR1 and IFNAR2 rely on the 74 members of the JAK family for signal transduction (131). The results published by Zhang suggested that pUL50 has dUTPase activity. dUTPase catalyzes the hydrolysis of dUTP into dUMP and inorganic pyrophosphate, providing the dUMP precursor for dTTP biosynthesis and inhibiting IFN signaling. dUTPase also has the ability to suppress type I IFN signaling by promoting the lysosomal degradation of IFNAR1, thereby contributing to innate immune evasion (130) (**Table 6**).

Cytokine Signaling Pathways

Cytokines are small molecular proteins with extensive biological activity stimulated by immune cells (such as monocytes, macrophages, T cells, B cells, and natural killer (NK) cells) and some non-immune cells (endothelial cells, epidermal cells, and fibroblasts). Cytokines are produced by many kinds of cells induced by immunogens, mitogens or other stimulants and have many functions such as regulating innate and adaptive immunity, hematopoiesis, cell growth, and damaged tissue repair. Aside from IFNs, other cytokines can be classified into the following categories according to their function: ILs, TNF-α, TNF-β, colony-stimulating factors, chemokines, and growth factors (**Table 8**) (132).

SOCS1 and SOCS3

Suppressor of cytokine signaling 1 (SOCS1) and SOCS3 contain kinase inhibitory regions (KIRs) that can inhibit JAK signal transduction through the SH2 domain and interact with the phosphotyrosines of JAK and GP130, respectively (138). In addition to IFNs, PAMPs are effective inducers of SOCS1 and SOCS3. Because SOCS proteins negatively regulate cytokine signal transduction, many viruses induce the expression of SOCSs to aid in their survival (139). The SOCS family has eight members and suppresses various cytokine signaling pathways, including the IFN signaling pathway. In one study, the expression of eight SOCS family members during HSV-1 infection was analyzed by q RT-PCR, revealing that the tegument protein pUL13 could induce SOCS1 and SOCS3. However, no such induction was observed in UL13-deficient virus-infected cells, suggesting that the UL13 protein kinase was involved in the induction of the two genes (136).

TNF-α

TNF-α is a cytokine with multipotent biological effects that are triggered by two types of TNF-α receptors on the cell surface (140). The TNF-α signaling transduction pathway primarily involves caspase family-mediated apoptosis and activation of the transcription factors NF-κB and JNK protein kinase mediated by TRAF (141). The expression of TNF-α has been observed to be increased in the spleens of mice infected with PRV UL41 mutant virus. TNF-α is considered to be an important cytokine in innate immune responses. In addition, PRV UL41 plays an important role in targeting host innate immune responses via its ribonuclease activity. Studies have suggested that pUL41 may contribute to the protection of organisms from viral damage mediated by TNF-α via degradation TNF-α mRNA (133).

SLPI

Secretory leukocyte protease inhibitor (SLPI), an anti-inflammatory mediator of mucosal immunity, can inhibit both human immunodeficiency virus (HIV) and HSV in cell culture. Epidemiological studies have shown that high

TABLE 7 | Immune evasion of tegument proteins through the JAK/ STAT signaling pathway.

Protein	Virus	Function	Reference
UL50	PRV	Promotes the lysosomal degradation of IFNAR1	(130)

TABLE 8 | Immune evasion of tegument proteins through cytokine signaling.

Protein	Virus	Function	References
UL49 (VP22)	HSV-1	Interacts with AIM2 and prevents its oligomerization Inhibits OAS	(115)
UL41	PRV	Reduces the expression of TNF-α	(133)
UL41	HSV-1	Suppresses cytokines such as IL-1β and IL-18	(134)
RL2 (ICP0)/RS1 (ICP4)	HSV	Downregulates SLPI or activates NF-κB	(135)
UL13	HSV-1	Induces SOCS1 and SOCS3	(136)
US10	DEV	Downregulates the transcript levels of IL-4, IL-6, and IL-10	(137)

concentrations of SLPI in mucosal secretions can inhibit HIV transmission. Whether the loss of SLPI caused by HSV allows the virus to evade the host's innate immune response is currently being studied, and the loss of SLPI may lead to an increased risk of HIV infection in the context of HSV infection (142). Reverse transcription PCR experiments have shown that SLPI is lost due to downregulating genes expression. The downregulation of SLPI is related to NF-κB signaling pathway activation and inflammatory cytokine upregulation. Fakioglu showed that the ICP4- or ICP0-induced expression of immediate-early genes can downregulate SLPI or activate NF-κB (135).

ILs

ILs are cytokines that are produced by and used in many types of cells. Currently, at least 38 ILs, named IL-1 to -IL38, have been identified. These ILs have complex functions, forming networks and exhibiting complex overlaps, and playing important roles in the maturation, activation, proliferation, and immune regulation of immune cells. In addition, they also participate in various physiological and pathological reactions in organisms. For example, the proliferation, differentiation and functions of immune cells are regulated by a series of cytokines. According to their structure, cytokines can be divided into several protein families, such as the IL-1, IL-6, IL-10, TNF, and hematopoietic factor families (143). ILs can lead to local inflammation and cause sterilization and cell damage. Suzutani showed that a UL41-deleted strain of HSV-1 exhibited 20- and 5-fold higher sensitivity to IFN-α and IFN-β than the wild-type strain, respectively. These results indicate that one important role of HSV-1 pUL41/VHS *in vivo* is the evasion of non-specific host defense mechanisms during primary infection by suppressing cytokines such as IL-1β and IL-18 (134). A study by Ma (137) showed that DEV pUS10, which plays an important role in viral replication, could upregulate the transcription of IL-4, IL-6, and IL-10 in US10-deleted DEV-infected duck embryonic fibroblasts (DEFs) at all assayed time points (**Table 7**).

NK Cells

Functional NK cells are essential for limiting herpesvirus transmission and disease symptoms. There are many types of receptors on the NK cell surface, and their functions can be divided into two categories: activation and inhibition of the proliferation system. By recognizing specific ligands, NK cells can sense changes of target cells surface properties. To prevent clearance from cytotoxic T lymphocytes, some viruses actively reduce the level of MHC class I (MHC-I), an important ligand of the KIR family on the cell surface inhibiting NK cell receptors (144). To benefit their survival, viruses can encode MHC-I-like proteins that activate KIR receptors and proteins that inhibit the exposure of NK cell receptor ligands.

CD300a, also known as IRP60, is a highly conserved inhibitory NK cell receptor that does not bind to MHC-I. CD300a is a 60 kDa protein belonging to the immunoglobulin (Ig) superfamily that is characterized by a single V-type Ig-like domain in its extracellular domain and several tyrosine-based immunoreceptor inhibition motifs (ITIMs) in its cytoplasmic domain (145). CD300a can identify aminophospholipids exposed on the cell surface, especially phosphatidylserine (PS) and phosphatidylethanolamine (PE), which can inhibit the cytolysis of NK cells by binding to ligands (146). CD300a inhibitory receptors and their lipid ligands have been specifically reported on mammals, birds and fish (147). To date, no descriptions of the NK cell evasion strategy involving CD300a have been reported. A study by Grauwet firstly indicated that the pUS3 protein kinase of the alphaherpesvirus PRV can trigger the inhibitory NK cell receptor CD300a binding to the surface of infected cells, thereby increasing the CD300a-mediated protection of the infected cells. In addition, the binding of pUS3 to CD300a is associated with the aminophospholipid ligand of CD300a and the IP21 activating kinase (148), thus representing a novel alphaherpesvirus strategy for escaping NK cells.

Other Innate Immune Responses

The DDR Response

The cellular DDR pathway monitors damage to genomic DNA. DNA-PK, ataxia telangiectasia mutated (ATM) kinase, and ATM- and Rad3-related (ATR) kinase are the primary signaling pathway mediators that initiate the DDR (48). Recently, cellular DNA repair machinery was demonstrated to recognize viral genetic material (149). The DDR plays an important role in viral infection, participating in the activation of many components of the ATM-dependent signaling pathway and inhibiting the DNA PKC- and ATR-dependent arms (150). Lilley and colleagues showed that RNF8 and RNF168, important mediators of ATM-dependent signaling pathways, are targeted for proteasome-mediated degradation by ICP0 (151).

ER Stress

The endoplasmic reticulum (ER) is a cytoplasmic eukaryotic organelle that has numerous functions, taking part in the transport of cellular materials, the provision of increased surface areas for cellular reactions, and the production of proteins, steroids and lipids (152). Mis- and unfolded proteins that can cause stress in the ER accumulate during viral replication and trigger the unfolded protein response (UPR) (153). The IRE1/XBP1 pathway is the most conserved component of the UPR branch in eukaryotic cells (154). IRE1 is a dual-activity enzyme that contains a serine-threonine kinase domain and a ribonuclease domain (155). Upon activation, IRE1 undergoes dimerization and transphosphorylation, which facilitates the removal of a 26-nucleotide (NT) intron from the XBP1 gene to form a spliced XBP1, which translated into a transcription factor. In the nucleus, XBP1 induces the expression of the genes that enhance the folding ability of ER proteins and functions in phospholipid biosynthesis and ER-associated protein degradation (ERAD) (156). During HSV-1 infection, the molecular mechanism by which the IRE1/XBP1 branch of the UPR is repressed remains unclear. Zhang and colleagues showed that the HSV-1 tegument protein pUL41, which has endoribonuclease activity, degrades XBP1 mRNA to inhibit its expression (157). These findings reveal a novel mechanism by which HSV-1 modulates the IRE1/XBP1 branch of the UPR. Interestingly, the HSV-1 ICP0 promoter can react to ER stress. Burnett and colleagues found that ICP0 can activate itself independently during HSV-1 infection, suggesting that HSV-1 regulates the ER stress response through ICP0 (153).

CONCLUSION

Over millions of years of coevolution between host and viruses, host species have developed a highly complex set of physiological immune mechanisms to block and eliminate viral infection. However, for every host immune response step, viruses have developed corresponding immune escape mechanisms to ensure their own survival. Successful immune escape is a primary factor underlying chronic herpesvirus infection. In recent years, substantial progress has been made in understanding the variety of cytoplasmic DNA sensors that enable resistance to the immune response. Tegument proteins play important role in alphaherpesvirus innate immune evasion. However, despite the mapping of antiviral defense signaling pathways between tegument proteins and host, a functional understanding of how tegument proteins work together to interfere with the innate immune system remains elusive. Further studies on the mechanisms of tegument proteins, capsid proteins, and glycoproteins will be helpful in the search for antiviral targets and development of antiviral drugs.

AUTHOR CONTRIBUTIONS

LY wrote the manuscript and constructed the figures. AC and MW contributed ideas for the review. QY, YWu, RJ, ML, DZ, SC, SZ, XZ, JH, YWa, ZX, ZC, LZhu, QL, YL, YY, LZha, BT, LP, MR, and XC edited and revised the manuscript.

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Human NK Cells and Herpesviruses: Mechanisms of Recognition, Response and Adaptation

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NK cells contribute to early defenses against viruses through their inborn abilities that include sensing of PAMPs and inflammatory signals such as cytokines or chemokines, recognition, and killing of infected cells through activating surface receptors engagement. Moreover, they support adaptive responses via Ab-dependent mechanisms, triggered by CD16, and DC editing. Their fundamental role in anti-viral responses has been unveiled in patients with NK cell deficiencies suffering from severe Herpesvirus infections. Notably, these infections, often occurring as primary infections early in life, can be efficiently cleared by NK, T, and B cells in healthy hosts. Herpesviruses however, generate a complicated balance with the host immune system through their latency cycle moving between immune control and viral reactivation. This lifelong challenge has contributed to the development of numerous evasion mechanisms by Herpesviruses, many of which devoted to elude NK cell surveillance from viral reactivations rather than primary infections. This delicate equilibrium can be altered in proportions of healthy individuals promoting virus reactivation and, more often, in immunocompromised subjects. However, the constant stimulus provided by virus-host interplay has also favored NK-cell adaptation to Herpesviruses. During anti-HCMV responses, NK cells can reshape their receptor repertoire and function, through epigenetic remodeling, and acquire adaptive traits such as longevity and clonal expansion abilities. The major mechanisms of recognition and effector responses employed by NK cells against Herpesviruses, related to their genomic organization will be addressed, including those allowing NK cells to generate memory-like responses. In addition, the mechanisms underlying virus reactivation or control will be discussed.

Keywords: NK cells, Herpesvirus, activating receptors, TLRs, memory responses, viral reactivation

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INTRODUCTION

Human NK cells are innate lymphocytes that rapidly provide defenses against tumors and viral infections allowing pathogen elimination or limiting viral spread (Vivier et al., 2011; Della Chiesa et al., 2014b). Their fast responses mainly rely on the expression of multiple germ-line encoded activating receptors among which natural cytotoxicity receptors (NCRs) and NKG2D play the

most relevant role in the recognition and killing of infected cells (Bottino et al., 2000; Moretta and Moretta, 2004; Lanier, 2015). The responses elicited by activating receptors are integrated and balanced by the engagement of inhibitory receptors mainly depending on those specific for HLA class I (HLA-I) molecules that include the Killer Ig-like Receptors (KIRs), able to distinguish among allotypic determinants of HLA-A, -B and -C (Bottino et al., 1996; Parham, 2005), the CD94/NKG2A heterodimer, specific for the non-classic HLA-I molecule HLA-E (Braud et al., 1998), and LILRB1 (or CD85j/ILT-2) broadly recognizing HLA-I alleles (Colonna et al., 1997).

Upon infection many viruses, including Herpesviruses, target T cell function via specific interactions with TCR and HLA-I molecules. Indeed, several viral products interfere with host TAP proteins and HLA-I expression, leading to reduced CTL-mediated recognition of infected cells, and decreased naïve T cell activation (Hill et al., 1995; Imai et al., 2013; Schuren et al., 2016). Conversely, downregulated HLA-I expression renders infected cells susceptible to NK-cell killing (Huard and Fruh, 2000; Tortorella et al., 2000). However, activating counterparts of HLA-I-specific receptors, namely activating KIRs (aKIRs), and CD94/NKG2C can also importantly contribute to defense against virus (Della Chiesa et al., 2015).

Human NK cells are usually divided in two major populations, the $CD56^{bright}$ subset expressing NKG2A, lacking KIRs and CD16 (i.e., a low affinity Fcγ Receptor) and the $CD56^{dim}$ subset expressing high CD16 and variable proportions of KIRs, NKG2A, LILRB1, CD57, and NKG2C (Cooper et al., 2001; Caligiuri, 2008; Freud et al., 2017). These two subsets differ in their proliferative potential, cytotoxic activity, cytokine production, and homing to peripheral tissues (Moretta, 2010; Castriconi et al., 2018) thus offering different anti-viral defenses. Notably, $CD56^{dim}$ NK cells, besides high cytotoxicity, can also rapidly produce IFN-γ and TNF-α upon receptor-induced cell triggering (De Maria et al., 2011).

The critical role of NK cells in viral defense has been disclosed by the higher susceptibility to viral infections, caused primarily by Herpesviruses, in individuals affected by congenital immunodeficiencies in which NK cells are absent or defective (Orange, 2002; Etzioni et al., 2005; Notarangelo and Mazzolari, 2006; Mace and Orange, 2019). Herpesviruses are a family of dsDNA viruses, divided in three subfamilies, i.e., α- (HSV-1, HSV-2, and VZV), β- (CMV, HHV6, and HHV7) and γ-Herpesvirus (EBV and KSHV), that differ for their genetic content, infection sites and pathogenesis, while sharing the ability to persist in the host in a latency status after resolution of a primary infection (De Pelsmaeker et al., 2018). The mechanisms by which Herpesviruses establish and maintain latency have not been completely elucidated.

In an evolutionary perspective, our immune system and Herpesviruses have co-evolved influencing reciprocally. During this process the generation of several viral immunoevasion mechanisms has been favored. Most of these mechanisms aim at limiting and suppressing NK-cell responses, which point again to the relevance of these lymphocytes in Herpesvirus control. Although viral immunoevasion strategies are crucial in NK-Herpesvirus interactions, they will not be specifically addressed here and have been exhaustively reviewed elsewhere (Corrales-Aguilar et al., 2014; De Pelsmaeker et al., 2018).

On the other hand, the host-Herpesvirus interaction has exerted a strong pressure on our immune system likely favoring the generation of unexpected memory responses by NK cells and their adaptation to Herpesviruses, in particular to CMV (Muntasell et al., 2013; Sun et al., 2014).

OVERVIEW OF THE MAIN ACTIVATING RECEPTORS REGULATING NK-MEDIATED RECOGNITION AND EFFECTOR RESPONSES TO HERPESVIRUS

The main mechanisms by which NK cells can recognize and eliminate virus-infected cells involve the employ of (i) activating receptors for cellular ligands often overexpressed upon infection, (ii) activating receptors for virus-derived ligands, (iii) activating receptors, i.e., NKG2C and aKIRs, recognizing virus-modified HLA-I molecules, and (iv) CD16-mediated antibody-dependent cellular cytotoxicity (ADCC) (Hammer et al., 2018b). Almost all these mechanisms can be applied to NK cells in Herpesvirus control (**Figures 1A–C**).

The importance of certain activating receptors in Herpesvirus elimination has been indirectly revealed by the numerous proteins encoded by the different Herpesviruses aimed at limiting activating receptors function, in most cases by downregulating the respective cellular ligands on infected cells. In this context, the activating receptor NKG2D that recognizes stress-induced cellular ligands often overexpressed upon viral infection or tumor transformation (i.e., MIC-A, MIC-B, and ULBPs) (Lanier, 2015), is central in NK-mediated immune responses against virtually all Herpesviruses, namely HSV-1, VZV, CMV, HHV6, HHV7, KSHV, and EBV, all of which encode molecules downregulating NKG2D ligands (Wu et al., 2003; Thomas et al., 2008; Nachmani et al., 2009; Schneider and Hudson, 2011; Campbell et al., 2015; Schmiedel et al., 2016). Besides NKG2D, other non-HLA-I-specific activating receptors can play a role against several Herpesviruses suggesting a common strategy to eliminate these pathogens. In particular, the three NCRs (i.e., NKp46, NKp30, and NKp44) are involved in killing HSV-1-infected fibroblasts. The upregulation of cellular NCR ligands upon HSV-1 infection is resulted responsible for the increased susceptibility to NK-mediated cytotoxicity (Chisholm et al., 2007). Interestingly, NK-mediated killing was appreciable even before HLA-I downregulation had occurred, suggesting that, in NK-HSV-1 interactions, activating signals can overcome inhibitory receptors signaling (Chisholm et al., 2007). In this context, increased NCRs expression and function have been observed in NK cells differentiating *in vitro* from $CD34^{+}$ precursors in the presence of HSV-infected myelomonocytes, further strengthening the relevance of the NCRs-NCR ligands axis against HSV (Costa et al., 2009).

The NCR NKp30 also participates in recognition and killing of CMV- and HHV6-infected cells. Its involvement is again

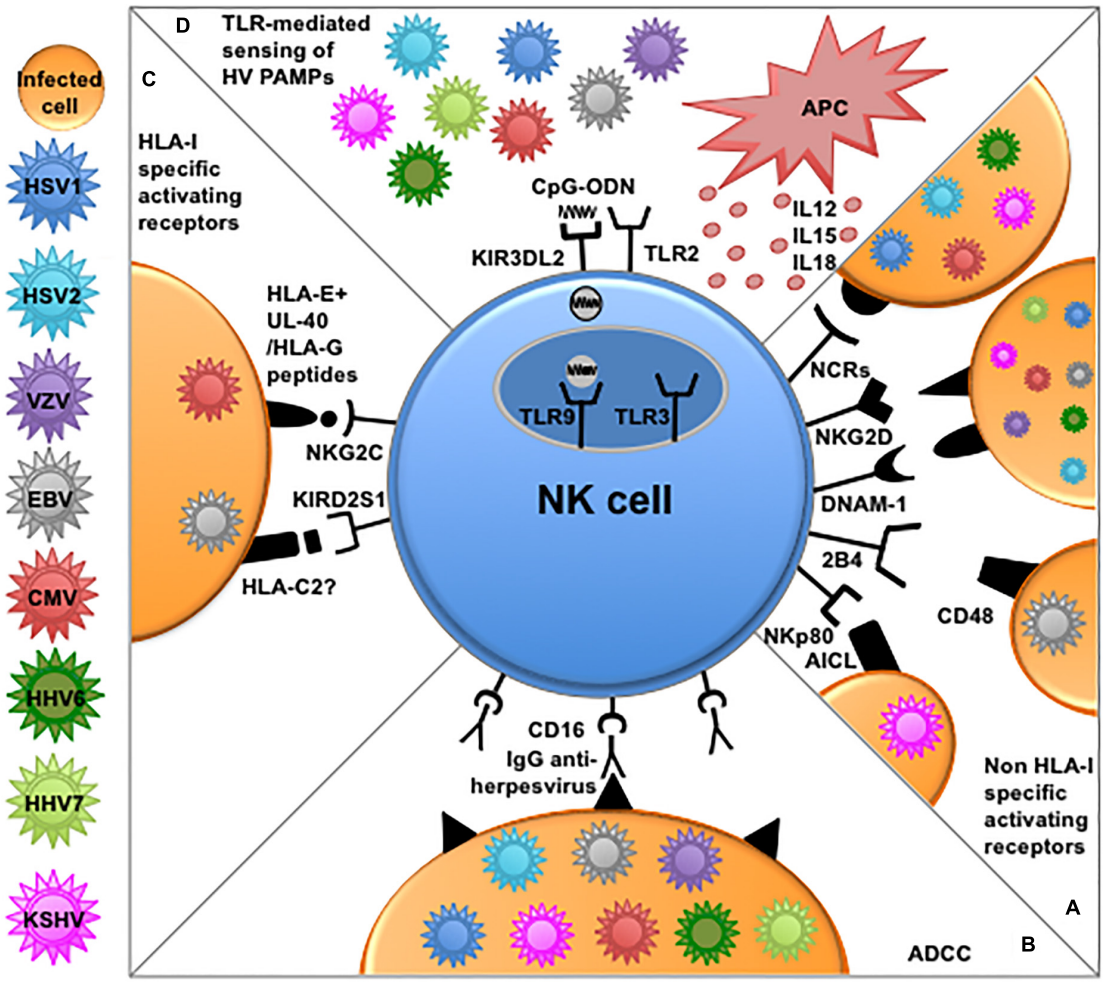

FIGURE 1 | NK cell-mediated mechanisms of recognition and responses to Herpesviruses. **(A)** Several non-HLA-I-specific activating receptors and co-receptors, i.e., NCRs, NKG2D, DNAM-1, 2B4, and NKp80 play an important role in the elimination of cells infected by different Herpesviruses through the recognition of cellular ligands expressed on target cells. **(B)** NK cells can efficiently kill opsonized Herpesvirus-infected cells through antibody-dependent cellular cytotoxicity (ADCC) via CD16 engagement by the Fc fragment of anti-viral immunoglobulins. **(C)** NKG2C and aKIRs play a role mainly in the recognition of CMV-infected cells. The underlying recognition mechanisms are based on interactions with cognate HLA-I molecules. NKG2C shows enhanced interaction to HLA-E presenting peptides derived from viral UL-40 or HLA-G leader sequences, while, among aKIRs, KIR2DS1 seems to better recognize HLA-C2, modified upon CMV infection or presenting EBV-derived peptides. **(D)** NK cells express different functional TLRs involved in the recognition of PAMPs derived from Herpesviruses. In particular TLR2 alllows NK-mediated recognition of envelope glycoproteins from HSV and CMV, while TLR9 can recognize viral CpG sequences shuttled by KIR3DL2 from the surface of NK cells to endosomes. APC-derived cytokines and reciprocal interactions with these immune cells (e.g., dendritic cells and macrophages) can further enhance NK cells effector function against Herpesviruses.

testified by viral evasion mechanisms that downregulate B7-H6, a major NKp30 cellular ligand (Brandt et al., 2009), possibly expressed on infected cells (Schmiedel et al., 2016; Charpak-Amikam et al., 2017). In addition, NKp30 itself is the target of a CMV-encoded protein, pp65, that by binding to this NCR can induce its dissociation from the signaling molecule CD3ζ, thereby inhibiting NK-mediated killing of CMV-infected fibroblasts and dendritic cells (DCs) (Arnon et al., 2005). Along this line, a role for the NKp44-NKp44 ligand signaling pathway against KSHV is suggested by NKp44 ligand downregulation during lytic infection in KSHV-infected cells (Madrid and Ganem, 2012).

Similar to NKG2D and NCRs, the activating co-receptor DNAM1 recognizing PVR and Nectin-2 (CD112) (Bottino et al., 2003), plays a role against different Herpesviruses, i.e., CMV, EBV, and HSV-2 as demonstrated by different evasion strategies reducing DNAM-1 signaling (Tomasec et al., 2005; Prod'homme et al., 2010; Grauwet et al., 2014; Williams et al., 2015).

While NKG2D, DNAM-1, and NCRs serve against several Herpesviruses, other activating NK receptors are specifically involved in the recognition/elimination of cells infected only by a given Herpesvirus. An example is the co-receptor 2B4 (or CD244) which requires the adaptor protein SLAM-associated protein (SAP) to deliver activating signals upon engagement with its ligand CD48 (Nakajima et al., 1999; Bottino et al., 2000). 2B4 engagement is crucial to NK-mediated killing of

EBV-infected B cells. Indeed, B cells that are CD48 high, represent a preferential target for this Herpesvirus (Chijioke et al., 2016). A role for 2B4 was actually revealed by the severe consequences of primary EBV infection in individuals suffering from X-linked lymphoproliferative disease (XLP-1), a congenital immunodeficiency in which SAP is absent or defective (Sayos et al., 1998), resulting in inhibitory signals from 2B4 impairing NK-mediated B-EBV elimination (Parolini et al., 2000). Interestingly, NK cells can respond efficiently to EBV-infected B cells in early lytic cycle and NK-mediated killing involves also NKG2D and DNAM-1 (Chijioke et al., 2013; Williams et al., 2015). However, EBV-infected B cells in latency or even in late lytic stages are resistant to NK attack, due to viral evasion mechanisms independent of NK cell function (Williams et al., 2015).

Finally, a role for the activating co-receptor NKp80 in the recognition of KSHV-infected cells was also proposed, based on the downregulation of its ligand AICL upon KSHV infection (Thomas et al., 2008).

Overall, in most instances, the activating receptors described above allow NK cells to eliminate infected cells by the recognition of cellular ligands expressed on target cells, while the engagement of activating receptors by virus-encoded ligands has not been demonstrated for Herpesviruses, at variance with influenza or vaccinia virus whose products hemagglutinin, and neuraminidase are directly recognized by NKp46 and NKp44 (Mandelboim et al., 2001; Ho et al., 2008). On the contrary, the HLA-I specific receptor NKG2C can recognize viral ligands although the mechanisms described so far are based on interactions with viral peptides bound to HLA-E molecules on CMV-infected cells. NKG2C is also involved in generating CMV-induced adaptive responses and will thus be discussed in more detail in the dedicated paragraph.

Another major mechanism employed by NK cells in controlling both primary viral infections, when adaptive immunity is already established, and secondary reactivations (either subclinical or clinical), relies on the activating receptor CD16 (FcγRIIIa), the low-affinity receptor for the immunoglobulin Fc fragment (Braud et al., 1998; Vivier et al., 2011). Upon CD16 engagement, NK cells can efficiently eliminate opsonized infected cells via ADCC. The relevance of this mechanism in providing defense against Herpesvirus is underlined by severe EBV and VZV infections associated to a dysfunctional mutated CD16 (de Vries et al., 1996; Grier et al., 2012). Furthermore, a polymorphism of the *CD16* gene resulting in the surface expression of a high affinity CD16 receptor (i.e., the CD16A-158V/V polymorphism) is associated to enhanced NK-mediated ADCC and confers protection from clinical HSV-1 reactivation (Moraru et al., 2012, 2015). Not unexpectedly, this highly effective anti-viral mechanism is targeted by multiple evasion strategies, as both HSV and CMV encode Fcγ-binding proteins that act as decoy receptors interfering with IgG binding to CD16 and thus attenuating ADCC (Johnson et al., 1988; Atalay et al., 2002; Corrales-Aguilar et al., 2014; Costa-Garcia et al., 2015). However, it has been recently described that a viral Fcγ-binding protein, gE, which is expressed on the cell surface by HSV-infected cells, can react with non-specific IgG thus generating a "Fc-bridge" that instead favors NK-mediated ADCC responses (Dai et al., 2017; Dai and Caligiuri, 2018).

NK cells can importantly contribute to early viral defense not only by exerting cytolytic activity against infected cells but also through their ability to sense pathogens via toll-like receptors (TLRs) (Sivori et al., 2004). NK cells express different functional TLRs among which TLR2, TLR3, and TLR9 seem to be primarily involved in the recognition of pathogen-associated molecular patterns (PAMPs) derived from Herpesviruses, such as double stranded viral nucleic acids or structural proteins (Adib-Conquy et al., 2014; Della Chiesa et al., 2014b). In particular, NK cells can directly recognize envelope glycoproteins from both CMV and HSV virions through TLR2 (Kim et al., 2012; Muntasell et al., 2013). Upon TLR2 engagement, NK cells become activated, and produce IFN-γ, further promoting anti-viral immune responses. Indeed, NK cells have been detected in herpetic lesions in close contact with CD4 T cells, thus possibly contributing to directly shaping adaptive responses (Kim et al., 2012). Interestingly, *TLR9* polymorphisms are associated with susceptibility to infection, with the T-1237C polymorphism that causes altered *TLR9* expression, being predictive of susceptibility to CMV infection (Carvalho et al., 2009). NK cells could thus play a role in TLR9-mediated defense to CMV, as they can efficiently respond to TLR9 agonists such as CpG-ODNs. Remarkably, these TLR9 ligands can be bound at the cell surface by KIR3DL2, a member of the KIR family, and then shuttled by receptor internalization to endosomes where TLR9 is localized (Sivori et al., 2010).

Thus, in a scenario where NK cells are recruited to viral infection sites, their effector function (e.g., cytotoxicity, IFN-γ, and chemokine production) can be enhanced by combined exposure to microbial products and cytokines available in the inflammatory milieu, such as IL-12 or IL-18. In this context, TLRs- and/or cytokine-activated NK cells can reciprocally interact with other immune cells responding to the same PAMPs via TLRs, such as DCs or macrophages (**Figure 1D**). This cross-talk can occur in the early phases of anti-viral responses (Andrews et al., 2005; Vogel et al., 2014) and can also contribute to DC editing and/or promote DC maturation (Della Chiesa et al., 2005, 2014b; Ferlazzo and Morandi, 2014), thus possibly amplifying and regulating adaptive responses to Herpesviruses.

It should be noted however, that TLR-mediated sensing of viral PAMPs by NK cells has not been definitively settled yet, similar to the contribution of TLRs on DC and macrophages to the response to NK cells. A more extensive review work and additional original work will be needed to appropriately address this issue.

"ADAPTIVE" NK-CELL RESPONSES TO CMV

The conventional view of NK cells as short-lived innate lymphocytes, unable to retain any kind of memory has been considerably challenged in the last years, based on several studies demonstrating that NK cells are capable of adapting to viruses and keep memory of past infections (Sun and Lanier, 2009; Sun et al., 2011, 2014; Della Chiesa et al., 2015, 2016). Interestingly, the first evidence that NK cells can develop memory responses

to pathogens was against the Herpesvirus CMV, initially in mice (Hadinoto et al., 2009) and later on in humans (Della Chiesa et al., 2012; Foley et al., 2012b; Muccio et al., 2016).

In CMV-seropositive individuals a striking expansion of NK cells expressing the HLA-E-specific activating receptor CD94/NKG2C was observed 15 years ago (Guma et al., 2004). Further studies on NK cells developing in hematopoietic stem cell transplation (HSCT) recipients showed that indeed CMV is a powerful driver of NK cell differentiation favoring the expansion of $KIR^+NKG2A^-LILRB1^+$ mature NK cells expressing the marker of terminal differentiation CD57 (Della Chiesa et al., 2012; Foley et al., 2012a,b; Locatelli et al., 2018).

In the HSCT setting the CMV-induced reconfiguration also revealed features typical of adaptive immunity, i.e., virus-induced clonal expansions and long-term persistence that led to the concept of "adaptive" or "memory" NK cells (Sun et al., 2011; Della Chiesa et al., 2016; Rolle and Brodin, 2016). This peculiar CMV-driven NK cell subset is characterized by epigenetic modifications, altered expression of signaling molecules and transcription factors that modulate their phenotype and function (Luetke-Eversloh et al., 2014; Lee et al., 2015; Schlums et al., 2015). The generation of this population likely involves interactions between NKG2C and its ligand HLA-E that usually binds peptides derived from HLA-I leader sequences. However, in CMV-infected cells, HLA-I molecules are downregulated by viral evasion mechanisms, while HLA-E can be stabilized and upregulated by peptides derived from the viral-encoded protein UL40 leader sequence, thus stimulating $NKG2C^+$ NK cells and favoring adaptive NK cells expansion (Guma et al., 2006; Rolle et al., 2014). Interestingly, recent studies demonstrated that $NKG2C^+$ NK cells can distinguish subtle differences between peptides bound to HLA-E molecules, showing stronger responses to a particular peptide derived from rare variants of CMV-encoded UL40, precisely mimicking the peptide derived from HLA-G leader sequence (Hammer et al., 2018a; Rolle et al., 2018). This peptide-specificity and the avidity selection of NK cells during CMV infection recently reported in mice (Adams et al., 2019), further support the concept that CMV recognition by NK cells can elicit responses akin to T cell-adaptive responses.

In addition to NKG2C-HLA-E interactions, CD2-costimulation, and different cytokines such as IL-12, IL-18, and IL-15 are involved in adaptive NK cells generation and proliferation (Hammer et al., 2018a; Rolle et al., 2018).

Upon CMV-induced reconfiguration, NK cells display specialized effector function, showing in particular enhanced ADCC abilities. This increased response to Ab-coated targets has been associated to the downregulated expression of the signaling protein FcεRγ which represents a common feature in CMV-adapted NK cells (Lee et al., 2015; Schlums et al., 2015; Muntasell et al., 2016; Muccio et al., 2018). Although the generation of this subset seems to be promoted exclusively by CMV, its increased ability to eliminate Ab-coated infected cells through enhanced ADCC could keep under control infections and reactivations caused by other viruses, as suggested by studies reporting efficient ADCC-mediated killing of opsonized EBV- and HSV-infected targets by adaptive $NKG2C^+$ NK cells (Costa-Garcia et al., 2015; Moraru et al., 2015).

Interestingly, adaptive $NKG2C^+$ NK cells are also capable of presenting CMV antigens through HLA-DR to autologous memory CD4 T cells (Costa-Garcia et al., 2019), regulating T-cell mediated adaptive responses to CMV and possibly contributing to control viral reactivations.

Besides the central role played by NKG2C, aKIRs are also involved in CMV recognition and generation of adaptive responses (Beziat et al., 2013; Della Chiesa et al., 2015). Indeed, CMV infection can promote the expansion of mature NK cells expressing aKIRs in patients receiving Umbilical Cord Blood transplants from $NKG2C^{-/-}$ donors, thus lacking NKG2C expression (Della Chiesa et al., 2014a). The involvement of aKIRs is in line with observations in mice where NK cells expressing the activating receptor Ly49H, homolog of aKIR, expand in response to MCMV infection and confer long-term protection to secondary challenges through the recognition of the viral-encoded ligand m157 (Arase et al., 2002; Hadinoto et al., 2009). Moreover, in humans, a reduced risk of CMV reactivation was associated to the presence of aKIRs in both hematological and solid organ transplant patients supporting their role in anti-viral defense (Stern et al., 2008; Zaia et al., 2009; Mancusi et al., 2015). The exact mechanisms underlying the recognition of infected cells by aKIRs has not been precisely elucidated, however a role for KIR2DS1 in the recognition of its ligand HLA-C2, modified by CMV in infected fibroblasts, has been recently reported (van der Ploeg et al., 2017). Interestingly, KIR2DS1 tetramers were also described to efficiently interact with EBV-infected B cells expressing HLA-C2 (Stewart et al., 2005; **Figure 1C**).

Notably, in individuals lacking both NKG2C and aKIRs, CMV infection can still favor NK cell reconfiguration indicating that additional unknown mechanisms are responsible for CMV recognition and adaptive NK cell differentiation (Muntasell et al., 2016).

While in mice it has been reported that NK cells can maintain memory of prior encounters with HSV-2 and protect from reactivations (Abdul-Careem et al., 2012), in humans few reports suggest that Herpesviruses other than CMV can induce the generation of specific NK cell subsets with memory properties. Upon EBV infection an expansion of $CD56^{bright}NKG2A^+CD62L^-$ NK cells was observed in tonsils (Lunemann et al., 2013), whereas $CD56^{dim}NKG2A^+KIR^-$ NK cells accumulated in peripheral blood during infectious mononucleosis and were involved in lytic EBV-infected B cells elimination (Azzi et al., 2014). However, at variance with CMV-induced expansions, EBV-induced NK cells were not bearing a specific activating receptor and evidences for their epigenetic reprograming has not been provided (Chijioke et al., 2016).

Further studies are necessary to investigate the impact of NK-Herpesvirus interactions in inducing adaptive NK cell subsets outside the CMV context. The possibility to generate virus-specific NK cell populations could help in designing novel vaccine protocols against Herpesviruses, considering that only anti-VZV vaccines have been successfully developed (Arnold and Messaoudi, 2017). However, in the generation of novel vaccines, it should be considered that prolonged exposure to both VZV and HSV-1 can directly impair NK-cell effector function,

through still unknown mechanisms, as recently described (Campbell et al., 2019).

CLINICAL AND BIOLOGICAL PERSPECTIVE AND CONCLUDING REMARKS

As mentioned above, major defects in NK cell function in respect to human Herpesviruses have been described and become overwhelmingly manifest during primary infections that may be lethal upon first host-virus encounter [e.g., SAP defects, NK cell deficiencies (Sayos et al., 1998; Orange, 2002; Etzioni et al., 2005; Notarangelo and Mazzolari, 2006; Mace and Orange, 2019)]. These cases represent a very limited part of Herpesvirus-induced clinical syndromes, since most primary infections are controlled by the immune system often as asymptomatic infections and latency ensues in the vast majority of patients without further clinical reactivations in >70% of infected subjects in the absence of secondary immunodeficiencies (e.g., HIV infection, transplantation, immunosuppression) (Clark and Griffiths, 2003; Ljungman et al., 2011; Locatelli et al., 2016). For this reason, most NK cell evasion mechanisms are less relevant during this acute phase of primary infection. Herpesvirus latency (e.g., HSV/VZV in neuronal ganglia, EBV in B cells and epithelial cells or CMV in organ and BM macrophages) has been long considered a period of antigenic eclipse to the immune system, while reactivation with clinical symptoms (e.g., recurrent HSV, Zoster or shingles, transformation by EBV or KSHV) represent a possible failure of the immune system to control viral latency. Most virus-induced strategies to evade NK cell (and/or T cell) control may be active during these "clinical escape" or reactivation phases. This perspective, however, needs to be carefully reevaluated in view of the overwhelming evidence showing that exit from latency or virus reactivation routinely occurs for all Herpesviruses in infected hosts at subclinical levels (Ling et al., 2003; Hadinoto et al., 2009; Schiffer et al., 2009; Tronstein et al., 2011). Thus, clinically latent Herpesvirus infection actually has a continuous component of persistent immune stimulation due to virus replication in part of the infected cells pool. In this context, virus evasion mechanisms are likely to occur continuously, and are quantitatively more frequent and relevant than during primary infection. Indeed, the magnitude of the specific T cell response during CMV clinical latency is surprisingly high, with 10–20% of CD4 and CD8 CMV-specific circulating T cells, and 5–15% of $NKG2C^+$ memory-like NK cells during clinical latency (Guma et al., 2004; Sylwester et al., 2005). For example, during latent EBV infection 5–10% of peripheral CD8 T cells are specific for latent or lytic epitopes (Tan et al., 1999; Hislop et al., 2002) and 20% of tonsil lymphocytes are EBV-specific (Hislop et al., 2005).

In view of these considerations, and of the participation of persistent Herpesvirus infection to the modulation of autoimmune, allergic, atopic and atherosclerotic events, Herpesviruses and the host may be regarded from an evolutionary-ecologic perspective as co-evolved symbionts with an evolutionary relationship (Virgin et al., 2009; Roossinck, 2011). It will be critical for future scientific focus to more precisely dissect which NK cell evasion mechanisms are functional to maintain this symbiontic equilibrium, from those that actually determine more severe, clinically relevant reactivations, particularly in immunosuppressed patients or in those with virus-induced tumor (e.g., NHL and KS).

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Host Intrinsic and Innate Intracellular Immunity During Herpes Simplex Virus Type 1 (HSV-1) Infection

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When host cells are invaded by viruses, they deploy multifaceted intracellular defense mechanisms to control infections and limit the damage they may cause. Host intracellular antiviral immunity can be classified into two main branches: (i) intrinsic immunity, an interferon (IFN)-independent antiviral response mediated by constitutively expressed cellular proteins (so-called intrinsic host restriction factors); and (ii) innate immunity, an IFN-dependent antiviral response conferred by IFN-stimulated gene (ISG) products, which are (as indicated by their name) upregulated in response to IFN secretion following the recognition of pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs). Recent evidence has demonstrated temporal regulation and specific viral requirements for the induction of these two arms of immunity during herpes simplex virus type 1 (HSV-1) infection. Moreover, they exert differential antiviral effects to control viral replication. Although they are distinct from one another, the words "intrinsic" and "innate" have been interchangeably and/or simultaneously used in the field of virology. Hence, the aims of this review are to (1) elucidate the current knowledge about host intrinsic and innate immunity during HSV-1 infection, (2) clarify the recent advances in the understanding of their regulation and address the distinctions between them with respect to their induction requirements and effects on viral infection, and (3) highlight the key roles of the viral E3 ubiquitin ligase ICP0 in counteracting both aspects of immunity. This review emphasizes that intrinsic and innate immunity are temporally and functionally distinct arms of host intracellular immunity during HSV-1 infection; the findings are likely pertinent to other clinically important viral infections.

Keywords: intracellular immunity, innate, intrinsic, HSV-1, ICP0, antiviral, interferons, PML-NBs

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INTRODUCTION

Intracellular immunity represents the front line of host defense against herpes simplex virus type 1 (HSV-1) infection, as for other invading pathogens. HSV-1 is a highly contagious virus that infects approximately 3.7 billion people under the age of 50 worldwide (Looker et al., 2015). It is mainly transmitted via direct contact with infected individuals but the virus can also pass from

infected pregnant mothers to their infants (Kriebs, 2008; Looker et al., 2017). The infection is usually asymptomatic or associated with mild symptoms (e.g., cold sores). However, it can lead to serious or even life-threatening outcomes (e.g., keratitis and encephalitis) in neonates and immunocompromised individuals (Simmons, 2002; Herget et al., 2005; Whitley and Baines, 2018). Epithelial cells are the primary sites for lytic replication. The virus is then transported to the trigeminal ganglia of infected hosts, where it establishes a lifelong latent infection. Periodic viral reactivation causes episodes of recurrent disease with variable severity, and this allows transmission to new hosts (Grinde, 2013). Due to its key role in determining the outcomes of infection, the molecular basis of host intracellular immunity during HSV-1 infection has been extensively studied. The current knowledge in the field enables this multifaceted system to be divided into two distinct branches: (i) intrinsic and (ii) innate immunity. However, by carefully reading the literature, these two terms (intrinsic and innate) have been found to be interchangeably and/or simultaneously used in many instances. Hence, the main aim of this review is to highlight the distinction and summarize the differences between intrinsic and innate immunity. The nature, orchestration, induction requirements, antiviral effects, and viral counteraction of these two arms of immunity are discussed. To delve into these concepts, it is important to initially start with a brief overview of the virion structure and replication cycle.

VIRION STRUCTURE

The HSV-1 virion is a spherical particle with an average diameter of 186 nm (Grunewald et al., 2003). It comprises four components: the core, capsid, tegument, and envelope (**Figure 1**). The core contains a linear double-stranded DNA (dsDNA) genome packaged as a toroid or spool (Furlong et al., 1972; Zhou et al., 1999). However, in the absence of protein synthesis, this linear DNA is circularized rapidly after nuclear entry (Poffenberger and Roizman, 1985). Complete genome sequencing revealed that the HSV-1 genome is approximately 152 kb in size, comprises 68.3% guanine and cytosine, and exhibits little variation among strains. The viral genome consists of two elements: unique long (UL) and unique short (US) regions bracketed by inverted repeats ab and b′a′, and ac and c′a′, respectively (Wadsworth et al., 1975). The core is surrounded by an icosahedral capsid composed of 162 capsomers (Schrag et al., 1989). The polyamines spermidine and spermine in the core neutralize the negative charge on the viral DNA (vDNA), which allows proper folding of the vDNA within the capsid (Gibson and Roizman, 1971). The protein matrix between the outer surface of the capsid and the undersurface of the envelope is called the tegument. It is highly unstructured and comprises more than 20 viral proteins that have been identified by biochemical assays and proteomics analysis (Roller and Roizman, 1992; Zhou et al., 1999; Loret et al., 2008). Tegument proteins regulate many aspects of viral infection, including entry into target cells, nuclear delivery of the viral genome, regulation of viral gene expression, assembly and egress of progeny virions, and host immune evasion (Kelly et al., 2009). The tegument is enclosed in the viral envelope, which consists of a lipid bilayer derived from host cells, with spike-like glycoprotein projections embedded in it (Spear and Roizman, 1967). Thirteen glycosylated envelope proteins (gB–E, and gG–N) and at least two non-glycosylated envelope proteins (UL20 and Us9) have been identified, and they are particularly important for HSV-1 attachment to target cells (Loret et al., 2008).

VIRAL LYTIC REPLICATION CYCLE

Epithelial cells represent the primary sites of HSV-1 lytic replication. The replication cycle is initiated when HSV-1 attaches to target cells via interactions between viral glycoproteins and cellular receptors (e.g., heparan sulfate glycosaminoglycans, nectin, herpesvirus entry mediator, and 3-O-sulfated heparin sulfate) (Spear et al., 1992; Geraghty et al., 1998; Warner et al., 1998; Shukla et al., 1999). These interactions enable fusion of the viral envelope with the cellular plasma membrane, and they thereby allow viral entry (Avitabile et al., 2007; Satoh et al., 2008; Gianni et al., 2009). HSV-1 also utilizes endocytosis to enter into some cell types (Nicola, 2016). In the cytoplasm of infected cells, the nucleocapsid is transported to a nuclear pore through the microtubular network. The vDNA remains encapsidated until it is released through a nuclear pore into the nucleus, where it initiates temporally regulated transcription/translation processes, leading to the production of viral immediate early (IE), early (E), and late (L) proteins (Miyamoto and Morgan, 1971; Honess and Roizman, 1974; Kristensson et al., 1986; Sodeik et al., 1997; Wolfstein et al., 2006; Radtke et al., 2010). IE protein (ICP0, ICP4, ICP22, ICP27, and ICP47) expression is mediated by a virion-associated tegument protein, VP16, and cellular factors [e.g., host cell factor 1 (HCF-1) and octamer-binding protein 1 (Oct-1)] (Triezenberg et al., 1988; Stern et al., 1989; Wysocka and Herr, 2003). *De novo* synthesis of IE proteins promotes the expression of E viral genes, which collectively provide the necessary components for triggering vDNA replication. Seven viral gene products have been shown to be essential for vDNA replication: the origin-binding protein UL9, vDNA polymerase catalytic subunit UL30 and its processivity factor UL42, the multifunctional single-stranded (ss) DNA-binding protein ICP8, and the helicase–primase complex (UL5, UL8, and UL52). vDNA replication starts with theta replication and then switches to a rolling circle mechanism, generating long concatemers (Rabkin and Hanlon, 1990; Wilkinson and Weller, 2003). vDNA replication in cooperation with IE proteins stimulates the expression of L proteins (e.g., capsid proteins VP5, VP21, VP23, VP24, and VP26), which enables nucleocapsid assembly and vDNA packaging. The long concatemers are cleaved into unit-length monomers and packaged into capsids (Heming et al., 2017). Newly synthesized nucleocapsids acquire their tegument and some glycoproteins during primary and secondary envelopment, enabling the release of mature infectious progeny virions (Skepper et al., 2001; Owen et al., 2015). An overview of the HSV-1 replication cycle is shown in **Figure 2**.

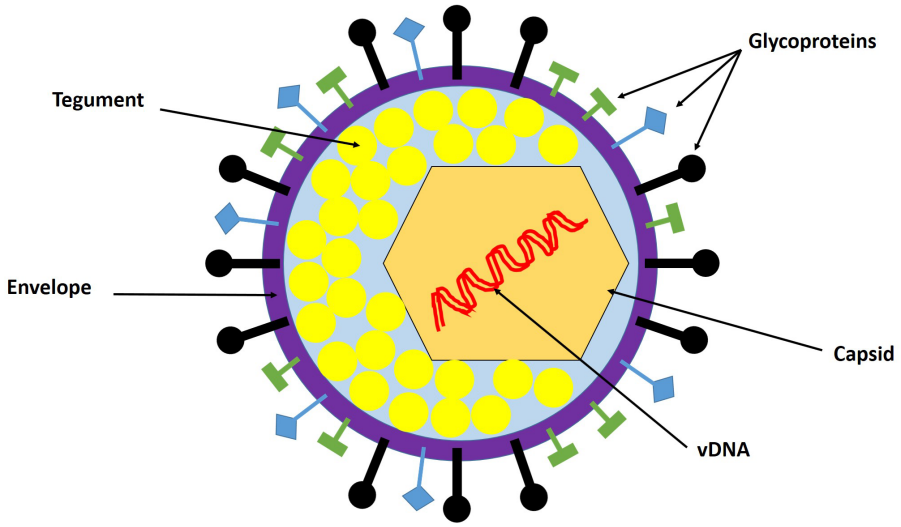

FIGURE 1 | HSV-1 virion structure. The virion is composed of the viral genome, capsid, tegument, and envelope. The viral genome is a linear double-stranded DNA (dsDNA) enclosed in the capsid. The tegument is the protein matrix between the capsid and the envelope. The envelope is a lipid bilayer membrane with glycoprotein projections embedded in it. Adapted with permission from Alandijany (2018).

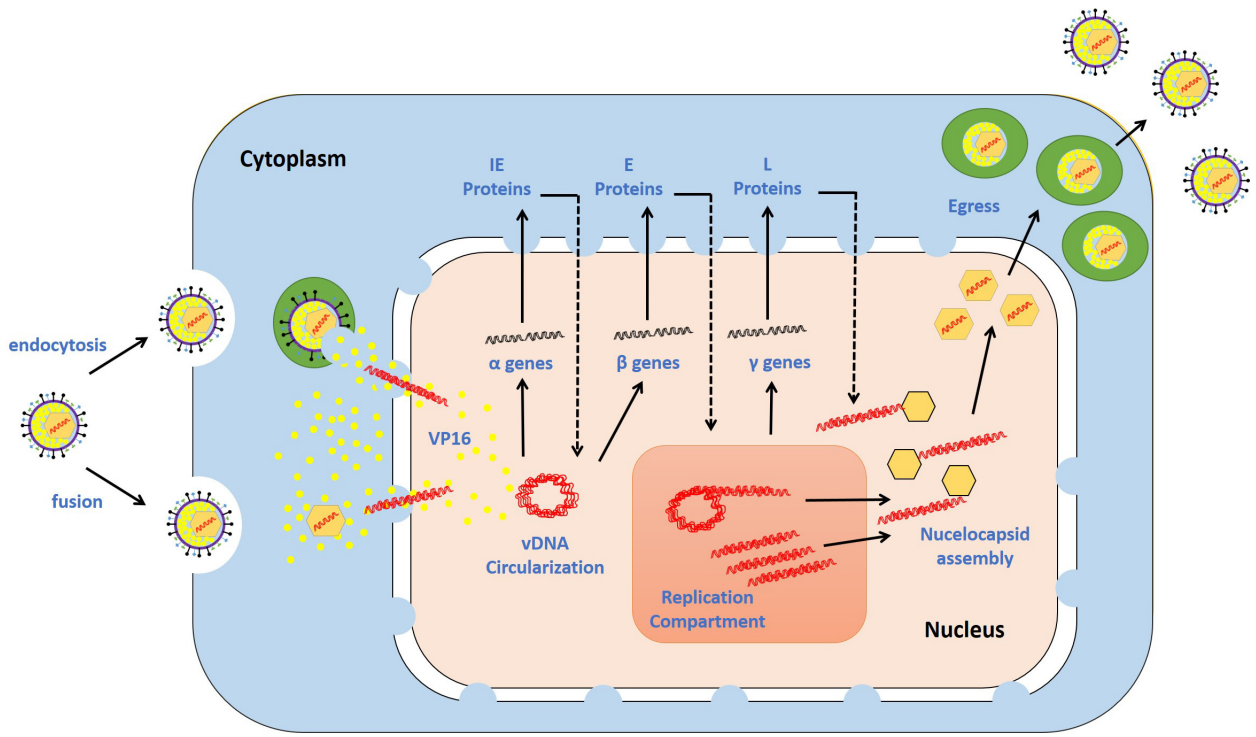

FIGURE 2 | HSV-1 replication cycle. The virus attaches via glycoproteins to cellular receptors. It enters the cells via the fusion of the viral envelope with the plasma membrane or endocytosis. The de-enveloped nucleocapsid is transported to the nuclear pores, and the viral DNA (vDNA) is ejected into the nucleus. The viral genes are transcribed in a temporal cascade: immediate early (IE), early (E), and late (L) proteins. IE protein expression is turned on by the virion-associated protein VP16. E proteins require IE protein synthesis for their expression and play critical roles in triggering vDNA replication. The hypothesized mechanisms underlying vDNA replication involve theta replication followed by rolling circle replication. L protein expression is dependent on vDNA replication. The capsid is assembled at sites adjacent to vDNA replication compartments, permitting the insertion of vDNA into the capsid. The nucleocapsid buds through the nuclear membrane, is transported through the cytoplasm, and fuses with the plasma membrane. During this journey, the nucleocapsid acquires tegument and envelope proteins. The release of mature progeny virions promotes attachment to new cells, and the cycle continues. Adapted with permission from Alandijany (2018).

HOST INTRACELLULAR IMMUNITY

During HSV-1 infection, intracellular immunity plays a central role in determining the fate of incoming virions and, therefore, the consequences of infection (Komatsu et al., 2016). Based on the nature of the effector proteins, induction requirements, and effects on viral replication, host intracellular immunity can be broadly divided into (i) intrinsic and (ii) innate immunity.

Intrinsic Immunity

Intrinsic immunity is mediated by constitutively expressed host cell restriction factors that can directly and immediately act to control the viral gene expression. The hallmarks of this arm of immunity include the high likelihood of being counteracted by viral proteins, cell specificity, and the potential to be saturated under high multiplicity of infection (MOI) conditions in the absence of viral countermeasures (Bieniasz, 2004; Yan and Chen, 2012; Boutell and Everett, 2013).

A breakthrough in studying intrinsic immunity during HSV-1 infection was the use of an HSV-1 mutant with a null mutation in the viral E3 ubiquitin ligase ICP0 (ΔICP0), which grows poorly under low MOI conditions in some cell types (Stow and Stow, 1986; Sacks and Schaffer, 1987). Indeed, compared with wild-type (WT) virus, ΔICP0 HSV-1 demonstrates a severe replication defect in fibroblast and keratinocytes (∼1000 fold). This replication defect is moderate in cells such as BHK and Vero cells (30–100 fold), while it is almost absent in U2OS and SAOS cells (in which it replicates as efficiently as WT virus). Cells are described as restrictive, semi-permissive, and permissive based on their ability to intrinsically restrict ΔICP0 HSV-1 replication (Yao and Schaffer, 1995; Everett et al., 2004a). Historically, permissive cell lines have been utilized to accurately determine the viral titer of both ΔICP0 and WT HSV-1 stocks, while restrictive cells have been used to investigate host immunity to HSV-1 infection. Importantly, the intrinsic antiviral response to ΔICP0 HSV-1 becomes saturated and no longer effective at increased MOI conditions (Everett et al., 2004a, 2013). A study conducted on human fibroblasts demonstrated that, under low MOI conditions (0.2–1 plaque forming unit (PFU)/cell based on the viral titer in U2OS), ΔICP0 HSV-1 was able to initiate plaque formation only in a minor proportion of infected cells, while the viral genomes remained quiescent in the majority of cells. Correspondingly, at equivalent genome input levels, the gene expression of ΔICP0 HSV-1 was severely restricted in comparison to the gene expression of WT virus. However, the restriction of ΔICP0 HSV-1 replication was relieved under higher MOI conditions (5–10 PFU/cell), leading to a level of replication that was similar to WT virus replication (Everett et al., 2004a). Combined, these studies demonstrated that intrinsic immunity renders some cell types restrictive to HSV-1 infection under low MOI conditions and in the absence of ICP0, which acts as a viral countermeasure.

Numerous studies were conducted on restrictive cell types to identify intrinsic restriction factors. Examples of these include promyelocytic leukemia protein-nuclear body (PML-NB) constituent proteins (e.g., promyelocytic leukemia, PML; speckled 100 kDa, Sp100; death domain associated protein, Daxx; alpha thalassemia/mental retardation syndrome X-linked, ATRX; and MORC family CW-type zinc finger 3, MORC3), E3 SUMO ligases [e.g., protein inhibitor of activated STAT (PIAS) 1 and 4; PIAS1 and PIAS4, respectively], DNA repair proteins (e.g., ring finger protein-8 and -168; RNF8 and RNF168, respectively), and epigenetic regulators (e.g., repressive histones) (**Figure 3**).

PML-NB Constituent Proteins

PML-NBs, as indicated by their name, are highly dynamic bodies found in the nuclei at about 1–30 PML-NBs per nucleus. They comprise over 70 permanent resident proteins in addition to many transient proteins actively associating and dissociating from these bodies. PML-NB constituent proteins (e.g., PML, SP100, Daxx, ATRX, and MORC3) are involved in the regulation of many cellular processes, including the cell cycle, DNA damage response (DDR), DNA repair, apoptosis, and metabolism (Hsu and Kao, 2018).

Over two decades ago, a disappearance of PML-NBs following HSV-1 infection was observed, which was linked to the expression of the viral IE protein ICP0 (Maul and Everett, 1994). Fluorescence *in situ* hybridization (FISH) experiments demonstrated that, upon nuclear entry, infecting HSV-1 genomes localize at or adjacent to PML-NBs (Maul et al., 1996). This localization was prominent during the initial stage of ΔICP0 HSV-1 infection of cells at the edge of developing plaques (Everett et al., 2004b). In these infected cells, rapid recruitment of PML-NB constituent proteins to dot-like complexes of the vDNA-binding protein ICP4 were observed, with an asymmetric distribution of PML-NB puncta that is distinct from that observed in non-infected cells (Everett et al., 2004b). These viral-induced complexes were shown to contain incoming viral genomes (Everett and Murray, 2005). It was initially unclear whether this phenotype reflected a beneficial or detrimental effect on viral infection. However, accumulating evidence has now conclusively revealed that multiple PML-NB constituent proteins act as host intrinsic restriction factors that restrict ΔICP0 HSV-1 infection.

Utilizing short hairpin RNA technology, individual knockdown of the PML-NB proteins PML, SP100, Daxx, ATRX, or MORC3 clearly enhanced the plaque-forming efficiency and viral protein expression of ΔICP0 HSV-1, but not WT HSV-1 (Everett et al., 2006, 2008a; Lukashchuk and Everett, 2010; Sloan et al., 2016). Although some of these PML-NB proteins are known to be upregulated in response to interferon (IFN), these proteins were found to mediate intrinsic viral restriction independently of IFN production and signaling (Everett et al., 2008b). Moreover, double and triple depletion of PML, SP100, and Daxx additively enhanced the plaque formation of ΔICP0 HSV-1, demonstrating cooperative action to restrict viral replication (Glass and Everett, 2013). However, this additive effect was not sufficient to fully rescue the plaque-formation efficiency of ΔICP0 HSV-1 to the WT virus level, indicating the presence of additional intrinsic restriction factors.

The mechanism underlying PML recruitment to infecting viral genomes is dependent on the SUMO pathway (Cuchet-Lourenco et al., 2011; Everett et al., 2013; Hannoun et al., 2016). Among PML isoforms (PML.I–VI), PML.VI fails to associate

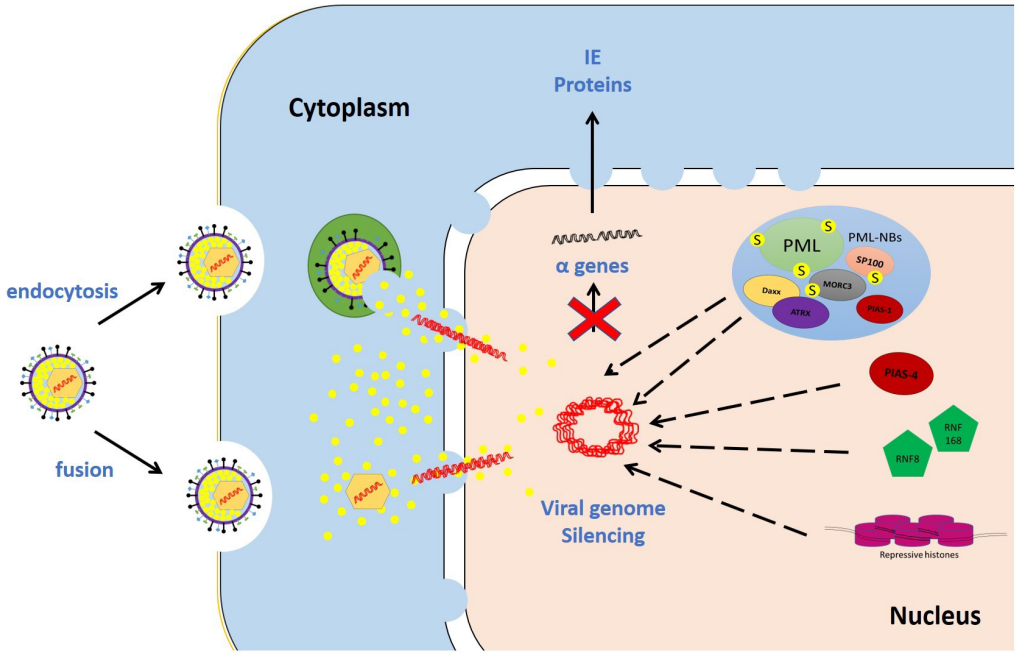

FIGURE 3 | Examples of intrinsic restriction factors that combat HSV-1 infection. As soon as the viral genomes are delivered to the nucleus, intrinsic restriction factors are rapidly recruited to the incoming viral genomes. In the absence of viral countermeasures and under low multiplicity of infection (MOI) conditions, these restriction factor recruitment events induce viral genome silencing. Promyelocytic leukemia protein-nuclear body (PML-NB) constituent proteins (e.g., promyelocytic leukemia, PML; speckled 100 kDa, Sp100; death domain associated protein, Daxx; alpha thalassemia/mental retardation syndrome X-linked, ATRX; and MORC family CW-type zinc finger 3, MORC3), E3 SUMO ligases (e.g., protein inhibitor of activated STAT 1 and 4; PIAS1 and PIAS4, respectively), DNA repair proteins (e.g., ring finger protein-8 and -168; RNF8 and RNF168, respectively), and repressive histones are examples of host intrinsic restriction factors.

with the viral-induced foci due to a lack of exon 7a that contains SUMO-interacting motif (SIM). Moreover, mutations of SIMs in PML.I and PML.IV negatively influenced their recruitment to viral-induced foci. Consistent with the correlation between PML recruitment to viral genomes and viral repression, the relief in PML-depleted cells of the restriction of ΔICP0 HSV-1 was reversed following reconstitution of WT PML.I, but not PML.I SIM mutants. Similar to PML SIM mutants, PML.I and PML.IV carrying a single or multiple mutation(s) at major SUMOylation sites (K65, K160, K490, and K616) were less efficiently recruited to viral genome foci. Sp100 and Daxx recruitment to viral foci were also found to occur in a SIM-dependent manner (Cuchet-Lourenco et al., 2011, 2012). Correspondingly, depletion of Ubc9, the sole SUMO E2 conjugating enzyme, impaired the recruitment of PML-NB restriction factors to ΔICP0 HSV-1 genomes and enhanced plaque formation (Boutell et al., 2011). These studies collectively demonstrated the key role of the host SUMO pathway in the regulation of PML-NB-mediated intrinsic antiviral immunity.

Protein Inhibitor of Activated STAT (PIAS) 1 and 4

PIAS is a family of SUMO E3 ligases that facilitate the third enzymatic step of the SUMO pathway (Rytinki et al., 2009). PIASs have been mainly known for their role as suppressors of innate immune signaling. Recently, novel roles for them as intrinsic restriction factors that combat HSV-1 infection have been identified (Brown et al., 2016; Conn et al., 2016). Among the members of the PIAS family, PIAS1 is the only type that has been shown to be a permanent constituent PML-NB protein (Brown et al., 2016). However, both PIAS1 and PIAS4 play a key role in mediating the intrinsic antiviral response to HSV-1 infection (Brown et al., 2016; Conn et al., 2016). By conducting the classic plaque edge assay, it was found that both PIAS1 and PIAS4 were recruited to the infecting viral genomes at the nuclear periphery of newly infected cells in a SIM-dependent manner. Depletion of PIAS1 or PIAS4 individually enhanced the plaque formation of ΔICP0 HSV-1 while WT HSV-1 plaque formation remained unaffected. Simultaneous depletion of PIAS1 and PIAS4, or PML with either one of the PIASs, additively enhanced the plaque formation of ΔICP0 HSV-1, demonstrating that PIAS1, PIAS4, and PML act cooperatively to mediate the intrinsic antiviral response to HSV-1 infection (Brown et al., 2016; Conn et al., 2016).

DNA Damage Response Proteins

The main function of the DDR machinery is to maintain the integrity of the host genomic DNA and ensure the fidelity of replication (Jackson and Bartek, 2009). Ring finger protein-8 and -168 (RNF8 and RNF168, respectively) play key roles in recruiting repair factors to sites of DNA damage (Mailand et al., 2007; Doil et al., 2009). During WT HSV-1 infection, RNF8 and RNF168 are targeted for degradation by the viral E3 ubiquitin ligase ICP0 (Lilley et al., 2010). In the absence of ICP0, however, RNF8 and RNF168 in addition to other DNA repair factors (e.g.,

p53-binding protein 1, and breast cancer-1) have been shown to be redistributed to sites adjacent to newly infecting viral genomes in cells at the edge of developing plaques (Lilley et al., 2011). This recruitment phenotype occurs independently of the presence of PML and Daxx, and it raises the hypothesis that RNF8 and RNF168 are involved in the intrinsic antiviral response. Indeed, regarding ΔICP0 HSV-1 infection of RNF8−/− mouse embryonic fibroblasts, the plaque-forming efficiency and viral gene expression were clearly reduced following transduction with retrovirus expressing human RNF8 compared to transduction with an empty retrovirus vector. Initial reduction in the gene expression of WT virus was observed as a result of RNF8 ectopic expression, but this effect was recovered as the infection progressed, probably due to ICP0-induced degradation of RNF8 (Lilley et al., 2010, 2011). These studies added RNF8 and RNF168 to the growing list of host restriction factors that mediate the intrinsic antiviral response to HSV-1 infection.

Epigenetic Regulators

Several studies have reported associations of the viral genomes with nucleosomes and their components during viral latency (Deshmane and Fraser, 1989; Maroui et al., 2016; Cohen et al., 2018). The nucleosome is the basic unit of chromatin and is composed of 146 bp of DNA wrapped around a histone octamer (an H3-H4 histone protein tetramer that interacts with two H2A-H2B dimers via two H2B–H4 associations). Linker histone (H1) variants also bind to nucleosomes and mediate chromatin compaction. These epigenetic regulators were found to confer an intrinsic antiviral response to HSV-1 lytic infection (Knipe, 2015).

As early as 1 h post-infection (hpi), core histones (H3) with repressive marks [e.g., H3 lysine 9-trimethylation (H3K9me3) and H3 lysine 27-trimethylation (H3K27me3)] were associated with the incoming viral genomes in a manner that increased over time (Kent et al., 2004; Oh and Fraser, 2008; Lee et al., 2016). Independent studies demonstrated mobilization and association of core histones (H2B and H4) and linker histones (H1 variants) with the vDNA upon entry into the nucleus. Histone mobilization occurs independently of viral gene expression. Nevertheless, the expression of IE and E genes, but not vDNA replication or L gene expression, promotes this process (Conn et al., 2008, 2011, 2013). In the absence of viral countermeasures (e.g., ICP0 and VP16), recruitment of repressive histones induced viral genome chromatinization and silencing. The histone chaperone HIRA and chromatin remodeling protein ATRX were found to be important in this process (Rai et al., 2017; Cabral et al., 2018). HIRA is localized to PML-NBs upon viral infection and deposits H3 variants (H3.3) onto incoming vDNA, and ATRX stably maintains vDNA heterochromatin, leading to intrinsic restriction of viral replication (Rai et al., 2017; Cabral et al., 2018).

Epigenetic regulators are also involved in impairing the viral infection during the transition from IE to E protein expression (Gu et al., 2005; Gu and Roizman, 2007). Indeed, many infected cells express IE proteins during ΔICP0 HSV-1 infection but the infection becomes stalled at this stage (Everett et al., 2004a). This phenotype is partly mediated by the RE1-silencing transcription factor (REST)/CoREST/histone deacetylases (HDAC) nuclear repressor complex. Inhibitors of HDAC and a mutant CoREST lacking the HDAC1 binding site both enhanced the transition of viral gene expression and viral replication in the absence of ICP0, again highlighting the key role of epigenetic regulators as intrinsic restriction factors that can combat HSV-1 infection (Gu et al., 2005; Gu and Roizman, 2007).

Innate Immunity

Innate immunity, unlike constitutive intrinsic immunity, is mediated by cellular proteins induced in response to IFNs, a family of proinflammatory cytokines that play central antiviral roles during HSV-1 infection (Mossman and Ashkar, 2005; Chew et al., 2009; Knipe, 2015). IFNs are classified into three main types depending on the receptors utilized for signaling: (i) IFN type I (IFN-I) comprises IFNα, β, ε, κ, and ω, which utilize IFNα receptors 1 and 2 (IFNAR1/2) (Gonzalez-Navajas et al., 2012), (ii) IFN type II (IFN-II) comprises IFNγ, which utilizes the IFNγ receptor (IFNGR) (Platanias, 2005), and (iii) IFN type III (IFN-III) comprises IFNλ1, λ2, and λ3 (IL-28A, IL28B, and IL29, respectively), which utilize the IFNλ receptor (IFNLR), also known as IL 28 receptor α (IL-28Rα) and IL-10 receptor β (IL-10Rβ) (Zanoni et al., 2017). Some cell types can produce and respond to more than one type of IFN while others are predominantly responsible for a specific type of IFN expression and signaling (Lee and Ashkar, 2018; Lazear et al., 2019). The induction of an IFN response during viral infections, including HSV-1 infection, involves two phases: (a) the first phase is initiated following sensing of pathogen-associated molecular patterns (PAMPs; e.g., viral particles or viral replication products) by pattern recognition receptors (PRRs), leading to the production of IFNs, and (b) the second phase starts when the secreted IFNs bind to their cognate receptors and subsequently activate IFN signaling cascades, resulting in the induction of IFN-stimulated genes (ISGs) whose products establish an antiviral state in the infected cells and neighboring uninfected cells to control the infection (Knipe, 2015; Kurt-Jones et al., 2017).

Importance of IFN Response in Controlling HSV-1 Replication

The role of IFN-I in controlling HSV-1 infection has been extensively studied. Historically, the resistance and susceptibility of different mouse strains to HSV-1 infection were linked to their abilities and efficiencies to induce an IFN-I response (IFNα and IFNβ) (Lopez, 1975; Zawatzky et al., 1982; Halford et al., 2004). Increased viral replication, severe pathogenesis, and reduced survival rates have been observed in mice lacking IFNAR in comparison to WT controls (Leib et al., 1999; Luker et al., 2003). Several *in vitro* studies also highlighted the important role of IFN-I in controlling the replication, spread, and cytopathic effect of HSV-1 (Domke-Opitz et al., 1986; Sainz and Halford, 2002; Rosato and Leib, 2014). Further studies discovered several PRRs and cellular factors required for IFN-I production, characterized the related signaling cascades, and identified ISG products with antiviral properties as well as the viral evasion strategies (reviewed below).

The IFN-II (IFNγ) signaling pathway plays crucial roles in controlling and minimizing the pathogenesis of HSV-1 infection during lytic infection (Bigley, 2014). Mice lacking IFNGR were more vulnerable to HSV-1 infection and had a higher mortality rate than WT mice (Cantin et al., 1995, 1999; Minami et al., 2002). IFNγ was found to synergize with IFN-I during HSV-1 infection, leading to a dramatic reduction in viral replication (Sainz and Halford, 2002; Vollstedt et al., 2004). Correspondingly, mice lacking both IFNAR and IFNGR had increased susceptibility to HSV-1 infection in comparison to mice lacking either one of the receptors individually (Luker et al., 2003). It is also known that IFNγ links the host innate and adaptive immune responses. It stimulates the expression of major histocompatibility complex class I to enhance antigen presentation to $CD8^+$ T cells, which plays a key role in the maintenance of viral latency. Indeed, mice lacking IFNGR displayed higher levels of viral gene expression and replication during reactivation than WT mice (Shaw et al., 1985; Cantin et al., 1999).

IFN-III (IFNλ1–3), the most recently discovered member of the IFN family, has unique receptors (IFNLRs) but utilizes the same signaling cascade as IFN-I (Zanoni et al., 2017; Lazear et al., 2019). Few studies have addressed the role of IFNλ during HSV-1 infection. Exogenous treatment of primary human astrocytes and neurons with IFNλ inhibited the viral gene expression and viral protein synthesis, probably by stimulating the induction of endogenous IFN-I production and ISG expression (Li et al., 2011). Similarly, the subset of plasmacytoid dendritic cells (pDCs) that can produce IFNλ in response to HSV-1 infection were shown to be associated with a higher level of IFNα and a more efficient antiviral response in comparison to cells that failed to produce IFNλ (Yin et al., 2012). Moreover, pretreatment of pDCs with IFNλ resulted in enhanced IFNα production following HSV-1 infection (Yin et al., 2012). These findings indicate that IFN-λ is an autocrine signaling factor that rapidly primes an IFN-I antiviral response in HSV-1-infected cells (Li et al., 2011; Yin et al., 2012). However, the underlying mechanism(s) of IFN-λ-mediated antiviral immunity remains far from being fully understood and requires further study.

Sensing and Recognition of HSV-1 by PRRs

The activation of the first phase of the IFN response is dependent on the ability of PRRs to recognize PAMPs in the infected cells. The interactions between PRRs and their viral ligands leads to the activation of TANK-binding kinase 1 (TBK1) in fibroblasts or inhibitor of nuclear factor kappa B (NFκB) epsilon (IKKε) in immune cells. These protein kinases induce the phosphorylation and activation of IFN regulatory factor 3 and 7 (IRF3 and IRF7) which, in cooperation with other transcription factors, bind to IFN gene promoters and stimulate IFN secretion (Chew et al., 2009). Numerous PRRs have been identified (**Figure 4**). They can recognize and sense virion components (e.g., viral glycoprotein and vDNA) as well as viral replication intermediates and products (e.g., cytosolic dsRNA) (Paludan et al., 2011). However, viral nucleic acid is likely the most potent PAMP inducer of host innate immunity (Paludan and Bowie, 2013; Knipe, 2015; Komatsu et al., 2016).

Toll-like receptors

Toll-like receptors (TLRs) were among the first PRRs to be studied and characterized. They can be found at the plasma membrane (e.g., TLR1, TLR2, and TLR4) and within endosomes (e.g., TLR3, TLR7, TLR8, and TLR9). TLR2, TLR3, and TLR9 have been shown to be critical in controlling HSV-1 replication and dissemination, and their functions appear to be redundant and cell type-specific (Krug et al., 2004; Rasmussen et al., 2007; Zhang et al., 2007; Sorensen et al., 2008; Guo et al., 2011; Cai et al., 2013; Mork et al., 2015; Zhang and Casanova, 2015; Uyangaa et al., 2018). It has been proposed that TLRs on the plasma membrane detect viral glycoproteins while TLRs within endosomes sense viral nucleic acids, though this has not been formally investigated during HSV-1 infection (Ma and He, 2014).

An initial *in vitro* study demonstrated TLR9-dependent IFN-I production in HSV-1-infected pDCs (Krug et al., 2004). A few years later, the antiviral role of TLR9 during HSV-1 infection both *in vivo* and *ex vivo* was confirmed (Rasmussen et al., 2007). WT and TLR9−/− mice were infected with HSV-1, and the level of IFN-I production was measured in the serum and in isolated conventional DCs, pDCs, macrophages, and fibroblasts. In WT mice, IFN-I was detectable in the serum at 8 hpi, peaked at 16 hpi, and diminished at 48 hpi. The initial induction of IFN-I expression (8 hpi) was dependent on the presence of TLR9. However, no difference was noticed at 16 hpi, suggesting a redundant role for TLR9 in cytokine production during HSV-1 infection. Consistently, viral infection induced IFN-I production in all isolated cell types, and only pDCs required TLR9 for this process (Rasmussen et al., 2007). TLR9 was found to synergize with the plasma membrane TLR2 to control viral replication and dissemination to the central nervous system (CNS), although other studies have suggested that TLR2 activation can be immunopathological (Kurt-Jones et al., 2004; Sarangi et al., 2007; Sorensen et al., 2008; Uyangaa et al., 2018). In addition to TLR2 and TLR9, the presence of functional TLR3 is believed to be key for an efficient antiviral response to HSV-1 infection. Several studies demonstrated that herpes encephalitis is associated with TLR3 deficiency or lack of a functional TLR3 (Zhang et al., 2007; Guo et al., 2011; Zhang and Casanova, 2015; Mielcarska et al., 2018). Collectively, these data highlight the important antiviral role of TLRs during HSV-1 infection.

Retinoic acid-inducible gene I (RIG-I) like receptors (RLRs) and DExD/H-box helicases (DHXs)

Melanoma differentiation-associated protein 5 (MDA5) belongs to the RLR family. It serves as a cytosolic double-stranded RNA (dsRNA) sensor and mediates cytokine signaling through its adaptor protein, mitochondrial antiviral signaling protein (MAVS) (Yoneyama et al., 2005). MDA5 preferentially recognizes long dsRNA (>1000 bp) and large RNA aggregates (Kato et al., 2008; Li et al., 2009; Pichlmair et al., 2009). Many viruses including HSV-1 produce dsRNA during replication (Weber et al., 2006). Research demonstrated that HSV-1 infection induced cytokine and chemokine production such as IFN-I, IFN-III, tumor necrosis factor α (TNFα), and C-C motif chemokine ligand 5) in human monocyte-derived macrophages (Melchjorsen et al., 2006, 2010). This process required virus entry

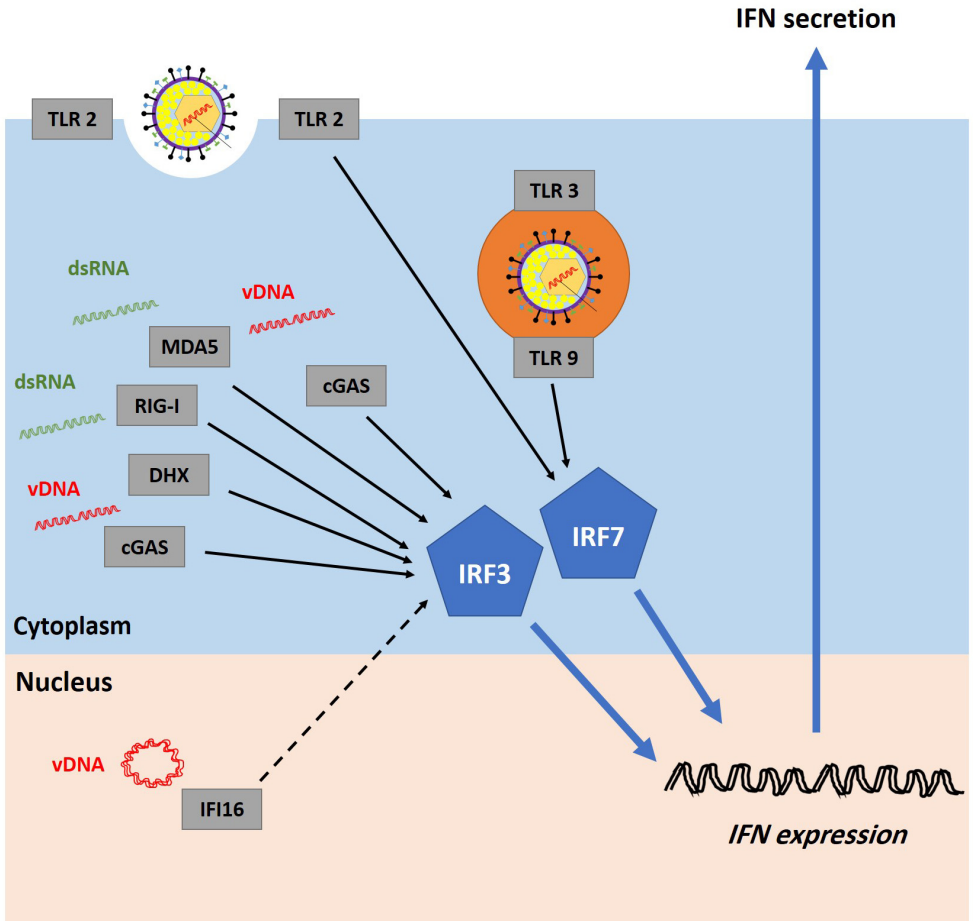

FIGURE 4 | Recognition of HSV-1 infection by pattern recognition receptors (PRRs). Host cells are equipped with several PRRs that can recognize virion components (e.g., glycoprotein and vDNA) and structures accumulated during vDNA replication (e.g., dsRNA). Examples of PRRs include cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS), DExD/H-box helicases (DHX), melanoma differentiation-associated protein 5 (MDA5), retinoic acid-inducible gene I (RIG-I), and toll-like receptors (TLRs). It remains highly controversial whether interferon-gamma-inducible protein 16 (IFI16) can sense incoming vDNA in the nucleus (the dashed line represents uncertainty). PRRs signal through the stimulator of interferon genes (STING)-TANK-binding kinase 1 (TBK1)-IFN regulatory factor 3 and 7 (IRF3/7) pathway to induce interferon (IFN) production. Adapted with permission from Alandijany (2018).

and replication but occurred independently of TLR2 and TLR9. MAVS or MDA5 knockdown led to significantly lower levels of HSV-1-induced IFN-I and IFN-III expression, while RIG-I knockdown did not affect this process (Melchjorsen et al., 2006, 2010). However, an independent study showed that RIG-I and MDA5 synergistically contribute to innate immune recognition of HSV-1 infection and upregulation of IFN-I genes (IFNα and IFNβ) in mouse embryonic fibroblasts and HeLa cells (Choi et al., 2009). Interestingly, this transfection-based study demonstrated that RIG-I and MDA5, known to be RNA sensors, serve as cytosolic DNA sensors and mediate IFN-I expression by activating the IRF3 pathway. Of note, RIG-I and MDA5 also belong to the DHX family. Further research identified other DHXs, namely DHX9 and DHX36, as cytosolic DNA sensors (Kim et al., 2010). The presence of DHX9 and DHX36 was crucial for efficient induction of cytokine and chemokine expression in HSV-1-infected pDCs. While DHX9-mediated sensing induced NFκB activity and TNFα expression, DHX36 activation was associated with IRF7 nuclear translocation and IFNα production (Kim et al., 2010).

Cyclic guanosine monophosphate-adenosine monophosphate (cyclic GMP-AMP, or cGAMP) synthase

Cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS), a member of the nucleotidyltransferase family, has been identified as a cytosolic DNA sensor during HSV-1 infection (Sun et al., 2013). Following the recognition of cytosolic DNA, cGAS promotes cGAMP production, which interacts with stimulator of interferon genes (STING), leading to IRF3 activation and IFNβ production (Wu et al., 2013). The post-translational modification status of cGAS plays a key role in its DNA sensing ability and innate immunity induction (Cui et al., 2017; Wang et al., 2017). cGAS is SUMOylated at different sites: K335, K372, and K382, which suppresses its DNA-binding capacity. Sentrin-specific protease 7 (SENP7) deSUMOylates cGAS and primes it for activation, leading

to efficient IRF3-dependent induction of innate immunity. Knockdown of SENP7 in infected mice impaired IFN secretion and ISG expression, rendering them more vulnerable to HSV-1 infection (Cui et al., 2017). It was also demonstrated that RNF185, the first E3 ubiquitin ligase identified for cGAS, binds and promotes polyubiquitination of cGAS at K27. Similar to SENP7 knockdown, RNF185 knockdown negatively influenced cGAS activity and innate immunity induction during HSV-1 infection (Wang et al., 2017).

Interferon-gamma-inducible protein 16

Interferon-gamma-inducible protein 16 (IFI16), which belongs to the pyrin domain and two DNA-binding hematopoietic interferon-inducible nuclear proteins with 200-amino acids repeat domains (PYHIN) protein family, was initially reported as a cytosolic DNA sensor of transfected foreign DNA derived from the HSV-1 genome (Unterholzner et al., 2010). Short-hairpin RNA-mediated depletion of IFI16 or the mouse ortholog of IFI16 (p204) inhibited IFNβ production in response to DNA transfection. Notably, stimulation of IFI16-mediated sensing was dependent on the foreign DNA length and structure but occurred independently of its nucleotide content (Unterholzner et al., 2010). An independent study also highlighted the key role of IFI16 as a cytosolic DNA sensor in HSV-1-infected macrophages (Horan et al., 2013). HSV-1 capsid proteins are ubiquitinated and targeted for proteasomal degradation in the cytoplasm of infected cells, exposing vDNA to IFI16-mediated sensing and innate immunity induction. At permissive and non-permissive temperatures, TsB7 (an HSV-1 temperature-sensitive mutant whose capsids accumulate in the cytoplasm and fail to release the viral genomes due to a defect in VP1-2 function) induced IFNβ and ISG56 to equivalent levels observed during WT HSV-1 infection. Localization of IFI16 near the TsB7 mutant DNA was also unaffected at the non-permissive temperature. Therefore, it was concluded that the nuclear import of viral genomes in human macrophages was not required for the induction of IFI16-mediated innate immunity (Horan et al., 2013).

Given that IFI16 is predominantly localized to the nucleus of many types of cells (e.g., fibroblast, endothelial, and epithelial cells), subsequent studies investigated whether IFI16 can serve as a nuclear DNA sensor. A multiphasic and dynamic subnuclear redistribution of IFI16 has been identified in HSV-1-infected fibroblasts (Orzalli et al., 2012; Cuchet-Lourenco et al., 2013; Diner et al., 2015; Everett, 2015). As early as 0.5–1 hpi, IFI16 puncta were transiently formed on the nuclear periphery of newly infected cells at the edge of developing plaques. As the infection progressed (approximately 3–4 hpi), IFI16 puncta were observed to assemble in the nucleoplasm of the infected cells. Soon after, these IFI16 signals were lost in WT HSV-1-infected cells, but they remained stable during ΔICP0 HSV-1 infection. This phenotype is believed to be crucial for IFI16-dependent IFNβ production and limitation of viral replication, although it also has a role in IFN-independent intrinsic repression of viral genomes (Orzalli et al., 2012, 2013; Johnson et al., 2014; Diner et al., 2015). Importantly, blocking of vDNA release into the nucleus using tosyl phenylalanyl chloromethyl ketone substantially inhibited the induction of IFNβ and ISG54 following infection, demonstrating that vDNA accumulation in the nucleus of fibroblasts, unlike in macrophages, is required for the induction of the innate immune response (Orzalli et al., 2012). Through its positively charged hematopoietic interferon-inducible nuclear proteins with 200-amino acids repeats (HIN) domain, nuclear IFI16 interacts with the sugar-phosphate backbone of foreign dsDNA, which releases the pyrin domain from its autoinhibited state (Jin et al., 2012). Following acetylation of its nuclear localization signal by acetyltransferase p300, activated IFI16 translocates to the cytoplasm and activates the STING pathway. STING associates with TBK1 and promotes IRF3 phosphorylation and nuclear translocation, eventually leading to IFNβ gene upregulation and cytokine production (Jin et al., 2012; Ansari et al., 2015).

Induction of ISG Products

The second phase of the IFN response starts when secreted IFNs bind to their cognate receptors. IFN-I and IFN-III, although utilizing distinct receptors, signal through the same pathways (Lazear et al., 2019). They activate Janus kinase 1 (JAK-1) and tyrosine kinase 2 (TYK-2), which subsequently induce the phosphorylation and accumulation of activated signal transducers and activators of transcription 1 and 2 (STAT-1 and STAT-2, respectively). Interaction between STAT1, STAT2, and IRF9 leads to the formation of the IFN-stimulated gene factor 3 (ISGF3) complex at ISG promoters, which induces their expression (Lazear et al., 2019). The IFN-II signaling cascade is triggered when IFNγ binds to IFNGR, followed by assembly of the IFNγ-IFNGR-JAK1-JAK2 complex. Activation of JAK1 and JAK2 induces IFNGR phosphorylation and STAT1 docking site formation. STAT1 molecules are first recruited to the complex and phosphorylated, and they then become dissociated and are eventually translocated to the nucleus where they act as ISG transactivators (Lee and Ashkar, 2018). The IFN signaling pathways function in both autocrine and paracrine fashions in order to inhibit viral replication in the infected cells and protect neighboring cells from infection (**Figure 5**).

Several cellular ISG products with antiviral effects against HSV-1 infection have been identified. Most of the studies have relied on utilizing viral mutants that lack IFN antagonists. For instance, the ISG products viperin, tetherin, and zinc finger antiviral protein (ZAP) have been found to restrict HSV-1 infection. However, their antiviral activities rely on the absence of HSV-1 virion host shutoff (vhs) protein (Zenner et al., 2013; Shen et al., 2014; Su et al., 2015). Indeed, ectopic expression of viperin, tetherin, and ZAP reduced the viral yield of a vhs-null mutant but not the WT virus, while depletion of these proteins enhanced the viral yield of the vhs-null mutant. The vhs protein was found to target the mRNAs of these ISG products for degradation, and it consequently counteracted their antiviral properties. Protein kinase R and 2′-5′-oligoadenylate synthetase were also shown to confer antiviral immunity to HSV-1 infection in a process that is efficiently counteracted by the viral protein Us11 (Sanchez and Mohr, 2007; Lussignol et al., 2013). Only two ISG products have been identified as antiviral factors during WT HSV-1 infection, namely ISG15 and MxB (Lenschow et al., 2007; Crameri et al., 2018). An *in vivo* study suggested that the presence of ISG15,

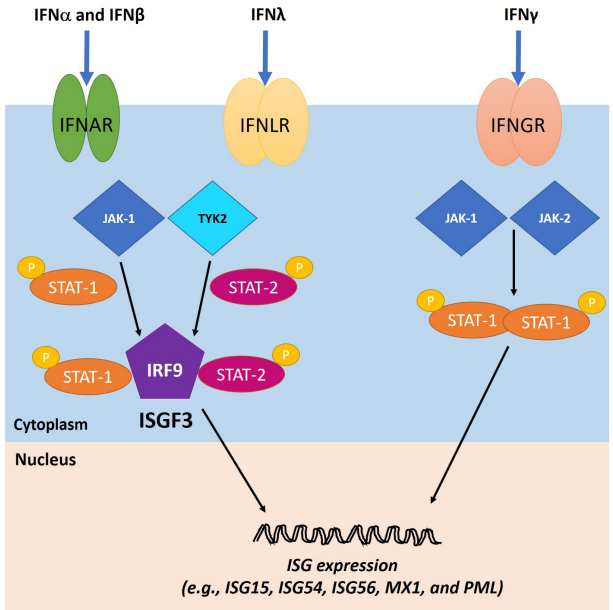

FIGURE 5 | IFN signaling cascades. The IFN type I (IFN-I) and III (IFN-III) bind to their cognate receptors, IFNα receptors (IFNAR) and IFNλ receptors (IFNLR), respectively, on the cell surface, leading to the activation of Janus kinase 1 (JAK-1) and tyrosine kinase 2 (TYK-2). As a result, activated signal transducers and activators of transcription 1 and 2 (STAT1 and STAT2, respectively) are phosphorylated and bind to IFN regulatory factor 9 (IRF9) to form ISGF3, which translocates to the nucleus to induce the expression of ISGs. The IFN type II (IFN-II) signaling cascade is triggered when IFNγ binds to IFNγ receptor (IFNGR) and activates JAK1 and Janus kinase 2 (JAK2). Consequently, STAT1 molecules are phosphorylated and localized to the nucleus to transactivate ISG expression. Adapted with permission from Alandijany (2018).

a ubiquitin-like molecule that is rapidly induced following viral infection, is key for mediating the IFN antiviral response against HSV-1 infection. In comparison with WT mice, ISG15-deficient mice showed increased susceptibility to HSV-1 infection and a decreased survival rate. However, the mechanism underlying this restriction process remains unknown (Lenschow et al., 2007). Recently, MxB was shown to restrict the HSV-1 life cycle in IFN-pretreated cells by interfering with vDNA delivery to the nucleus (Crameri et al., 2018). These studies shed light on ISG products with antiviral effects against HSV-1 infection, but this crucial area of research remains largely understudied, especially regarding WT HSV-1 infection, which is known to be impaired by IFN pretreatment of cell lines and animal models (Domke-Opitz et al., 1986; Taylor et al., 1998; Mossman et al., 2000; Sainz and Halford, 2002; Everett and Orr, 2009).

Temporal Regulation of Host Intrinsic and Innate Intracellular Immunity

It is clear that both intrinsic (constitutive) and innate (inducible) antiviral responses play key roles in the intracellular restriction of HSV-1 infection. Rapid recognition of viral nucleic acids is key for both arms of immunity (Paludan and Bowie, 2013; Knipe, 2015; Komatsu et al., 2016). Until recently, several questions had remained unanswered with regard to how intrinsic and innate immune responses are regulated. For example, (1) are they simultaneously or sequentially triggered in response to infection, (2) do they similarly or distinctly impair viral replication, and (3) does the permissiveness and vulnerability of certain cell types to ΔICP0 HSV-1 infection correlate with the lack of ability to mount an efficient intrinsic and/or innate immune response in these cells?

One of the main reasons why the temporal regulation of intrinsic and innate immunity remains poorly defined is the fact that most microscopy-based studies of host factor recruitment to HSV-1 genomes have utilized indirect methods to detect vDNA (e.g., immunostaining or fluorescent tagging of vDNA-binding proteins) (Everett et al., 2003, 2004b; Everett and Murray, 2005; Orzalli et al., 2015; Diner et al., 2016; Komatsu et al., 2016). These methods allowed identification of many intrinsic and innate immune factors. However, as these approaches necessitate the onset of viral gene expression, our understanding of the viral–host interactions that occur immediately upon nuclear entry of viral genomes (prior to the expression of viral proteins) was limited. The onset of viral gene expression may also displace host factors recruited or bound to viral genomes. Several studies have utilized direct methods for vDNA detection (e.g., FISH and bromodeoxyuridine-labeling of vDNA) (Maul et al., 1996; Everett et al., 2007; Glauser et al., 2007). However, these methods require harsh denaturation conditions and substantial sample processing, which can be incompatible with immunofluorescent staining of host factors (Jensen, 2014). Moreover, these experiments were conducted under high MOI conditions (≥10 PFU/cell) due to the technical difficulties associated with detection of low genome copy numbers. Utilizing high MOI conditions is suboptimal or even unsuitable for studying the regulation of intrinsic and innate immunity, given that intrinsic immunity has a threshold MOI above which it becomes saturated and no longer effective (Everett et al., 2004a). In addition, high MOI conditions trigger an IFN response, and several intrinsic immune factors (e.g., PML and Sp100) are considered as ISG products, which makes it difficult to distinguish their intrinsic from their innate antiviral roles (Lavau et al., 1995; Grotzinger et al., 1996).

Recently, to track the subcellular localization of vDNA, there has been an increase in the use of pyrimidine deoxynucleotide analog labeling of HSV-1 DNA in combination with click chemistry (Wang et al., 2013; Sekine et al., 2017; Alandijany et al., 2018; Cabral et al., 2018; Sun et al., 2019). This technique, which is not detrimental to viral infectivity, enables direct visualization of input viral genomes under low MOI conditions (as low as 0.1 PFU/cell) immediately upon nuclear entry (∼30 min post-virus addition). Furthermore, it is sensitive and specific to vDNA and compatible with indirect immunofluorescence staining protocols, providing a valuable method to investigate the temporal recruitment of intracellular immune regulators to infecting viral genomes at single-cell and single-molecule levels.

Utilizing this technique, it was found that PML-NB restriction factors (e.g., PML, SP100, Daxx, and ATRX) were rapidly recruited to infecting viral genome foci upon the entry of the vDNA into the nucleus of infected human foreskin fibroblasts (∼30 min post-virus addition) (Alandijany et al., 2018;

Cabral et al., 2018). This process occurred in a PML-dependent manner and led to genome entrapment and silencing within PML-NBs. Interestingly, genome entrapment was observed during both WT and ΔICP0 HSV-1 infection. However, during WT infection, ICP0 induced PML degradation and the dispersal of the PML-NB restriction factors, highlighting the importance of ICP0 in the release of viral genomes entrapped within PML-NBs to stimulate the onset of lytic HSV-1 replication. In contrast, during ΔICP0 HSV-1 infection, vDNA remained stably entrapped within PML-NBs, leading to repression of viral gene expression and restriction of plaque formation. Importantly, the host PRR and DNA sensor IFI16 was not stably recruited to vDNA entrapped within PML-NBs, and ISG expression was not induced under low MOI conditions that did not saturate the PML-NB intrinsic host defenses (Alandijany et al., 2018). An independent study on MRC-5 human embryonic lung fibroblasts that utilized mass spectrometry analysis supported these findings regarding the WT virus, demonstrating the association of PML, but not IFI16, with WT viral genomes prior to viral gene expression immediately upon nuclear entry (Dembowski and DeLuca, 2018). Instead, IFI16 was associated with the viral genomes that successfully initiated gene expression, as demonstrated by ICP4 expression. Data from these studies indicate that vDNA entry into the nucleus alone stimulates the recruitment of intrinsic restriction factors (such as PML) to the infecting genomes, but it is not sufficient for nuclear PRRs to recognize vDNA (Alandijany et al., 2018; Dembowski and DeLuca, 2018).

Saturation of intrinsic host defenses under higher MOI conditions (1 PFU/cell) stimulated the stable recruitment of IFI16 to infecting viral genomes, and induced ISG expression in an IFI16- and JAK-dependent manner. The induction of this innate immune response was dependent on the onset of viral gene expression and vDNA replication, as treatment of infected cell monolayers with phosphonoacetic acid (a vDNA polymerase inhibitor) inhibited ISG induction in a dose-dependent manner (Alandijany et al., 2018). Unlike intrinsic immune factor depletion, inhibition of JAK signaling failed to relieve the plaque formation defect of ΔICP0 HSV-1, and instead significantly enhanced the virus yield. These findings led to the conclusion that the intrinsic and innate arms of intracellular immunity are temporally and functionally distinct from one another (Alandijany et al., 2018). Intrinsic immunity acts immediately upon vDNA delivery to the nucleus to induce viral genome silencing. Escape from this immune response and initiation of vDNA replication trigger innate immunity in the infected and neighboring uninfected cells, which constricts viral propagation and limits the spread of infection (**Figure 6**). Cell types such as U2OS and SAOS, which fail to efficiently recruit intrinsic restriction factors to viral genomes and/or to induce a robust innate immune response, are highly permissive to HSV-1 infection even in the absence of ICP0 (Deschamps and Kalamvoki, 2017; Alandijany et al., 2018).

Nevertheless, the authors of previous studies that utilized ultraviolet (UV)-inactivated WT virus, WT virus in the presence of cycloheximide, or replication-incompetent viral mutants defective in multiple genes argued that initiation of vDNA replication is not required for innate immunity induction (Nicholl et al., 2000; Preston et al., 2001; Eidson et al., 2002; Collins et al., 2004). On the surface, these findings appear contradictory to the recent report described above (Alandijany et al., 2018). However, it is important to note that higher MOI conditions (5–50 PFU/cells) were used in these studies. In some cases, information about the particle-to-PFU ratio of viral stocks was missing, which is particularly critical in the case of viral mutants as they are usually associated with incredibly high particle-to-PFU ratios (Everett, 1989; Preston et al., 2001; Collins et al., 2004; Everett et al., 2004a). Additionally, UV inactivation of HSV-1 may have detrimental effects on viral capsids (e.g., degradation or permeabilization), as observed for other viruses (Miller and Plagemann, 1974; De Sena and Jarvis, 1981; Smirnov et al., 1983). The experimental settings used might be problematic and physiologically irrelevant, as they may deliver or generate PAMPs (e.g., accumulation of a large number of capsids, premature DNA release in the cytoplasm, and aggregation of high-order vDNA structures in the nucleus) that allow PRR detection.

More recent studies proposed that vDNA entry into the nucleus is not required for innate immunity induction in immune cells (e.g., macrophages) (Horan et al., 2013; Sun et al., 2019). Both IFI16 and cGAS can sense vDNA in the cytoplasm in these cells, leading to induction of an IFN response and the

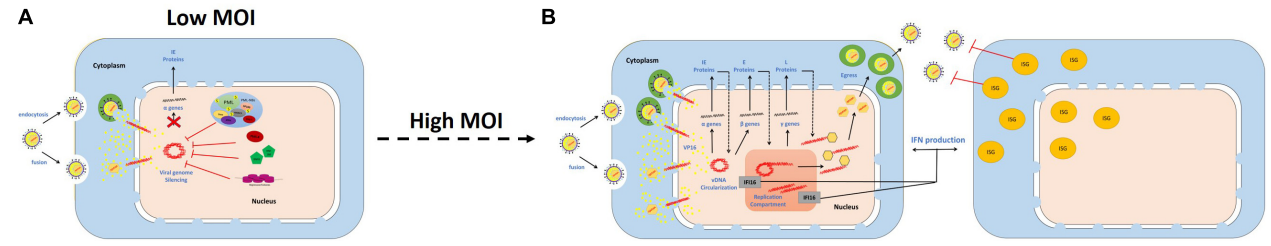

FIGURE 6 | Temporal regulation of intrinsic and innate immunity during HSV-1 infection. Schematic diagrams demonstrate the sequential recruitment of intrinsic restriction factors and pattern recognition receptors (PRRs) to viral DNA (vDNA), and the associated antiviral effects. **(A)** Under low multiplicity of infection (MOI) conditions, intrinsic restriction factors are recruited to vDNA upon nuclear entry to induce viral genome silencing. **(B)** Under high MOI conditions, escape from the intrinsic immune response and initiation of vDNA replication enables PRR recruitment and induction of interferon (IFN) production. Innate immunity constricts viral propagation and limits the spread of infection.

elimination of the infecting viral genomes. This is quite intriguing as viral genomes should, in theory, remain encapsulated within capsids during cytoplasmic transportation and only be released upon ejection through the nuclear pores, which makes vDNA inaccessible to DNA sensors. One explanation is that HSV-1 capsids are targeted for proteasomal degradation in the cytoplasm of infected macrophages, allowing IFI16 sensing of naked vDNA (Horan et al., 2013). Independent of proteasomal degradation, premature release of vDNA into the cytoplasm of infected monocytes has been reported (Sun et al., 2019). This premature release is believed to enable cGAS-mediated sensing of viral genomes, induction of an IFN response, and clearance of the cytosolic viral genomes and capsids (Sun et al., 2019). Importantly, however, high MOI conditions (10 PFU/cell, and sometimes up to 100 PFU/cell) were utilized in these studies (Horan et al., 2013; Sun et al., 2019). Thus, the discrepancies in observations among the studies with regard to cellular and viral requirements underlying pathogen sensing and innate immunity induction may again be due to the MOI conditions used. It is also important to be aware that immune and non-immune cells differ strikingly in their abilities to mount an IFN response to viral infections. Hence, it is possible that the temporal regulation of intrinsic and innate immunity observed in fibroblasts does not apply in macrophages. However, this hypothesis remains to be investigated using a side-by-side comparison involving physiologically relevant low MOI conditions.

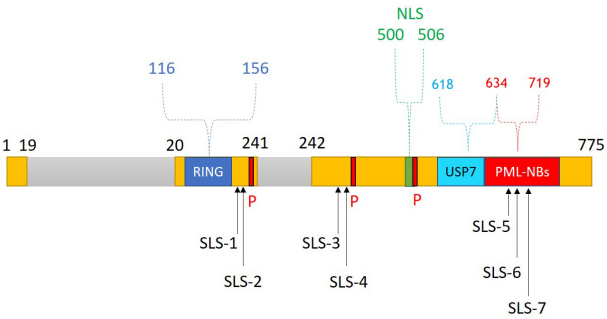

FIGURE 7 | ICP0 structure and functional domains. ICP0 comprises 775 amino acids and is encoded by the *IE-0* gene located within the inverted repeats sequences ab and b′a′. ICP0 is composed of three exons (1–19, 20–241, and 242–775 nucleotides; yellow), and two introns (765 and 136 nucleotides; gray). Several functional domains and interacting motifs have been identified in ICP0. A zinc-binding really interesting new gene (RING) finger domain is located within the N-terminal region of ICP0 (residues 116–156). The C-terminal region contains a nuclear localization signal (NLS; residues 500–506), a ubiquitin-specific protease 7 (USP7)-binding motif (residues 618–634), and sequences required for localization at promyelocytic leukemia protein-nuclear bodies (PML-NBs; residues 634–719). Three major phosphorylation sites (P; 224–234, 365–371, and 508–518) of ICP0 have been identified. ICP0 contains several SUMO interaction motif (SIM)-like sequences (SLS-1 to SLS-7). Reproduced with permission from Alandijany (2018).

HSV-1 E3 UBIQUITIN LIGASE ICP0, A KEY ANTAGONIST FOR HOST INTRACELLULAR IMMUNITY

HSV-1 has evolved multiple strategies to antagonize and evade the host immune response. In particular, the viral IE protein ICP0 has received significant attention due to its central roles in counteracting both the intrinsic and innate arms of intracellular immunity (Boutell and Everett, 2013; Lanfranca et al., 2014; Gu, 2016; Tognarelli et al., 2019). ICP0 is a multifunctional IE protein that enhances the viral lytic infection and promotes genome reactivation from quiescence/latency (Everett, 2000). It is encoded by the *IE-0* gene (also known as α*0*), which is located within the inverted repeats sequence ab and b′a′ (Wadsworth et al., 1975). Several functional domains and interacting motifs have been identified within ICP0 (Everett, 1988). The zinc-binding really interesting new gene (RING) finger domain located in the N-terminal region between amino acids 116 and 156 within exon 2 is considered the most important functional domain of ICP0 (**Figure 7**). Indeed, HSV-1 mutants that express a catalytically inactive RING domain had equivalent replication defects as ΔICP0 HSV-1 and failed to reactivate quiescent/latent viral genomes (Everett, 1989; Lium and Silverstein, 1997; Everett et al., 2004a, 2009; Ferenczy et al., 2011; Grant et al., 2012). This RING finger domain confers E3 ubiquitin ligase activity, facilitating the conjugation of ubiquitin molecules to the lysine residues of target proteins and thereby promoting their proteasome-dependent degradation (Boutell et al., 2002; Vanni et al., 2012). Importantly, many ICP0-targeted proteins are key regulators of host intrinsic and innate immunity (**Figure 8**). Targeting these immune factors using ICP0, directly or indirectly, at the early stages of infection provides a favorable environment for viral replication (Davido et al., 2005; Boutell and Everett, 2013; Gu, 2016).

ICP0-Mediated Counteraction of Host Intrinsic Immunity

ICP0 Deploys Multiple Strategies to Antagonize PML-NB Restriction Factors

PML-NB constituent proteins (e.g., PML, SP100, Daxx, ATRX, MORC3, and PIAS1) failed to restrict WT HSV-1 infection due to the presence of ICP0 (Everett et al., 2006, 2008a; Lukashchuk and Everett, 2010; Brown et al., 2016; Sloan et al., 2016). During the initial stages of infection, ICP0 localizes to PML-NBs prior to mediating their disruption. ICP0 employs multiple mechanisms to do so (Boutell and Everett, 2013; Gu et al., 2013). It shares many features with SUMO-targeted ubiquitin ligases (STUbL), which are a family of enzymes that contain SIMs that mediate interactions with SUMO-modified proteins (Boutell et al., 2011). Seven SIM-like sequences (SLS1–7) have been identified within the ICP0 open reading frame. During the initial stages of infection, ICP0 localizes to SUMO1 and SUMO2/3 conjugates and preferentially targets them for proteasomal degradation in a RING finger-dependent manner. Proteomics analysis has identified 124 proteins that showed reductions (≥threefold) in the levels of their SUMO-modified forms during HSV-1 infection (Sloan et al., 2015). SUMO-modified PML and SP100 are well-known target substrates for ICP0 (Chelbi-Alix and de The, 1999; Boutell et al., 2003, 2011). SLS4 has been

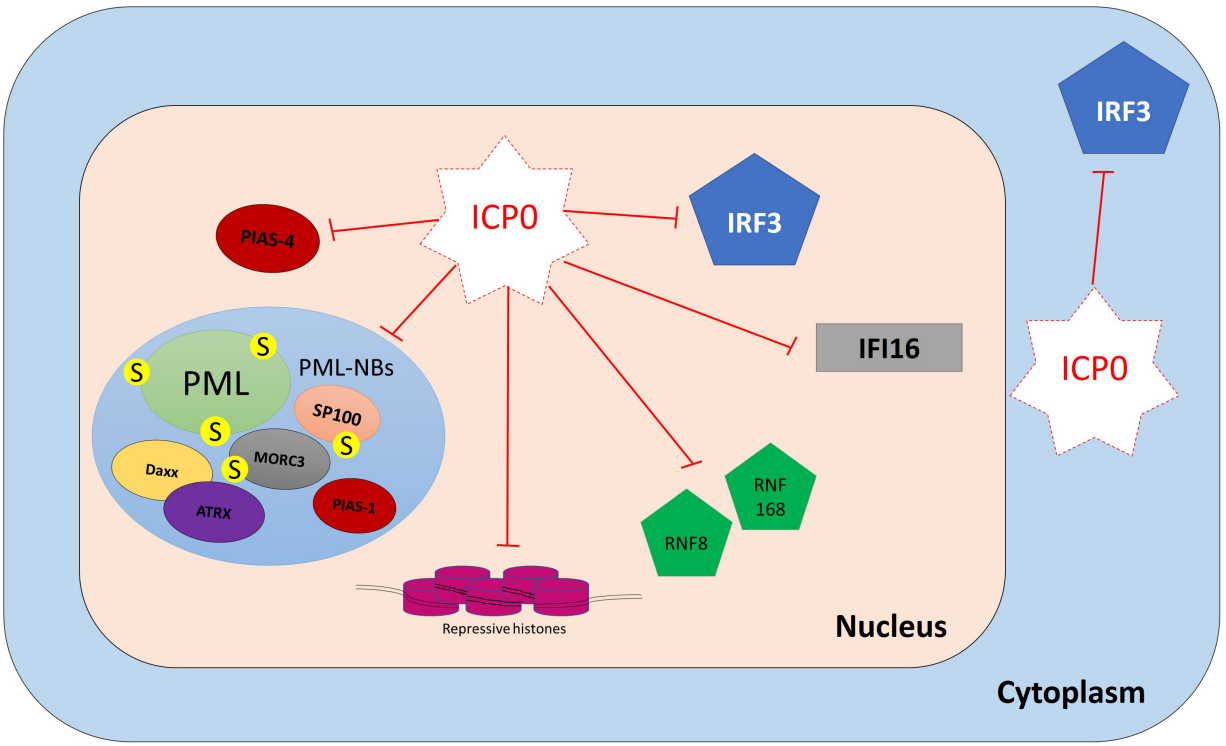

FIGURE 8 | ICP0 counteracts host intrinsic and innate immunity. ICP0 employs multiple strategies to antagonize host intracellular immunity. It targets promyelocytic leukemia protein (PML), speckled 100 kDa (SP100), MORC family CW-type zinc finger 3 (MORC3), protein inhibitor of activated STAT 1 (PIAS1), and interferon-gamma-inducible protein 16 (IFI16) for degradation. It interferes with the recruitment of death domain associated protein (Daxx), alpha thalassemia/mental retardation syndrome X-linked (ATRX), protein inhibitor of activated STAT 4 (PIAS4), and epigenetic repressors to viral DNA (vDNA). It prevents IFN-regulatory factor 3 (IRF3) activity and impairs its function.

shown to be necessary for ICP0 interaction with SUMO2/3 and targeting of SUMO-modified PML for degradation. Moreover, multiple mutations within ICP0 SLSs (SLS4–7) rescued SUMO-conjugated proteins from degradation and reduced the ability of ICP0 to rescue the plaque formation ability of ΔICP0 HSV-1 (Boutell et al., 2011). However, ICP0 also employs a SUMO-independent mechanism for PML targeting (Cuchet-Lourenco et al., 2012). It directly interacts with PML isoform I (PML.I) and induces its degradation. This process occurs independently of the PML.I SIM, and it instead depends on the PML.I-specific exon 9 in the N-terminal half of ICP0 (Cuchet-Lourenco et al., 2012).

Recently, MORC3 was identified as a target substrate for ICP0 (Sloan et al., 2015, 2016). During WT HSV-1 infection, a high degree of colocalization between ICP0 and MORC3 was observed during the initial stages of infection prior to the degradation of SUMO-modified and unmodified MORC3. This process occurred in an ICP0 RING finger-dependent manner, but independently of SLS4–7 (Sloan et al., 2016). Whether ICP0 directly interacts with MORC3 remains to be determined.

Other PML-NB restriction factors (e.g., Daxx, ATRX, and PIAS1) are not degraded during WT HSV-1 infection (Lukashchuk and Everett, 2010; Brown et al., 2016). In fact, ICP0 failed to directly interact with these proteins, as demonstrated by co-immunoprecipitation assays (Lukashchuk and Everett, 2010; Brown et al., 2016). However, the presence of ICP0 blocks their recruitment to infecting viral genomes and efficiently counteracts their repressive antiviral activity, possibly by degrading other PML-NB restriction factors such as PML and MORC3, leading to PML-NB disruption (Lukashchuk and Everett, 2010; Brown et al., 2016; Sloan et al., 2016; Alandijany et al., 2018).

Thus, HSV-1 can efficiently counteract PML-NB-mediated silencing of the viral genomes. The viral E3 ubiquitin ligase ICP0 employs SUMO-dependent and -independent targeting mechanisms to mediate the degradation and dispersal of host restriction factors away from viral genomes to promote the onset of lytic infection.

ICP0 Impairs the Intrinsic Restriction Mediated by DDR Proteins

During WT HSV-1 infection, the formation of irradiation-induced foci (IRIF) and the accumulation of DNA repair proteins at IRIF are disrupted by the viral E3 ubiquitin ligase ICP0 (Lilley et al., 2010, 2011). Indeed, ICP0 induces the degradation of the RNF8 and RNF168 ubiquitin ligases required for the accumulation of DNA repair proteins in an ICP0 RING finger- and cellular proteasome-dependent manner. In infected cells, cellular CK1 kinase phosphorylates ICP0, thereby creating a "mimic" of a cellular phosphosite, which promotes ICP0 interaction with RNF8, eventually leading to the degradation of RNF8 (Chaurushiya et al., 2012).

Degradation of these cellular ubiquitin ligases (RNF8 and RNF168) leads to a substantial loss of ubiquitinated H2A and H2AX, which impairs DNA repair protein recruitment and IRIF formation. Therefore, the plaque formation of WT virus, unlike ΔICP0 HSV-1 and ICP0 RING finger mutants, was not affected by RNF8 ectopic expression, which highlights the key role of ICP0 in evading intrinsic repression mediated by DNA repair proteins (Lilley et al., 2010, 2011; Chaurushiya et al., 2012).

Epigenetic Repression of Viral Genome Is Also Counteracted by ICP0

In addition to VP16, the viral protein ICP0 is key in reversing the association of epigenetic repressors with viral genomes (Herrera and Triezenberg, 2004; Lee et al., 2016). VP16 initially promotes the removal of histone H3 from IE promoters and enhances the recruitment of transactivation factors (e.g., HCF-1 and Oct-1) to stimulate viral gene expression, including ICP0 expression (Herrera and Triezenberg, 2004). Thereafter, ICP0 mediates heterochromatin (H3K9me3) removal on viral E gene promoters (e.g., ICP8), and induces the degradation of free histones (H2B) to minimize their availability to bind the vDNA (Lee et al., 2016). Furthermore, ICP0 disrupts the REST/CoREST/HDAC nuclear repressor complex in order to enhance the transition from IE to E protein expression. ICP0 binds to CoREST, translocates CoREST and HDAC to the cytoplasm, and promotes the dissociation of HDAC (Gu et al., 2005; Gu and Roizman, 2007, 2009). As the infection progresses, encapsidation of viral genomes further contributes to the removal of core histones and makes the vDNA inaccessible to epigenetic repressors.

ICP0-Mediated Impairment of Host Innate Immunity

HSV-1 efficiently counteracts many aspects of host innate immunity, including evasion of PRR recognition (e.g., IFI16 degradation and inhibition of the cGAS-mediated signaling pathway), modulation or blocking of immune signaling cascades (e.g., TRIM29-mediated degradation of STING and disruption of TBK1-IRF3 interaction), and interference with effector protein functions (e.g., degradation of ISG mRNAs) (Verpooten et al., 2009; Zenner et al., 2013; Shen et al., 2014; Su et al., 2015; Orzalli et al., 2016; Xing et al., 2017; Xu et al., 2017; Tognarelli et al., 2019). Many HSV-1 proteins have been found to be involved in the innate immune evasion. ICP0 plays a central role in this process (Lanfranca et al., 2014; Orzalli and Knipe, 2014). In fact, the presence of ICP0 has been shown to inhibit both IFN-induced and viral-induced ISG expression (Harle et al., 2002; Mossman and Smiley, 2002; Melroe et al., 2004; Paladino et al., 2010). Correspondingly, robust induction of ISGs was only observed during infection with HSV-1 mutants that failed to express ICP0, and these mutants were hypersensitive to IFN pretreatment compared to WT virus (Mossman et al., 2000; Eidson et al., 2002; Harle et al., 2002; Everett and Orr, 2009).

Multiple mechanisms for ICP0-mediated inhibition of innate immunity have been proposed (Lanfranca et al., 2014). As discussed above, ICP0 induces PML degradation at the early stage of infection to release the viral genomes entrapped within PML-NBs. By doing so, ICP0 also counteracts host innate immunity because the presence of PML is important for efficient induction of ISG expression (Alandijany et al., 2018; McFarlane et al., 2019). ICP0 has also been shown to induce the degradation of the vDNA sensor IFI16 in a RING-dependent manner (Orzalli et al., 2012; Cuchet-Lourenco et al., 2013; Johnson et al., 2013). However, IFI16 degradation occurred at a slower kinetic rate in comparison to PML degradation (Cuchet-Lourenco et al., 2013). Also, it remains controversial whether ICP0 is directly required and sufficient for IFI16 degradation (Cuchet-Lourenco et al., 2013; Orzalli et al., 2016).

Impairment of IRF3 function is another strategy employed by ICP0 to counteract innate immunity. Nuclear ICP0 binds to IRF3 and its binding partner CBP, leading to the formation of the ICP0/IRF3/CBP nuclear complex. This interaction sequesters IRF3 away from the host chromatin and prevents ISG expression (Melroe et al., 2004, 2007). Although ICP0 expression is predominantly nuclear, ICP0 translocates to the cytoplasm as the infection progresses (Lopez et al., 2001). A study suggested that cytoplasmic ICP0 promotes viral replication by blocking the activation of IRF3 and preventing the induction of innate immunity (Taylor et al., 2014).

ICP0 does not only impair the host IFN response, but it also interferes with the NF-κB signaling pathway via several mechanisms, including degradation of TLR2 and p50, and blocking of p65 nuclear import (van Lint et al., 2010; Zhang et al., 2013). Collectively, these studies demonstrate that ICP0 impedes the induction of host innate immunity during HSV-1 infection in addition to its key role in antagonizing intrinsic antiviral restriction.

CONCLUSION

Intrinsic and innate immunity are two distinct arms of host intracellular antiviral responses to HSV-1 infection. The differences between these two arms include the induction requirements, the nature of the effector proteins, and the antiviral effects on viral replication. The intrinsic antiviral response is mediated by pre-existing host cell restriction factors that immediately recognize vDNA upon nuclear entry and directly repress the onset of viral replication by inducing viral genome silencing. On the other hand, the induction of the innate immune response is triggered following the escape of viral genomes from intrinsic silencing and the initiation of viral gene expression and DNA replication. It is mediated by PRRs, which recognize viral components and replication intermediates/products, leading to IFN production and ISG expression. The induction of ISG products with antiviral properties in the infected and neighboring uninfected cells inhibits viral propagation and limits the spread of infection. The presence of the viral countermeasure ICP0 initially antagonizes the intrinsic repression of viral genomes and it subsequently impairs innate immunity induction. This sequential

regulation of intracellular immunity remained unidentified for many years, and it would not have been possible to characterize this regulation process without studies utilizing physiologically relevant low MOI conditions. Click chemistry-mediated detection of vDNA prelabeled with pyrimidine deoxynucleotide was key in advancing our understanding of these temporally regulated very early events of the cellular antiviral response. These findings on HSV-1 infection likely apply to other viral infections and are worthy of further investigation.

AUTHOR CONTRIBUTIONS

TA: conceptualization, writing the original draft, reviewing, and editing.

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REVIEW
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Post-translational Control of Innate Immune Signaling Pathways by Herpesviruses

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Herpesviruses constitute a large family of disease-causing DNA viruses. Each herpesvirus strain is capable of infecting particular organisms with a specific cell tropism. Upon infection, pattern recognition receptors (PRRs) recognize conserved viral features to trigger signaling cascades that culminate in the production of interferons and pro-inflammatory cytokines. To invoke a proper immune response while avoiding collateral tissue damage, signaling proteins involved in these cascades are tightly regulated by post-translational modifications (PTMs). Herpesviruses have developed strategies to subvert innate immune signaling pathways in order to ensure efficient viral replication and achieve persistent infection. The ability of these viruses to control the proteins involved in these signaling cascades post-translationally, either directly *via* virus-encoded enzymes or indirectly through the deregulation of cellular enzymes, has been widely reported. This ability provides herpesviruses with a powerful tool to shut off or restrict host antiviral and inflammatory responses. In this review, we highlight recent findings on the herpesvirus-mediated post-translational control along PRR-mediated signaling pathways.

Keywords: herpesviruses, immune evasion, pattern recognition receptors, signaling pathways, post-translational modification

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INTRODUCTION

Herpesviruses constitute a broad family of DNA viruses that cause a wide spectrum of diseases in humans, other vertebrates and non-vertebrates as well. These viruses are characterized by a common structure consisting of linear double-stranded DNA packaged in an icosahedral nucleocapsid with a size ranging from 115 to 130 nm in diameter. The nucleocapsid is surrounded by a protein layer called tegument and a lipid bilayer envelope anchored with various glycoproteins. A hallmark of all herpesviruses is the ability to establish and maintain a lifelong latent infection in the infected host. Based on the genome sequence and biological properties, the herpesviridae family is divided into three subfamilies: alpha-herpesvirinae, beta-herpesvirinae and gamma-herpesvirinae (**Table 1**; for review, see Davison et al., 2009). Despite the presence of a competent immune response, each herpesvirus strain has the ability to infect specific cell types within their target organisms. Therefore, they likely have evolved various strategies to subvert and exploit antiviral and inflammatory responses. Comprehension of the innate immune responses and the corresponding viral countermeasures is crucial to understanding viral pathogenesis.

TABLE 1 | Classification of human herpesviruses.

Subfamily	Taxonomic name	Common name
Alpha-herpesvirinae	HHV-1	Herpes simplex virus 1 (HSV-1)
	HHV-2	Herpes simplex virus 2 (HSV-2)
	HHV-3	Varicella-zoster virus (VZV)
Beta-herpesvirinae	HHV-5	Human cytomegalovirus (HCMV)
	HHV-6	HHV-6 vairant A or B
	HHV-7	HHV-7
Gamma-herpesvirinae	HHV-4	Epstein-Barr virus (EBV)
	HHV-8	Kaposi's sarcoma-associated herpesvirus (KSHV)

HHV, human herpesvirus.

During the infection, the herpesvirus faces several lines of host defense starting with the physical and mechanical mucosal/epithelial barrier (Huard et al., 1996; Rahn et al., 2017; Thier et al., 2017). At the cellular level, the virus encounters the host immune defense, i.e., the intrinsic immunity orchestrated by restriction factors suppressing/preventing the infection and the pursuit of the viral replication cycle (Tavalai and Stamminger, 2009); and the innate immunity which allows the discrimination of "non-self" components from "self" ones and triggers signaling pathways leading to pro-inflammatory and antiviral responses through the production of interferons (IFNs). Interestingly, a few years ago, Iversen et al. (2016), reported the activation of an IFN-independent innate antiviral pathway through the detection of viral O-linked glycans that leads to the production of CXCR3 chemokines which stimulate a neutrophil-dependent antiviral response.

The innate immunity process is initiated by the detection of microbial determinants or pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) which activate various spatially localized adaptor molecules. These receptor-adaptor pairs ultimately converge to trigger inflammatory responses and/or antimicrobial gene expression *via* two crucial families of transcription factors, nuclear factor-kappa B (NF-κB) and interferon regulatory factors (IRFs) (for review, see Thaiss et al., 2016). A wide range of PRRs have been described and classified based on their subcellular location and corresponding PAMPs. The first category includes the membrane-bound receptors, such as the Toll-like receptors (TLRs) and the C-type lectin receptors (CLRs). The second group includes the cytosolic receptors, i.e., the nucleotide oligomerization domain (NOD)-like receptors (NLRs) and the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). Recently, intracellular DNA sensors, including cyclic GMP-AMP synthase (cGAS), interferon gamma-inducible protein 16 (IFI16), DDX41, and hnRNPA2B1 were reported to activate innate immune responses against invading DNA viruses (Sun et al., 2013; Wu et al., 2013; Almine et al., 2017; Wang et al., 2019). These PRRs reside in anatomically distinct subcellular locations to patrol for microbial infection and provoke a highly conserved signaling cascade to defeat microbial propagation.

Post-translational modifications (PTMs) are mostly enzyme-mediated, sometimes enzyme-independent or even spontaneous modifications that regulate the folding, function, subcellular localization, stability and protein-protein interactions of the target protein. In the midst of the reported PTMs, phosphorylation and ubiquitination are among the best studied. A wide range of PTMs have been described, including SUMOylation, acetylation, deamidation, methylation, ISGylation, succinylation, carbonylation, glycation, glutamylation, hydroxylation, citrullination, nitration, palmitoylation, and sulfation. The broad spectrum of PTMs and their targets enables a dynamic and tight regulation of diverse signal transduction pathways to re-establish homeostasis under stressed conditions, such as microbial infection. Innate immunity and particularly the sensing of pathogens through the PRRs is regulated by numerous PTMs to ensure efficient signal transmission and antimicrobial response. However, a growing number of studies have reported microbe-encoded enzymes that post-translationally control cellular proteins involved in innate immunity to promote the replication and/or survival of the pathogen.

Herpesviruses have acquired the ability to dampen the innate immune inflammatory and antiviral responses by regulating the proteins involved in these pathways post-translationally. In particular, herpesviruses have been shown to regulate their phosphorylation, ubiquitination, SUMOylation, acetylation, deamidation and ISGylation, which are crucial for proper signal transmission and viral eradication. In this review, we summarize the current findings on the herpesvirus-mediated direct (i.e., *via* viral-encoded enzymes) or indirect (i.e., *via* targeting cellular enzymes) post-translational control at each level of the PRR-mediated innate immune signaling pathways.

PRR-MEDIATED SENSING OF HERPESVIRUSES

Each herpesvirus contains various PAMPs that can be detected by most of PRR families, including TLRs, NLRs, RLRs, CLRs, and intracellular DNA sensors (**Table 2**).

TLR Signaling Pathway

TLRs, the first discovered and best characterized PRRs, are transmembrane proteins found on the cell surface and in the endosomal membrane. TLRs constitute a family of receptors sharing sequence homology. To date, 10 TLRs in human and 13 in mouse have been described (for review, see Botos et al., 2011). Among these, TLR2, TLR3, TLR4, TLR7, and TLR9 have been reported to directly sense or indirectly participate in innate immune defense against herpesvirus infection (**Table 2**; Ma and He, 2014; Reuven et al., 2014). These receptors contain two key domains, a leucine-rich repeat (LRR) domain located in the extracellular or endosomal compartment, and a Toll/interleukin-1 receptor (TIR) domain in the cytoplasm. Upon the LRR-dependent binding to a ligand, TLRs transmit signal across the membrane through the TIR domain, recruiting downstream adaptor proteins such as myeloid differentiation primary

TABLE 2 | PRRs involved in the sensing of herpesviruses.

	PRR	Herpesvirus	PAMPs	References
TLRs	TLR2	EBV	dUTPase	Gaudreault et al., 2007; Ariza et al., 2009
		HCMV	gB and/or gH	Compton et al., 2003; Boehme et al., 2006
		HSV-1, HSV-2	gH/gL and gB	Kurt-Jones et al., 2004; Sorensen et al., 2008; Leoni et al., 2012
		mHV68	?	Michaud et al., 2010
		VZV	?	Wang et al., 2005
	TLR3	EBV	EBV-encoded small RNA	Iwakiri et al., 2009
		HCMV	dsRNA	Nahum et al., 2012
		HSV-1	dsRNA	Zhang et al., 2007; Guo et al., 2011
		KSHV	dsRNA	West and Damania, 2008
		MCMV	dsRNA	Tabeta et al., 2004
	TLR4	HSV-2	?	Liu et al., 2014
		KSHV	?	Lagos et al., 2008
	TLR7	EBV	ssRNA	Martin et al., 2007
		HSV-1	ssRNA	Li et al., 2006
		MCMV	ssRNA	Zucchini et al., 2008
	TLR9	EBV	Genomic DNA	Lim et al., 2007; Fiola et al., 2010
		HCMV	Genomic DNA	Varani et al., 2007
		HSV-1, HSV-2	Genomic DNA	Lund et al., 2003; Krug et al., 2004; Rasmussen et al., 2009
		KSHV	Genomic DNA	West et al., 2011
		MCMV	Genomic DNA	Krug et al., 2004; Tabeta et al., 2004; Delale et al., 2005
		VZV	Genomic DNA	Yu et al., 2011
NLR and PYHIN families	IFI16*	BoHV-1	Genomic DNA	Wang J. et al., 2014
		EBV	Genomic DNA	Ansari et al., 2013
		HCMV	Genomic DNA	Horan et al., 2013
		HSV-1, HSV-2	Genomic DNA	Unterholzner et al., 2010; Horan et al., 2013; Johnson et al., 2013
		KSHV	Genomic DNA	Kerur et al., 2011; Singh et al., 2013; Roy et al., 2016
	NOD1, NOD2	HCMV	?	Kapoor et al., 2014; Fan et al., 2016
	NLRP3	BoHV-1	?	Wang J. et al., 2014
		HSV-1	?	Muruve et al., 2008; Johnson et al., 2013
		MCMV	DAMP (ROS)	Zhuang et al., 2018
		MHV68	?	Sun et al., 2015
		VZV	?	Nour et al., 2011
	AIM2	HCMV	Genomic DNA	Huang et al., 2017
		MCMV	Genomic DNA	Rathinam et al., 2010
	NLRC3	HSV-1	Genomic DNA	Li et al., 2019
RLRs	RIG-I/RNA PolIII	EBV	EBV-encoded small RNA	Samanta et al., 2006, 2008; Ablasser et al., 2009
		HSV-1	dsRNA	Chiu et al., 2009; Rasmussen et al., 2009
		KSHV	Viral transcripts	Zhang Y. et al., 2018
	MDA5	HSV-1	dsRNA	Melchjorsen et al., 2010
CLRs	DC-SIGN	HCMV	gB	Halary et al., 2002
		HSV-1, HSV-2	gB and gC	de Jong et al., 2008
		KSHV	?	Rappocciolo et al., 2006
Intracellular DNA sensors	IFI16*	BoHV-1	Genomic DNA	Wang J. et al., 2014
		EBV	Genomic DNA	Ansari et al., 2013
		HCMV	Genomic DNA	Horan et al., 2013
		HSV-1, HSV-2	Genomic DNA	Unterholzner et al., 2010; Horan et al., 2013; Johnson et al., 2013
		KSHV	Genomic DNA	Kerur et al., 2011; Singh et al., 2013; Roy et al., 2016
	cGAS	EBV	Genomic DNA	Wu et al., 2015
		HCMV	Genomic DNA	Paijo et al., 2016

(Continued)

TABLE 2 | Continued

PRR	Herpesvirus	PAMPs	References
	HSV-1	Genomic DNA	Li et al., 2013; Orzalli et al., 2015
	KSHV	Genomic DNA	Wu et al., 2015
DAI (ZBP1)	HCMV	Genomic DNA	DeFilippis et al., 2010
	HSV-1	Genomic DNA	Takaoka et al., 2007

*The different PRRs responsible for the sensing of herpesviruses are presented, along with the ligand/PAMP detected. AIM2, absent in melanoma 2; BoHV-1, Bovine herpesvirus-1; cGAS, cyclic GMP-AMP (cGAMP) synthase; CLR, C-type lectin receptor; DAI, DNA-dependent activator of interferon-regulatory factors; DAMP, danger-associated molecular pattern; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin dsRNA, double-stranded RNA; EBV, Epstein-Barr virus; HCMV, human cytomegalovirus; HSV, herpes simplex virus; IFI16, gamma interferon-inducible protein 16; KSHV, Kaposi sarcoma–associated herpesvirus; MCMV, murine cytomegalovirus; MDA5, melanoma differentiation-associated protein 5; NLR, nucleotide oligomerization domain (NOD)-like receptor; NLRC3, NOD-like receptor family CARD domain-containing 3; NLRP3, NOD-like receptor family pyrin domain-containing 3; PAMP, pathogTable 2en-associated molecular pattern; RIG-I, retinoic acid-inducible gene-I; RLR, RIG-I-like receptor; RNA Pol III, RNA polymerase III; ROS, reactive oxygen species; ssRNA, single-stranded; TLR, Toll-like receptor; VZV, varicella zoster virus; ZBP1, Z-DNA binding protein 1; ?, unindentified PAMP. *The intracellular DNA sensor IFI16 also belongs to the PYHIN family.*

response protein 88 (MyD88), MyD88 adaptor-like protein (Mal), Toll/interleukin 1 receptor domain-containing adaptor protein (TIRAP), Toll/interleukin 1 receptor domain-containing adaptor-inducing IFN-β (TRIF), and/or TRIF-related adaptor molecule (TRAM). TLR-mediated adaptor oligomerization triggers the formation and activation of two kinase complexes, i.e., TANK-binding kinase 1 (TBK1) and inhibitor of kappa B (IκB) kinase (IKK). TBK1 and IKK in turn activate the transcription factors IRFs and NF-κB *via* phosphorylation, which trigger the production of pro-inflammatory cytokines (for review, see Vidya et al., 2018). Activated IRFs and NF-κB, along with activating transcription factor-2 (ATF-2)/c-Jun, the histone acetyltransferase p300 and CREB-binding protein (CBP), are part of the IFN-β enhanceosome complex responsible for the transcriptional activation of the IFN-β gene (Falvo et al., 2000; Li et al., 2000; Lin et al., 2001; Panne et al., 2007). Dendritic cells (DCs) including plasmacytoid and conventional DCs were also shown to be critical in the production of IFNs and cytokines in response to microbial infection. Hence, many studies have reported a critical role of DCs in the control of herpesviruses such as herpes simplex virus 1 (HSV-1; Siegal et al., 1999), HSV-2 (Stout-Delgado et al., 2008), human cytomegalovirus (HCMV; Kvale et al., 2006; Schneider et al., 2008; Cederarv et al., 2009), Epstein-Barr virus (EBV; Lim et al., 2007) and murine cytomegalovirus (MCMV; Dalod et al., 2002; Andoniou et al., 2005; Puttur et al., 2016). These cells sense the presence of herpesviruses through TLR7 and/or TLR9 on endosomes to induce the production of type I IFNs and other cytokines.

NLR Signaling Pathway and Inflammasomes

NLRs are intracellular sensors located in the cytoplasm and can be activated by either PAMPs or danger-associated molecular patterns (DAMPs). The NLR family is composed of 23 members sharing a similar structure with a central NOD domain, a C-terminal LRR domain and an N-terminal binding region which may be a caspase-recruitment domain (CARD), a pyrin domain (PYD) or a baculovirus inhibitor of apoptosis protein repeat domain (BIR) (for review, see Kersse et al., 2011). The NLRs can be divided into two subfamilies known as the "inflammasome" and the "non-inflammasome" NLRs, depending on their ability to induce the formation of multiprotein complexes called inflammasomes. Inflammasomes consist of the apoptosis-associated speck-like protein containing a CARD (ASC), pro-caspase 1 and an oligomerized member of the NLR family. Inflammasome activation ultimately leads to the proteolytic cleavage and activation of caspase-1, which processes the pro-inflammatory cytokines IL-1β and IL-18 and promotes their secretion (for review, see Lamkanfi and Dixit, 2014). Several PRRs, most of them belonging to the NLR family such as NOD-like receptor family pyrin domain-containing 3 (NLRP3), or the IFI20X/IFI16 (PYHIN) family such as absent in melanoma 2 (AIM2) and IFI16, can trigger the assembly of inflammasomes. During herpesvirus infection, the assembly and activation of NLRP3, AIM2 and IFI16 inflammasomes has been reported upon the detection of various viral PAMPs or DAMPs (**Table 2**). Moreover, a recent study showed that dsDNA from HSV-1 binds to NOD-like receptor family CARD domain-containing 3 (NLRC3), leading to the detachment of NLRC3 from stimulator of interferon genes (STING) that is then available to activate the IFN pathway (Li et al., 2019). Among the "non-inflammasome" NLRs, NOD1, and NOD2 are the most studied and have been shown to induce the formation of another multiprotein complex, the NODosome, which in turn activates IRF, NF-κB, and MAPK in response to viral infection (for review, see Ting et al., 2010). To date, only HCMV has been shown to be detected by NOD1 and NOD2 though the ligand has not been identified yet (Kapoor et al., 2014; Fan et al., 2016).

RLR Signaling Pathway

RLRs are intracellular sensors responsible for the detection of viral dsRNA in the cytoplasm (for review, see Reikine et al., 2014). The RLR family is composed of three homologous members, RIG-I or DExD/H-Box helicase 58 (DDX58), melanoma differentiation-associated protein 5 (MDA5) or IFN-induced with helicase domain 1 (IFIH1) and laboratory of genetics and physiology 2 (LGP2) or DExH-Box helicase 58 (DHX58). These proteins share a similar structure with a central DExH/D-box RNA helicase domain responsible for binding dsRNA and a C-terminal domain (CTD). However, unlike LGP2, RIG-I, and MDA5 possess two additional N-terminal CARD domains that can dimerize with the CARD domain of RIG-I, MDA5 or

mitochondrial antiviral signaling protein (MAVS). Despite their sequence and structural similarities, these proteins recognize distinct features of viral dsRNA. RIG-I detects short dsRNA with a 5′-tri- or di-phosphate moiety, while MDA5 recognizes long dsRNA with no end-specificity (for review, see Bruns and Horvath, 2014). Interestingly, some herpesviruses such as HSV-1, EBV and Kaposi sarcoma-associated herpesvirus (KSHV), produce dsRNA that can be detected by RIG-I, as the RNA-Pol III converts viral DNA into RNA containing 5′ tri-phosphate moiety (Samanta et al., 2006, 2008; Ablasser et al., 2009; Chiu et al., 2009; Rasmussen et al., 2009; Melchjorsen et al., 2010; Zhang Y. et al., 2018). Recently, several studies showed that RIG-I is able to directly recognize viral or host dsRNA species which are not of DNA origin (Samanta et al., 2006; Cheng et al., 2007; Rasmussen et al., 2007; Chiu et al., 2009; West et al., 2014; Liu et al., 2016; Zhang Y. et al., 2018; Zhao et al., 2018; Lee et al., 2019). In resting cells, RIG-I is maintained in an autoinhibited conformation characterized by the intramolecular interaction between the CARD domain and the helicase 2 insertion (Hel2i) domain (Kowalinski et al., 2011; Luo et al., 2011), thereby exposing the CTD to patrol the cytoplasm for microbial dsRNA (Jiang et al., 2011). The binding of dsRNA by CTD triggers an overall conformation change of RIG-I, which coils around the dsRNA helix to form a "O" ring-like structure. In doing so, RIG-I exposes the CARD domain, so it is free to interact with the CARD domain of MAVS, promoting the transcription and production of IFNs and inflammatory cytokines (Kawai et al., 2005; Seth et al., 2005; Xu et al., 2005; Cui et al., 2008; Takahasi et al., 2008). Interestingly, dsRNA binding to MDA5 induces CTD rotation, which triggers the formation of an oligomeric MDA5 filament on the dsRNA. Filament formation of MDA5 releases its CARD domains, which heterodimerize with the CARD of MAVS (Peisley et al., 2011; Berke and Modis, 2012; Reikine et al., 2014). Lacking CARD domains, LGP2 cannot induce downstream innate immune signaling. Perplexingly, LGP2 has been reported as being able to act either as a negative regulator of the recognition of viral RNA by RIG-I or as a positive regulator of RIG-I- and MDA5-mediated viral dsRNA recognition and antiviral signaling (Yoneyama et al., 2005; Satoh et al., 2010; Childs et al., 2013; Bruns and Horvath, 2015). The opposing effect of LGP2 on dsRNA-mediated innate immune signaling may be context-dependent. Nevertheless, these results suggest that LGP2 is a regulatory homolog of RIG-I and MDA5.

CLRs Signaling Pathway

CLRs are selectively expressed on the surface of immune cells such as Langerhans cells, monocytes, macrophages and DCs. These proteins specifically recognize carbohydrate moieties to sense pathogens. Binding to carbohydrates triggers the internalization and typically the degradation of CLRs *via* the lysosomal pathway (for review, see Bermejo-Jambrina et al., 2018). Given their presence on immune cells, these CLRs are able to trigger both innate and adaptive (*via* the presentation of microbial antigens) immune responses. Some CLRs such as immunoreceptor tyrosine-based activation motifs (ITAMs) in Dectin-2, DC immune-activating receptor (DCAR) and myeloid DAP12-associated lectin-1 (MDL-1) and immunoreceptor tyrosine-based inhibition motifs (ITIMs) in DC immunoreceptor (DCIR), contain distinct signaling motifs. Other CLRs such as DC-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin (DC-SIGN), mannose receptor (MR) and lymphocyte antigen 75 (LY75), contain no signaling motifs in their cytoplasmic tails at all. These motifs provide a structural and physical platform to enable the crosstalk with the immune pathways triggered by other PRRs. Very few studies have implicated roles of CLRs during herpesvirus infection. DC-SIGN and the related receptor DC-SIGNR have been reported to be activated by HSV-1, HSV-2, KSHV, and HCMV (**Table 2**; Halary et al., 2002; Rappocciolo et al., 2006; de Jong et al., 2008). To date, the post-translational control of CLR-mediated recognition of herpesviruses remains unknown.

Intracellular DNA Sensors

Recently, several intracellular DNA sensors have been identified, including cGAS, IFI16, DNA-dependent activator of IFN-regulatory factors (DAI), AIM2, DEAD-box helicase 41 (DDX41), Z-DNA binding protein 1 (ZBP1), and RNA polymerase III (Takaoka et al., 2007; Chiu et al., 2009; Hornung et al., 2009; Unterholzner et al., 2010; Parvatiyar et al., 2012). Further studies have reported a pivotal role for cGAS in sensing cytosolic DNA. Upon detection of cytoplasmic DNA, cGAS undergoes dimerization and a structural rearrangement centered on the catalytic core that synthesizes cGAMP, a second messenger that in turn triggers a conformational change and activation of STING (Li et al., 2013; Sun et al., 2013; Zhang X. et al., 2013). Activated STING recruits TBK1 and IRF3 to facilitate the phosphorylation of IRF3 by TBK1 and the subsequent production of IFN-β (Ishikawa and Barber, 2008; Unterholzner et al., 2010; Abe et al., 2013; Unterholzner, 2013; Wu et al., 2013; Almine et al., 2017). Compared to other nucleic acid sensors, such as RIG-I for dsRNA, the DNA-binding affinity of cGAS appears to be low; the potent enzymatic activity in cGAMP synthesis may compensate for this (Li et al., 2013). Alternatively, a growing amount of studies highlighted the importance of the post-translational control of cGAS in the establishment of a proper and regulated antiviral response. Hence, cGAS stability, enzymatic activity and binding to dsDNA is tightly regulated by phosphorylation (Seo et al., 2015), ubiquitination (Bhoj and Chen, 2009; Chen et al., 2016; Wang et al., 2017), SUMOylation (Hu et al., 2016; Cui et al., 2017) and glutamylation (Xia et al., 2016). Unlike the other DNA sensors, IFI16 is primarily located in the nuclei of resting cells. Upon HSV-1 infection, IFI16 interacts with the viral genome in the nucleus and is acetylated by the histone acetyltransferase p300, thus triggering its translocation into the cytoplasm where it binds to STING to induce IFN production (Unterholzner et al., 2010; Ansari et al., 2015). IFI16 can also activate a caspase 1-dependent inflammasome that processes and promotes the production of IL-1β and IL-18 (for review, see Dempsey and Bowie, 2015). cGAS, IFI16 and DAI have been shown to detect the genomic dsDNA of herpesviruses and in particular EBV, HCMV, HSV-1, HSV-2, KSHV, and bovine herpesvirus-1 (BoHV-1; **Table 2**; Takaoka et al., 2007; DeFilippis et al., 2010; Unterholzner et al., 2010; Kerur et al., 2011; Ansari et al., 2013; Horan et al., 2013;

Johnson et al., 2013; Li et al., 2013; Singh et al., 2013; Wang J. et al., 2014; Orzalli et al., 2015; Wu et al., 2015; Paijo et al., 2016; Roy et al., 2016).

Interestingly, several studies revealed a dynamic interaction between DNA- and RNA- sensing pathways. Hence, upon HSV-1 infection and recognition of the viral 5′ tri-phosphorylated RNA by RIG-I, RIG-I upregulates STING through the NF-κB and JAK/STAT cascades (Liu et al., 2016; Zevini et al., 2017). This study also demonstrated that a proper antiviral response to HSV-1 infection *in vivo* through RIG-I signaling requires STING, revealing STING as a central molecule of RNA- and DNA-sensing pathways. However, Wu et al. (2017), demonstrated that the transfection of dsDNA into human diploid cells leads to the proteasome-mediated degradation of STING associated with an upregulation of RIG-I and IL-6 expression. In this study, the authors also showed that RIG-I and IL-6 are responsible for STING degradation, providing a negative feedback mechanism to limit the activation of STING-mediated innate immune signaling (Wu et al., 2017). This mechanism, however, seems to be limited to human diploid cells as it was not observed in HEK293 cells, suggesting that it might be a cell type-specific regulatory mechanism.

POST-TRANSLATIONAL CONTROL OF PRR-MEDIATED SIGNALING PATHWAYS BY HERPESVIRUSES

Herpesviruses prevent pro-inflammatory and antiviral responses by regulating the phosphorylation, ubiquitination, SUMOylation, acetylation, deamidation and ISGylation of proteins involved in PRR-mediated signaling pathways. Phosphorylation, the most common PTM, is induced by kinases and reversed by phosphatases. Phosphorylation is critical for the regulation of numerous biological processes including cell cycle, cell growth, apoptosis, metabolism and signal transduction (for review, see Ardito et al., 2017). Phosphorylation is required for a proper signal transduction along innate immune signaling pathways. Therefore, herpesviruses have evolved to regulate the phosphorylation of critical signaling proteins either by expressing kinases inducing aberrant phosphorylation or by inhibiting cellular kinases (**Table 3**).

Ubiquitination corresponds to the attachment of ubiquitin moiety to lysine residues of a target protein and requires the sequential action of three enzymes: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase. The ubiquitination process starts with the attachment of a monoubiquitin to the target residue and can remain as monoubiquitination or be further extended with the attachment of additional ubiquitin molecules, forming elongated polyubiquitin chains. The conventional linkage of ubiquitin occurs on lysine residues of the target protein although unconventional linkages to cysteine residues have also been identified (for review, see McDowell and Philpott, 2013). Both monoubiquitination and polyubiquitination are essential in the regulation of biological processes as they define the fate of a target protein depending on the lysine residue conjugated to the carboxyl terminal di-glycine of the ubiquitin. Hence, for instance, mono-ubiquitination has been associated with the regulation of protein trafficking and subcellular localization, or chromatin regulation while polyubiquitination has been associated with protein clearance though the proteasomal or autophagic pathways and with protein signaling (for review, see Husnjak and Dikic, 2012). The fate of a target protein is further regulated by ubiquitin chain linkage or topology. Ubiquitin itself contains seven lysine residues (K6, K11, K27, K29, K33, K48, K63, and M1) that can be ubiquitinated and the role of K48 and K63, to a less extent K27, is well established. Specifically, the K48-linked polyubiquitin chain generally targets proteins for proteasomal degradation, while a polyubiquitin chain of the K63-linkage can regulate signal transduction or protein trafficking (for review, see Pickart and Eddins, 2004). Given the critical role of this PTM in the regulation of innate immune signaling pathways, it is not surprising that herpesviruses exploit ubiquitination to dampen the cellular antiviral response. Notably, herpesviruses express proteins that possess the intrinsic activities of either E3 ubiquitin ligases or deubiquitinases (**Table 3**).

SUMOylation has emerged as a critical player in the regulation of signaling pathways. SUMOylation occurs after the binding of a protein containing a SUMO-interacting motif (SIM). Similar to ubiquitination, SUMOylation is a cascade of reactions catalyzed by three distinct enzymes, a SUMO E1 activating enzyme, a SUMO E2 conjugating enzyme, and a SUMO E3 ligase which links a SUMO moiety to a lysine residue in the target protein. SUMOylation has diverse consequences on the target protein and plays an important role in regulating protein localization, trafficking and signal transduction (for review, see Wilkinson and Henley, 2010). Studies have reported strategies evolved by herpesviruses to dampen SUMOylation by host SUMO E3 ligases or to directly SUMOylate proteins involved in antiviral signaling (**Table 3**).

Acetylation is one of the major PTMs and consists of the attachment of an acetyl group from the donor acetyl-coenzyme A (Acetyl-CoA) to the target protein. This reaction is catalyzed by acetyltransferases and can either occur at the N-terminus of the target protein or at the α-amino group of lysine residues (for review, see Drazic et al., 2016). Along with the other PTMs, acetylation allows the tight regulation of numerous biological processes by regulating the function and localization of the target proteins including those involved in innate immune signaling pathways. Hence, acetylation is also controlled by herpesviruses to restrict antiviral responses (**Table 3**). Notably, upon infection by KSHV, EBV and HSV-1, the innate DNA sensor IFI16 interacts with the histone acetyltransferase p300 and CREB-binding protein (CBP) in the nucleus, which leads to the acetylation of IFI16. This acetylation triggers the translocation of IFI16 to the cytoplasm, where it then interacts with ASC, resulting in inflammasome assembly and increased interaction with STING. Ultimately, these signaling events activate IFN-β induction (Wathelet et al., 1998; Ansari et al., 2015; Dutta et al., 2015). Acetylation often targets lysine residues for modification like ubiquitination and SUMOylation. These PTMs may compete and target the same lysine residue to dictate the fate of the target protein. Acetylation and Acetyl-CoA provide a point of

TABLE 3 | Post-translational control of PRR-mediated signaling pathways by herpesviruses.

	Target protein	Herpesvirus	Viral regulators	Regulation	Signaling function	References
PRRs	RIG-I	EBV	BPLF1	Reduced ubiquitination	EBV BPLF1 interacts with TRIM25 and promotes its autoubiquitination and inactivation, thereby blocking RIG-I activation	Gupta et al., 2018
		HSV-1	UL37	Deamidation	The HSV-1 tegument protein UL37 deamidates RIG-I, blocking its activation	He et al., 2015; Zhao J. et al., 2016
		KSHV	ORF64	Reduced ubiquitination	KSHV deubiquitinase ORF64 inhibits RIG-I activation by reducing its ubquitination	Inn et al., 2011
		KSHV	k-vGAT	Deamidation	KSHV-encoded vGAT induces the deamidation and activation of RIG-I	He et al., 2015
		MHV68	vGAT	Deamidation	MHV68 vGAT recruits the cellular GAT PFAS to deamidate and activate RIG-I, which is necessary for RelA degradation and to avoid the production of inflammatory cytokines	He et al., 2015
	DC-SIGN, DC-SIGNR	KSHV	K3, K5	Ubiquitination/ proteasome-dependent degradation	KSHV ubiquitin ligases K3 and K5 target DC-SIGN and DC-SIGNR for ubiquitination and proteasome-dependent degradation	Lang et al., 2013
	IFI16	HSV-1	ICP0	Ubiquitination/ proteasome-dependent degradation	HSV-1 E3 ubiquitin ligase ICP0 promotes the ubiquitin/ proteasome-dependent degradation of IFI16	Orzalli et al., 2012
	cGAS	HSV-1	UL37	Deamidation	The HSV-1 tegument protein UL37 deamidates cGAS to block cGAS-mediated signaling	Zhang J. et al., 2018
Adaptor proteins	MyD88/Mal/ TIRAP	HSV-1	ICP0	Ubiquitination/ proteasome-dependent degradation	HSV-1 E3 ligase ICP0 ubiquitinates MyD88 and Mal (TIRAP), leading to their proteasome-mediated degradation	van Lint et al., 2010
		KSHV	RTA	Ubiquitination/ proteasome-dependent degradation	KSHV protein RTA induces the uniquitin/ proteasome-dependent degradation of MyD88	Zhao et al., 2015
	TRAF3	HSV-1	UL36USP	Reduced ubiquitination	HSV-1 UL36USP deubiquinates TRAF3 ubiquitination, which is no longer recruited to TBK1	Wang et al., 2013a
	TRAF6	EBV	LMP1	Reduced ubiquitination	EBV LMP1 deubiquitinates TRAF6, blocking its activation and NF-κB signaling	Saito et al., 2013
		HSV-1	US3	Reduced ubiquitination	HSV-1 US3 reduces TRAF6 ubiquitination required for its activation and for TLR2-mediated signaling	Sen et al., 2013
		HSV-1	ICP0	Reduced ubiquitination	HSV-1 ICP0 triggers the translocation of the E3 ubiquitin ligase USP7 from the nucleus to the cytoplasm which will deubiquitinate TRAF6, inhibiting the TLR-mediated NF-κB signaling	Daubeuf et al., 2009
	STING	HSV-1	$\gamma_1$34.5	Reduced phosphorylation	HSV-1 γ134.5 protein inhibited STING phosphorylation and activation, likely via a targeted phosphatase activity	Pan et al., 2018
		KSHV	vIRF1	Reduced phosphorylation	KSHV vIRF1 directly interacts with STING, blocking its interaction with TBK1, inhibiting TBK1 phosphorylation and activation of STING and the subsequent production of IFN-β	Ma et al., 2015
		HCMV	UL48	Reduced ubiquitination	HCMV UL48 deubiquitinase enzyme reduces K63-linked ubiquitination of STING, thus blocking STING activation	Kumari et al., 2017
		MHV68	ORF64	Reduced ubiquitination	MHV68 deubiquitinase ORF64 prevents the activation of the STING signaling pathway	Sun et al., 2015
IRFs	IRF3	EBV	BGLF4	Phosphorylation	EBV BGLF4 phosphorylates IRF3 at Ser123, Ser173, and Thr180, inhibiting its recruitment to ISREs	Wang et al., 2009
		HSV-1	US11	Reduced phosphorylation	HSV-1 US11 directly interacts with RIG-I and MDA5, impeding IRF3 activation by reducing its phosphorylation and dimerization	Xing et al., 2012
		HSV-1	US3	Hyperphosphorylation	HSV-1 US3 interacts with and hyperphosphorylates IRF3 at Ser175 to prevent its activation	Wang et al., 2013b

(Continued)

TABLE 3 | Continued

	Target protein	Herpesvirus	Viral regulators	Regulation	Signaling function	References
		HSV-1	ICP34.5	Reduced phosphorylation	HSV-1 ICP34.5 directly interacts with TBK1, blocking the phosphorylation of IRF3	Verpooten et al., 2009; Ma et al., 2012
		HSV-1	VP24	Reduced phosphorylation	HSV-1 VP24 inhibits the interferon stimulatory DNA-mediated phosphorylation and dimerization of IRF3	Zhang et al., 2016
		HSV-1	ICP27	Reduced phosphorylation	HSV-1 ICP27 interacts with STING, blocking TBK1-mediated phosphorylation of IRF3	Christensen et al., 2016
		KSHV	miR-K12-11	Reduced phosphorylation	KSHV-encoded miR-K12-11 inhibits IRF3 phosphorylation by targeting IKKε	Liang et al., 2011
		HSV-1	ICP0	Ubiquitination/ proteasome-dependent degradation	HSV-1 E3 ubiquitin ligase ICP0 promotes the ubiquitin/ proteasome-dependent degradation of IRF3	Melroe et al., 2004
		VZV	ORF61	Ubiquitination/ proteasome-dependent degradation	VZV ORF61 directly interacts with activated IRF3, and IRF3 is ubiquitinated and downregulated in the presence of ORF61	Zhu et al., 2011
	IRF7	EBV	LMP1	SUMOylation	EBV LMP1 inhibits IRF7 by inducing its SUMOylation	Bentz et al., 2012
		KSHV	RTA	Ubiquitination/ proteasome-dependent degradation	KSHV RTA promotes the ubiquitin/ proteasome-dependent degradation of IRF7	Yu et al., 2005
		EBV	LMP1	Ubiquitination/ proteasome-dependent degradation	EBV LMP1 promotes the TRAF6-mediated ubiquitination/ proteasome-dependent degradation of IRF7	Ning et al., 2008
	IRF9	VZV	ORF63	Ubiquitination/ proteasome-dependent degradation	VZV ORF63 reduces the levels of IRF9 in a proteasome degradation-dependent pathway	Verweij et al., 2015
	IRF1, IRF2, IRF7	KSHV	K-bZIP	SUMOylation	KSHV encodes a SUMO E3 ligase named K-bZIP potentially targeting the transcription factors IRF1, 2, and 7	Chang et al., 2010, 2013
	IRFs	KSHV	vIRF1	Reduced acetylation	vIRF1 directly interacts with p300 and reduces its histone acetyltransferase activity, affecting the formation of the CBP/p300 enhanceosome complex responsible for the cellular IRF transcriptional activity	Li et al., 2000; Lin et al., 2001
NF-κB	IKKβ	HCMV	UL26	Reduced phosphorylation	HCMV UL26 blocks the phosphorylation and activation of IKKβ, which is the key step for IκB phosphorylation and NF-κB activation	Mathers et al., 2014
	IκB	VZV	ORF61	Reduced ubiquitination	VZV ORF61 blocks IκB ubiquitination, inhibiting NF-κB signaling	Whitmer et al., 2015
		HSV-1	UL36USP	Reduced ubiquitination	HSV-1 UL36USP deubiquitinates IκBα, preventing it from degradation and blocking NF-κB in an inactivated form	Ye et al., 2017
	p65/RelA	HSV-1	US3	Hyperphosphorylation	HSV-1 US3 hyperphosphorylates p65 at Ser75, blocking its nuclear translocation	Wang K. et al., 2014
		MHV68	n.d.	Phosphorylation	MHV68 hijacks RIG-I and MAVS to activate IKKβ, inducing RelA phosphorylation at Ser468 and subsequent RelA degradation by the proteasomal pathway	Dong and Feng, 2011
		MHV68	ORF73	Ubiquitination/ proteasome-dependent degradation	MuHV-4 ORF73 interacts *via* its SOCS box motif with ElonginC and Cullin5 to mediate p65/RelA ubiquitination and degradation	Rodrigues et al., 2009
	p50	HSV-1	ICP0	Ubiquitination/ proteasome-dependent degradation	HSV-1 ICP0 interacts directly with p50 and p65/RelA, blocks the nuclear translocation of p65/RelA and induces the ubiquitination and proteasome-dependent degradation of p50	Zhang J. et al., 2013
JAK/STAT	TYK2	EBV	LMP1	Reduced phosphorylation	EBV LMP-1 directly interacts with TYK2, blocking its phosphorylation/activation and subsequent IFNα signaling	Geiger and Martin, 2006

(Continued)

TABLE 3 | Continued

	Target protein	Herpesvirus	Viral regulators	Regulation	Signaling function	References
	JAK	HSV-1	VR3, UL13, UL41	Reduced phosphorylation	HSV-1 VR3, UL13, and UL41 induce the expression of SOCS1 and SOCS3 protein to inhibit JAK phosphorylation	Chee and Roizman, 2004; Yokota et al., 2004; Sato et al., 2017
	STAT1	EBV	BZLF1	Reduced phosphorylation	EBV BZLF1 inhibits IFN-gamma-induced STAT1 tyrosine phosphorylation	Morrison et al., 2001
		HSV-1	ICP27	Reduced phosphorylation	HSV-1 ICP27 downregulates STAT1 phosphorylation and its accumulation in the nucleus	Johnson et al., 2008
	STAT2	MCMV	pMP27	Ubiquitination/ proteasome-dependent degradation	pM27 induces STAT2 ubiquitination and degradation by the proteasome, likely through its interaction with DDB1	Trilling et al., 2011
		VZV	ORF63	Reduced phosphorylation	VZV ORF63 interferes with JAK-STAT signaling by reducing the IFN-induced STAT2 phosphorylation	Verweij et al., 2015
ISGylation	UL26	HCMV	IE1, UL26	Reduced ISGylation	In response to infection, HCMV pUL26 is ISGylated, destabilizing the protein and inhibiting its ability to restrict the NF-κB response. HCMV IE1 and pUL26 can suppress infection-induced ISGylation	Kim et al., 2016
	pUL50	HCMV	UBE1L	Reduced ISGylation	HCMV IE1 and UL26 are able to suppress the infection-induced ISGylation	Lee et al., 2018
	Global ISGylation	KSHV	vIRF1	Reduced ISGylation	vIRF1 interacts with the ISG15 E3 ligase HERC5, leading to a global decreased ISGylation of proteins in infected cells	Jacobs et al., 2015

The target protein, the viral protein responsible for the regulation, the PTM involved, and the signaling function/consequence of the regulation are presented. CBP, CREB-binding protein; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin; DC-SIGNR, DC-SIGN receptor; DDB1, DNA binding protein 1; EBV, Epstein-Barr virus; GAT, glutamine amidotransferase; vGAT, viral GAT; HSV, herpes simplex virus; HERC5, HECT and RLD domain containing E3 ubiquitin protein ligase 5; HCMV, human cytomagalovirus; IFI16, gamma interferon-inducible protein 16; IFN, interferon; IκB, inhibitor of kappa B; IKKe, IκB kinase; IRF, interferon regulatory factor; ISG, interferon-stimulated gene; ISREs, interferon-sensitive response elements; JAK, janus kinase; K-bZIP, KSHV basic region-leucine zipper; KSHV, Kaposi sarcoma–associated herpesvirus; LMP1, latent membrane protein 1; Mal, MyD88 adaptor-like protein; MDA5, melanoma differentiation-associated protein 5; miR, microRNA; MyD88, myeloid differentiation primary response protein 88, NF-κB, nuclear factor-kappa B; ORF, open reading frame; PFAS, phosphoribosylformylglycinamidine synthetase; RIG-I, retinoic acid-inducible gene-I; RTA, replication and transcription activator; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; STING, stimulator of IFN genes; TBK1, TANK-binding kinase 1; TIRAP, Toll/interleukin 1 receptor domain-containing adaptor protein; TRAF, tumor necrosis factor (TNF) receptor-associated factor; TRIM, tripartite motif; TYK2, non-receptor tyrosine kinase 2; UL36USP, UL36 ubiquitin-specific protease; USP7, ubiquitin specific peptidase 7; VZV, varicella zoster virus.

crosstalk with key cellular metabolic pathways since Acetyl-CoA intersects with glycolysis, tricarboxylic acid cycle (TCA) and lipid synthesis. A growing amount of studies demonstrated a metabolic reprogramming upon herpesvirus infection and on glycolysis, TCA cycle and lipid synthesis in particular (for review, see Thaker et al., 2019), giving insight on the critical role that Acetyl-CoA and acetylation might play in herpesvirus pathogenesis.

Post-translational Control of PRRs

RIG-I

The mechanism of RIG-I activation requires K63-ubiquitination by E3 ubiquitin ligases including tripartite motif 25 (TRIM25; Gack et al., 2007) and RIPLET (Cadena et al., 2019). To fully activate RIG-I, both covalent conjugation of a polyubiquitin chain to RIG-I and free polyubiquitin chains are required to induce RIG-I oligomerization (Zeng et al., 2010; Peisley et al., 2014). RIG-I ubiquitination is a critical checkpoint to regulate its dsRNA engagement and subsequent activation. Therefore, herpesviruses demonstrate the ability to counteract this activation mechanism. For instance, KSHV open reading frame (ORF) 64 is able to disassemble these polyubiquitin chains through its deubiquitinase activity to suppress RIG-I activation and the subsequent antiviral signaling, leading to an increased lytic replication of the virus (Inn et al., 2011). Recently, Gupta et al. (2018), reported that BPLF1, encoded by the EBV genome, interacts with TRIM25 to promote its auto-ubiquitination and inactivation, thereby blocking RIG-I activation and the IFN induction. This study also revealed that the effect of BPLF1 is conserved in homolog proteins encoded by other herpesvirus families such as HSV, HCMV, and KSHV. Controlling RIG-I deamidation enabled herpesviruses to evade the immune system as well. Indeed, murid gamma-herpesvirus 4 (MuHV-4) also known as murine gamma herpesvirus 68 (MHV68), a murine model gamma-herpesvirus closely related to human KSHV and EBV, encodes homologs of cellular glutamine amidotransferases (GATs), dubbed viral GATs or vGATs. vGAT encoded by the ORF75c open reading frame lacks key active sites required for enzyme catalysis and is a pseudo-enzyme. Despite missing intrinsic enzymatic activity, vGAT can recruit cellular phosphoribosyl-formylglycinamidine synthetase (PFAS) to deamidate and activate RIG-I (He et al., 2015). While KSHV and EBV encode one vGAT, herpesvirus saimiri (HVS; a strain infecting primates) and MHV68 encode two and three vGAT homologs, respectively. Whether these additional vGAT homologs possess a similar function remains unknown. HSV-1

encodes no homolog of vGAT, however, the infection induces a shift of RIG-I toward the positive side on a two-dimensional gel electrophoresis which is an indication of deamidation. A functional screen identified that the tegument protein UL37 demonstrates intrinsic enzymatic activity to deamidate RIG-I and that this deamidation abolishes the ability of RIG-I to bind and sense dsRNA (Zhao J. et al., 2016). Retrospectively, it appears that UL37 of alpha-herpesviruses shares a similar repertoire of function with vGAT proteins of gamma-herpesviruses, despite the lack of sequence homology (Gaspar et al., 2008; Liu et al., 2008; Pitts et al., 2014; He et al., 2015). Whether beta-herpesviruses express viral proteins with similar properties is unclear. These observations reveal that herpesviruses utilize protein phosphorylation and deamidation to evade host innate immune activation upon recognition of viral RNA by RIG-I.

DC-SIGN and DC-SIGNR

The post-translational regulation of CLRs by herpesviruses is not well understood. However, a study revealed that the KSHV-encoded ubiquitin ligases K3 and K5 are able to target the receptors DC-SIGN and DC-SIGNR for ubiquitination and subsequent proteasome-dependent degradation (Lang et al., 2013) implying the defensive role of these receptors against KSHV infection.

cGAS and IFI16

cGAS and IFI16 are important in host innate immune defense and are targeted by herpesviruses to shut off the inflammatory and antiviral responses. For instance, HSV-1 infection is sensed in the nucleus by IFI16 upon release of viral DNA, which presumably triggers IFI16 acetylation and subsequent nuclear export. Cytoplasmic IFI16 then induces IFN production or inflammasome activation (Unterholzner et al., 2010; Horan et al., 2013; Johnson et al., 2013). Interestingly, IFI16 was also reported to act in the nucleus to directly suppress viral gene expression against KSHV and HCMV (Kerur et al., 2011; Singh et al., 2013; Roy et al., 2016). An unanswered question is how IFI16 distinguishes cellular from viral genomic DNA in the nucleus. Additionally, the dichotomy of IFI16 action in antiviral defense at two distinct subcellular locations against herpesvirus infection requires further investigation.

HSV-1 has evolved to regulate the ubiquitination of IFI16 and the deamidation of cGAS. Hence, the HSV-1 E3 ubiquitin ligase ICP0 promotes the ubiquitination and proteasome-dependent degradation of IFI16, thus inhibiting viral DNA detection and the consequent IRF3-mediated signaling (Orzalli et al., 2012). However, Cuchet-Lourenco et al. (2013), later demonstrated that HSV-1 ICP0 is not sufficient to destabilize IFI16 and that IFI16 seems to be required for the recruitment of the restriction factor promyelocytic leukemia protein (PML) to decrease the viral replication, suggesting the existence of an IFN-independent antiviral activity of IFI16. Interestingly, HSV-1 UL37 was recently shown to deamidate human and mouse cGAS, but not cGAS from non-human primates, to antagonize cGAS-mediated immune activation (Zhang J. et al., 2018). Deamidated cGAS, although able to interact with dsDNA and dimerize, fails to synthesize cGAMP and induce downstream signaling, allowing an increased replication of the virus. Thus, deamidation of RIG-I and cGAS conveyed by HSV-1 UL37 inactivates host innate immune activation by dsRNA and dsDNA, respectively. Importantly, a recombinant HSV-1 carrying an active site mutation within UL37 is highly attenuated in its viral replication and pathogenesis in mice. The loss of cGAS and STING restored the replication and pathogenesis of the deamidase-deficient HSV-1 in mice, demonstrating a specific role of UL37-mediated evasion from the cGAS-STING signaling pathway *in vivo*. The role of UL37-mediated evasion of RIG-I by HSV-1 remains to be determined. These studies revealed immune evasion mechanisms *via* regulating the ubiquitination and deamidation of IFI16 and cGAS respectively to restrict the recognition and subsequent antiviral signaling by these DNA sensors in the context of HSV-1 infection. However, the use of similar mechanisms by other herpesviruses is yet to be determined. Moreover, whether herpesviruses exploit or modulate cGAS-regulating PTMs i.e., phosphorylation, ubiquitination, SUMOylation, and glutamylation would be an interesting study that paves the way for identifying new immune evasion strategies of herpesviruses.

Post-translational Control of PRR Adaptors

MyD88/Mal/TIRAP

Mal is a bridging adapter molecule responsible for the specific recruitment of MyD88 to the TLR2 and TLR4 complexes (Yamamoto et al., 2002; Fitzgerald et al., 2003; Kagan and Medzhitov, 2006). By targeting these adaptor proteins for degradation by the proteasomal pathway, HSV-1 and KSHV were shown to be able to restrict TLR-mediated antiviral signaling. HSV-1 ICP0 inhibits TLR2-mediated NF-κB activation by inducing the ubiquitination and subsequently the proteasomal-mediated degradation of MyD88 and Mal/TIRAP (Hagglund and Roizman, 2004; van Lint et al., 2010). KSHV can also induce the ubiquitin/proteasome-mediated degradation of MyD88 *via* its replication and transcription activator (RTA) protein and thereby inhibit TLR4 signaling (Zhao et al., 2015). Both ICP0 and RTA are expressed immediately after viral entry and belong to the immediate-early category of viral genes, consistent with the putative evasion from host defense. Unfortunately, their effect on viral lytic replication was not assessed under those conditions and remains an open question. A related study, although independent of the post-translational control of MyD88, showed that MyD88 is required for the efficient establishment of a latent MHV68 infection in B cells in a mouse model, suggesting that herpesviruses may evolve to usurp this molecule for persistent infection (Forrest and Speck, 2008). These findings highlight the function of viral proteins in the regulation of TLR signaling *via* targeting the adaptor molecules MyD88 and Mal/TIRAP.

TRAFs

Tumor necrosis factor (TNF) receptor-associated factor (TRAF) is a family of proteins that act as adaptor molecules in various signaling pathways. TRAF3 and TRAF6 are components of the TIR signaling complexes and are activated by conjugation of polyubiquitin chains upon TLR activation. TRAFs are critical

for the induction of the type I IFN response and are recruited to the adaptors TRIF, interleukin 1 receptor-associated kinase 1 (IRAK1), and downstream of TBK1 and IKKε (for review, see Xie, 2013). Several studies have demonstrated a critical role of TRAF3 in TLR-dependent and -independent antiviral responses (Hacker et al., 2006; Oganesyan et al., 2006). As central components of TIR signaling complexes, TRAF proteins are often targeted by herpesviruses to derail the host immune defense, thus enabling their lifelong persistence. In particular, since the ubiquitination of TRAF proteins is required for their activation and proper TLR signaling, herpesviruses were shown to induce their deubiquitination. For instance, the HSV-1 US3 kinase inhibits TLR2 signaling pathway and the subsequent production of inflammatory cytokines by reducing TRAF6 ubiquitination, which is critical for its function in TLR2 signaling, and this requires the kinase activity of US3 (Sen et al., 2013). It is not clear how the US3-dependent kinase activity inhibits TRAF6 autoubiquitination. Understanding this molecular detail may reveal a mechanism mediating a crosstalk between phosphorylation and ubiquitination. The largest tegument protein of HSV-1, UL36, which is conserved among all herpesviruses, contains a N-terminal de-ubiquitinase motif known as UL36 ubiquitin-specific protease (UL36USP; Kattenhorn et al., 2005; Abaitua and O'Hare, 2008). This protein has been shown to be critical for HSV-1 replication as it deubiquitinates TRAF3, leading to the inhibition of TBK1 recruitment and ultimately a diminished IFN-β induction (Wang et al., 2013a). Furthermore, the HSV-1 ICP0 protein can trigger the translocation of the cellular E3 ubiquitin ligase ubiquitin specific peptidase 7 (USP7) from the nucleus to the cytoplasm, where it deubiquitinates TRAF6 to terminate the TLR-mediated NF-κB activation (Daubeuf et al., 2009). Also, EBV BPLF1 deubiquitinates TRAF6 to downregulate NF-κB signaling (Saito et al., 2013). These findings characterize viral proteins that counteract the ubiquitinating activity of TRAF molecules either *via* their intrinsic enzyme activity or *via* engaging cellular enzymes.

STING

STING is critical for the induction of the IFN response upon detection of viral DNA by intracellular DNA sensors. Therefore, herpesviruses acquired the ability to prevent STING-mediated antiviral responses. For instance, KSHV encodes four IRF homologs, namely vIRF1-4, which inhibit the host immune response by acting as transcriptional activators (for review, see Jacobs and Damania, 2011). KSHV vIRF1 was shown to interact with STING, blocking its interaction with TBK1. This negates STING phosphorylation and activation, and the subsequent induction of IFN-β, promoting KSHV replication (Ma et al., 2015). Similarly, HSV-1 ICP27 interacts with STING to block TBK1-mediated phosphorylation and activation of IRF3 (Christensen et al., 2016). A recent report showed that HSV-1 ICP34.5, a known neurovirulence factor, inhibits STING phosphorylation and activation likely *via* a targeted phosphatase activity, resulting in an increased viral replication (Pan et al., 2018). Given that multiple viral proteins from the same virus, e.g., HSV-1, target the cGAS-STING-IFN pathway for interference, it is a burning question how each individual viral protein contributes to the smoldered immune response and the elevated viral replication and pathogenesis thereof. Recombinant herpesvirus strains with exquisitely designed mutations will provide a powerful tool to answer this question.

Deubiquitination and subsequent inactivation of STING has also been shown to be triggered by herpesviruses to favorize their persistence. Sun et al. (2015), revealed that the absence of the deubiquitinase activity of the MHV68 ORF64 tegument protein is associated with an impaired delivery of the viral DNA into the nucleus. This ORF64 deficiency also triggers a STING-dependent antiviral signaling blocking the establishment of a latent infection *in vivo* (Sun et al., 2015). Interestingly, the gamma-herpesvirus ORF64 is a homolog of the alpha-herpesvirus UL36 protein that, in addition to its deubiquitinase activity, is also crucial for viral capsid maturation and subsequent tegumentation. A regulation of STING activation by HCMV has also been reported. Indeed, the HCMV-encoded UL48 deubiquitinase reduces the K63-linked ubiquitination of STING, thus blocking its activation and downstream antiviral signaling, while promoting HCMV-associated carcinogenesis (Kumari et al., 2017). These studies revealed that herpesviruses acquired the ability to inhibit STING activation to restrict the antiviral response upon viral DNA detection. However, in the case of HSV-1, a study reported an ambivalent link between STING and IFI16 and the virus-encoded proteins ICP0, ICP4, and US3, because these proteins appear to be required to stabilize IFI16 and STING in HSV-1-infected cells (Kalamvoki and Roizman, 2014). The observation that most herpesvirus-encoded deubiquitinating enzymes target STING for inactivation suggests a central role of STING in innate immune activation. Furthermore, the unique dynamic regulation of STING in trafficking from the ER membrane to the trans-Golgi network in innate immune signaling activation provides an opportunity to investigate the integration of organelles and PTMs in signal transduction.

Post-translational Control of IRFs

IRF transcription factors are activated through phosphorylation by IKK-related kinases (TBK1 and IKKε), which induce their dimerization and subsequent translocation into the nucleus. Collaborating with other transcription factors of the NF-κB and AP-1 families, IRFs bind to interferon-sensitive response elements (ISRE) to activate the transcription of their target genes (e.g., IFN-β), establishing a potent antiviral response (for review, see Honda and Taniguchi, 2006). Given the pivotal role of the post-translational control of IRFs in the PRR-mediated antiviral signaling pathways, it is not surprising that herpesviruses subvert IRF activation or transcriptional activity by controlling their phosphorylation, ubiquitination, SUMOylation or acetylation.

In order to block the activation of IRFs by phosphorylation, herpesviruses adopted various strategies. For example, some herpesvirus strains encode kinases that are able to directly and aberrantly phosphorylate IRFs, which inhibits their transcriptional activity. The HSV-1-encoded kinase US3 interacts with and hyper-phosphorylates IRF3 at serine 175 and

this abnormal phosphorylation blocks its activation (Wang et al., 2013b). Similarly, the EBV BGLF4 kinase phosphorylates IRF3 at Ser123, Ser173, and Thr180, in a region between the DNA binding and IRF association domains, suppressing the IRF3 signaling pathway (Wang et al., 2009). Varicella-Zoster Virus (VZV) ORF47 also aberrantly phosphorylates IRF3, preventing IRF3 phosphorylation at S396 and subsequent homodimerization (Vandevenne et al., 2011). Interestingly, this study also showed that the ORF47-mediated phosphorylation and inhibition of IRF3 dampens the IFN-β induction but not NF-κB activation, consistent with the selective inhibition on IRF3. Herpesviruses were also shown to deregulate cellular kinases responsible for the phosphorylation and activation of IRFs. The HSV-1 tegument protein US11 downregulates RLR signaling by interacting with RIG-I and MDA5, while impeding IRF3 activation by reducing its phosphorylation, dimerization and nuclear translocation (Xing et al., 2012). The HSV-1 protein ICP34.5 has been shown to directly interact with TBK1, blocking IRF3 phosphorylation immediately downstream of TBK1 and impeding the induction of type I IFNs to promote viral replication (Verpooten et al., 2009; Ma et al., 2012). Another study revealed that the MHV68-encoded ORF36 kinase selectively inhibits IRF3 by binding to its activated and phosphorylated form, facilitating the establishment of viral splenic latency *in vivo* (Hwang et al., 2009). Interestingly, the HSV-1-encoded serine protease VP24 downregulates the interferon stimulatory DNA-mediated phosphorylation and dimerization of IRF3 downstream of the cGAS-STING pathway, resulting in the inhibition of the IFN induction but not of the NF-κB activation (Zhang et al., 2016). KSHV also has adopted strategies to dampen the transcriptional activity of IRFs. For instance, the KSHV-encoded microRNA miR-K12-11 inhibits IRF3 phosphorylation by targeting and lowering the protein levels of the kinase IKKε (Liang et al., 2011). Noticeably, this study also showed that the absence of this microRNA enhanced KSHV reactivation induced by the infection with vesicular stomatitis virus. These studies demonstrated that viral proteins target IRF3 phosphorylation to block its activation and downstream signaling. Concerning IRF7, few studies revealed the control of IRF7 phosphorylation by herpesviruses. In particular, KSHV ORF45 competitively inhibits IRF7 phosphorylation by IKKε and TBK1, impairing IRF7 nuclear translocation and type I IFN induction (Liang et al., 2012; Zhu et al., 2002). These findings highlight the checkpoint role of IRF phosphorylation in regulating the antiviral IFN production.

In addition to inhibiting the phosphorylation-mediated activation of IRFs, herpesviruses also developed the ability to induce their ubiquitination and proteasomal degradation. The HSV-1 E3 ubiquitin ligase ICP0 promotes the ubiquitin/proteasome-mediated degradation of IRF3, thereby preventing IFN induction and subsequent antiviral immune defense (Melroe et al., 2004). Another study reported that VZV ORF61 directly interacts with activated IRF3 to promote the ubiquitination and downregulation of IRF3 (Zhu et al., 2011). Moreover, KSHV RTA possesses an intrinsic E3 ligase activity that ubiquitinates and induces the proteasomal degradation of IRF7 (Yu et al., 2005). The EBV latent membrane protein 1 (LMP1) was also shown to promote the TRAF6-mediated ubiquitination/proteasome-dependent degradation of IRF7 (Ning et al., 2008). The VZV protein ORF63 was shown to induce a reduction of IRF9 levels in a proteasome degradation-dependent manner (Verweij et al., 2015). These findings highlight the concept that phosphorylation and ubiquitination of IRFs are coupled by various viral and cellular proteins, forming a PTM-based signaling network.

The role of SUMOylation in the inhibition of IRFs activation by herpesviruses is not well understood yet. However, a study showed that EBV LMP1 induces IRF7 SUMOylation, reducing its turnover, increasing its nuclear retention, DNA binding and transcriptional activity (Bentz et al., 2012). Moreover, KSHV encodes a SUMO E3 ligase named basic region-leucine zipper (K-bZIP) that potentially targets the transcription factors IRF1, 2, and 7 (Chang et al., 2010; Chang et al., 2013). Other SUMO E3 ligases including EBV BRLF1, HSV-1 UL54/ICP27, and CMV UL69 were recently identified (De La Cruz-Herrera et al., 2018). These results emphasize that the SUMOylation of IRFs by herpesviruses can result in a loss of their transcriptional activity, suggesting that SUMOylation is a negative regulatory mechanism of IRF-mediated gene expression.

KSHV-encoded IRFs homologs have been shown to interfere in the assembly of the IFN-β enhanceosome. In support of this notion, vIRF1 directly interacts with p300 and reduces its histone acetyltransferase activity, leading to altered expression of cellular cytokines and consequently a diminished antiviral response (Li et al., 2000). vIRF1 can also block the interaction of cellular IRF3 with the enhanceosome, thus impeding the IFN induction. vIRF3 does not block IRF3 interaction with the IFN-β enhanceosome but interacts directly with it without impacting the acetylation of histones (Lubyova et al., 2004). These findings highlight the consensus of herpesvirus-mediated inhibition of IRF acetylation, to impair the assembly of a functional IFN-β enhanceosome and dampen the antiviral IFN response.

Post-translational Control of NF-κB

The NF-κB family is composed of five transcription factors that underpin numerous innate immune signaling pathways and trigger the production of pro-inflammatory cytokines and chemokines (for review, see Zhang et al., 2017). The prototype NF-κB dimer is composed of two subunits, namely p50 and p65 (also known as RelA), which are often targeted by herpesviruses to control the inflammatory response. The HSV-1 kinase US3 is able to hyper-phosphorylate p65/RelA at serine 75, which blocks its nuclear translocation and consequently its transcriptional activity, leading to a reduced production of IL-8 (Wang K. et al., 2014). Another study showed that MHV68 hijacks RIG-I and MAVS to activate IKKβ that in turn phosphorylates RelA, thus facilitating the proteasome-mediated degradation of RelA and negating the antiviral cytokine production (Dong and Feng, 2011). Furthermore, HCMV UL26 blocks the phosphorylation and activation of IKKβ, which is the key step for IκB phosphorylation and NF-κB activation (Mathers et al., 2014).

NF-κB activation is a classic example in which kinase activity regulates the ubiquitination and subsequent degradation of a

suppressor of a key transcription factor. Hence, upon reception of the upstream signal, the kinase IKKβ phosphorylates IκB, triggering IκB ubiquitination and its detachment from NF-κB which is now free to translocate into the nucleus and activate the transcription of its target genes (for review, see Chen and Chen, 2013). The activation mechanism of NF-κB constitutes a feed-forward mechanism involving these two modifications that is exploited by herpesviruses to reduce the inflammatory response. For instance, the E3 ligase of HSV-1 ICP0 was shown to dampen TNF-α-mediated NF-κB activation. To do that, ICP0 interacts directly with p50 and p65, blocks the nuclear translocation of p65, and induces the ubiquitination and proteasome-dependent degradation of p50, thus preventing the production of NF-κB-regulated pro-inflammatory cytokines (Zhang J. et al., 2013). Interestingly, ICP0 was also shown to trigger NF-κB activation by ubiquitinating IκBα during HSV-1 infection (Diao et al., 2005). The discrepancies between these studies are not self-evident, suggesting that the role of ICP0 may be contingent on the context of infection, e.g., temporal and viral parameters. Recently, Whitmer et al. (2015), reported that VZV ORF61 inhibits NF-κB activation by blocking IκB ubiquitination and degradation, thus retaining the p50–p65 dimer in the cytoplasm. Similarly, HSV-1 UL36USP deubiquitinates IκBα, preventing it from degradation and trapping p50–p65 in the cytoplasm (Ye et al., 2017). Another study showed that the MHV68-encoded ORF73, latency-associated nuclear antigen (LANA), interacts with ElonginC and Cullin5 *via* its suppressor of cytokine signaling (SOCS) motif to promote the ubiquitination and degradation of p65 (Rodrigues et al., 2009). The downregulation of NF-κB activation by MHV68 LANA is required for its latent infection. These studies expose cellular proteins that are specifically targeted by viral evasion proteins, reflecting their pivotal roles in NF-κB activation and inflammation.

Post-translational Control of Cytokine- and IFN-Mediated Signaling

Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT)

Innate immune activation culminates in the production of type I IFNs and other inflammatory cytokines which, when bound to their cognate receptors, recruit and promote the activation of the non-receptor tyrosine kinase 2 (TYK2) and JAK1 kinases *via* proximity-induced autophosphorylation. Activated TYK2 and JAK1 kinase phosphorylate the transcription factors STAT1 and STAT2. Phosphorylated STAT molecules can undergo homo- or hetero-dimerization, in either case resulting in a functional nuclear localization signal that mediates their nuclear translocation. When STAT2 heterodimerizes with STAT1, the heterodimer binds to IRF9 and forms the ISGF3 complex, which transactivates the promoters containing ISREs (for review, see Villarino et al., 2017). Not surprisingly, herpesviruses deploy diverse strategies to interfere with the phosphorylation-mediated activation of the JAK/STAT pathway. HSV-1 infection induces the expression of SOCS1 and SOCS3 proteins to inhibit JAK phosphorylation, and this process requires the viral proteins VR3, UL13 and UL41 (Chee and Roizman, 2004; Yokota et al., 2004; Sato et al., 2017). Moreover, HSV-1 ICP27 downregulates STAT1 phosphorylation and subsequent accumulation in the nucleus in response to IFN-α (Johnson et al., 2008). VZV ORF63 has also been shown to interfere with JAK/STAT signaling by reducing the IFN-induced phosphorylation of STAT2 and mediating the degradation of IRF9 (Verweij et al., 2015). In human B cells, the EBV LMP1 directly interacts with TYK2, which blocks its phosphorylation and activation, thereby inhibiting the IFN response (Geiger and Martin, 2006). Another EBV protein, BZLF1, was shown to inhibit IFN-gamma-induced STAT1 tyrosine phosphorylation and nuclear translocation (Morrison et al., 2001). Finally, HCMV antagonizes STAT2 phosphorylation and activation (Le et al., 2008). Overall, these viral proteins primarily target the phosphorylation and nuclear translocation of STAT transcription factors to derail the IFN response.

The disruption of the JAK/STAT pathway by HCMV was reported 20 years ago when Miller et al. (1998), reported a virus-associated alteration of JAK levels. Subsequent studies revealed the ability of CMV to induce STAT ubiquitination and proteasome-dependent degradation. Indeed, MCMV pM27 was shown to induce STAT2 ubiquitination and degradation by the proteasome, likely through its interaction with the cellular protein damage specific DNA binding protein 1 (DDB1), known to be part of a ubiquitin-ligase complex (Trilling et al., 2011). In addition, this study showed that HCMV also induces the proteasome-mediated degradation of STAT2 but UL27, the HCMV-encoded homolog of MCMV pM27, is insufficient to downregulate STAT2 and is incapable of binding to DDB1. In MCMV-infected mice, pM27-mediated inhibition of STAT2 is essential for efficient MCMV replication, while the remaining activity of STAT2 is critical for the survival of the host (Zimmermann et al., 2005; Le-Trilling et al., 2018). These studies highlight the fact that herpesviruses acquired the ability to prevent further cytokine- and IFN-mediated viral clearance by manipulating the JAK/STAT pathway.

Post-translational Control by ISGylation

PRR-mediated innate immune activation culminates in the production of type I IFNs that trigger the activation of a transcriptional program of so-called interferon-stimulated genes (ISGs). ISG15 is a ubiquitin-like 17 kDa protein that can be free in the cytoplasm or covalently conjugated to its target proteins in a reversible process termed ISGylation (Haas et al., 1987; Loeb and Haas, 1992; Narasimhan et al., 1996; Der et al., 1998; Potter et al., 1999; Hemelaar et al., 2004). ISGylation relies on the action of three enzymes catalyzing the conjugation and ligation of ISG15, named ISG15-activating enzyme (E1), ISG15-conjugating enzyme (E2), and ISG15-ligating enzyme (E3) (for review, see Durfee and Huibregtse, 2012). It has been established that ISGylation targets primarily, but not only newly translated proteins (Durfee et al., 2010). The exact consequences of ISGylation on the function of its target proteins remain elusive because it has been shown that ISGylation can restrict the ubiquitin system and downregulate protein degradation by the proteasomal pathway, thereby increasing protein stability (Okumura et al., 2008; Fan et al., 2015). However, ISGylation can also increase protein degradation by selective autophagy

(Nakashima et al., 2015). In the context of antiviral innate immune signaling, it is now established that ISGylation plays an important role since ISGylation can target cellular or viral proteins involved in these pathways. Consistent with this, ISG15 and enzymes catalyzing ISGylation are upregulated by IFNs (Malakhov et al., 2003; Thaiss et al., 2016; Cai et al., 2017; Tecalco Cruz and Mejia-Barreto, 2017).

The role of ISGylation in antiviral signaling and immune evasion has been clearly established in the context of Influenza virus infection in particular. Hence, numerous studies revealed the importance of the ISGylation of viral proteins in antiviral signaling and the evasion mechanisms adopted by the virus to promote its replication. The non-structural protein 1 of Influenza B virus (NSB1) was identified as the first viral protein able to bind ISG15 (Yuan and Krug, 2001). Then, several virus-encoded proteins were shown to be able to bind ISG15 or remove it from target proteins (for review, see Zhao et al., 2013). Interestingly, NS1B was shown to restrict the ISGylation-mediated antiviral activity by sequestering ISGylated viral nucleoproteins which are responsible for the inhibition of viral protein synthesis and replication (Zhao C. et al., 2016).

ISG15 has been shown to target key components of innate immunity signaling pathways, e.g., RIG-I, STAT1, and IRF3, which promotes innate immune activation and thwart the replication of diverse viruses. Within the herpesvirus family, these include HSV-1, HCMV, and MHV68, prototypical herpesviruses of the α, β, and γ subfamilies, respectively (for review, see Morales and Lenschow, 2013). Specifically, free ISG15 was shown to promote the interaction between RIG-I and the autophagic protein p62, targeting RIG-I for degradation by selective autophagy (Du et al., 2018). In contrast, ISGylation stabilizes IRF3 by inhibiting its ubiquitination and degradation (Shi et al., 2010), and a similar effect of ISGylation on STAT1 was reported (Ganesan et al., 2016). These studies highlight distinct roles of ISG15 in early (RIG-I activation, IFN induction) and late (STAT1, IFN stimulation) stages of the IFN response, i.e., restricting early IFN induction and boosting IFN-stimulated effect. Although little is known about the mechanism by which herpesviruses exploit or evade the ISGylation-mediated immune defense system, a study carried out in ISG15-deficient mice showed that ISG15 has a protective effect against HSV-1 infection, suggesting its antiviral role against herpesviruses (Lenschow et al., 2007). Later, Jacobs et al. (2015) demonstrated that, upon TLR3 activation, KSHV vIRF1 interacts with the ISG15 E3 ligase, HECT and RLD domain-containing E3 ubiquitin ligase 5 (HERC5), leading to a global decrease in ISGylation of proteins in infected cells. Interestingly, vIRF1 is itself a target of ISGylation. The diminished level of cellular ISGylation found in this study was associated with reduced levels of IRF3, another known target of ISG15, supporting the conclusion that ISGylation stabilizes proteins (Jacobs et al., 2015). During HCMV infection, ISG15 inhibits HCMV growth by downregulating viral gene expression. Specifically, ISGylation of UL26 modifies its stability and suppresses its ability to dampen NF-κB activation. Conversely, HCMV IE1 and UL26 suppress the infection-induced ISGylation, to counteract the ISG15-mediated immune defense (Kim et al., 2016). HCMV UL50 interacts with and induces the proteasomal degradation of UBE1L, an E1-activating enzyme for ISGylation. Furthermore, RNF170, an endoplasmic reticulum-associated ubiquitin E3 ligase, interacts with UL50 and promotes UL50-mediated UBE1L degradation *via* ubiquitination (Lee et al., 2018). As such, knockdown of ISG15 increases HCMV productive infection in cultured cells (Bianco and Mohr, 2017). Collectively, these studies showed that ISGylation promotes the host innate immune response and that herpesviruses target various components to suppress ISGylation. Despite the difficulty of apprehending and studying ISGylation process since it occurs predominantly during the translation of proteins, the importance of ISGylation in the context of Influenza virus infection could provide further working hypotheses to get a better understanding of the role of this PTM in innate immune responses and in the pathogenesis of herpesvirus infection.

CONCLUDING REMARKS

The regulation of protein function is chiefly achieved *via* PTMs and components of the innate immune response are of no exception. Retrospectively, the process of activation of the NF-κB pathway is powered by two key PTMs, phosphorylation and ubiquitination. While the networks of innate immune signaling pathways are established, new components are being discovered at an accelerating pace. Studies from microbial infection and herpesviruses in particular, offer great opportunities to understand the regulation of these components in addition to the roles of those pathways in host defense and microbial pathogenesis.

In the realm of innate immune defense, conventional approaches employ the established pathways and components to identify microbial modifiers. These approaches have yielded fruitful findings and advanced enormously our understanding of virus-host interactions. However, new cutting-edge technologies such as CRISPR screen and high throughput sequencing may be integrated to revolutionize how we discover new players in host immune defense using herpesvirus infection as a model system. This has the potential to unravel new components and to a less extent, new pathways in innate immune defense. Enzymes catalyzing PTMs possess diverse substrates, thus forging biological links between multiple processes that are otherwise unconnected. The discovery of cellular GATs in innate immune defense is an example that may provide an intrinsic link between metabolism and innate immunity, given that GATs catalyze the synthesis of key cellular metabolites such as nucleotides, amino acids and glycoproteins. On the other hand, acetylation of proteins such as histones is highly dependent on the cellular Acetyl-CoA pool, linking gene expression to the metabolic status as well. How the innate immune response connects with other fundamental biological processes *via* PTMs is in its infancy at best. To date, most of the studies on herpesvirus-mediated modulation of PTMs have focused on phosphorylation, ubiquitination, SUMOylation, deamidation, acetylation and ISGylation, leaving the modulation of other PTMs such as methylation, succinylation, carbonylation,

glycation, glutamylation, hydroxylation, citrullination, nitration, palmitoylation and sulfation poorly understood or yet to be studied. The advancement of high-resolution mass spectrometric analyses should soon allow the sensitive detection of these PTMs and their regulation by herpesviruses.

AUTHOR CONTRIBUTIONS

JC and PF conceived and designed the study. JC, YR, QL, XL, JZ, and PF wrote the manuscript.

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REVIEW
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A Tug of War: DNA-Sensing Antiviral Innate Immunity and Herpes Simplex Virus Type I Infection

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Cytosolic DNA sensors are the most recently described class of pattern recognition receptors (PRRs), which induce the production of type I interferons (IFN-I) and trigger the induction of a rapid and efficient innate immune response. Herpes simplex virus type I (HSV-1), a typical DNA virus, has displayed the ability to manipulate and evade host antiviral innate immune responses. Therefore, with an aim to highlight IFN-I-mediated innate immune response in a battle against viral infection, we have summarized the current understandings of DNA-sensing signal pathways and the most recent findings on the molecular mechanisms utilized by HSV-1 to counteract antiviral immune responses. A comprehensive understanding of the interplay between HSV-1 and host early antiviral immune responses will contribute to the development of novel therapies and vaccines in the future.

Keywords: herpes simplex virus type I, DNA sensor, immune evasion, interferon, antiviral immunity

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INTRODUCTION

Herpes simplex virus type I (HSV-1), a member of the alphaherpesvirus subfamily, has already co-evolved with human beings for thousands of years and is well known for its high prevalence in the population worldwide (Davison, 2010; Sharma et al., 2016; Ibanez et al., 2018). With a large linear double-stranded DNA that encodes over 80 proteins, HSV-1 can produce lifelong infections in the host, and this is achieved thanks to its capacity to infect epithelial cells, neurons, and other cell types, including immune cells *in vivo* and *in vitro* (Wu et al., 2016; Koelle et al., 2017; Tognarelli et al., 2019).

Clinically, HSV-1 is mainly associated with orofacial lesions, yet it is also the leading cause of infectious blindness in developed countries and viral encephalitis in adults (Horowitz et al., 2010; Farooq and Shukla, 2012; Bernstein et al., 2013). After the initial infection, HSV-1 becomes latent in the trigeminal ganglion, and recurrent reactivation leads to different immunopathology, which may cause neuronal damage and Alzheimer's disease (AD) (Devanand, 2018).

Early detection of viral invasion by pattern recognition receptors (PRRs) is crucial for the induction of a rapid and efficient innate immune response. Cytosolic DNA sensors are the most recently described class of PRRs, which also include the Toll-like receptors (TLRs), certain RNA sensors, such as RIG-I-like receptors and melanoma differentiation-associated gene 5 (Wu and Chen, 2014; Su et al., 2016). Viral nucleic acids of HSV-1, recognized by various PRRs, can act as strong activators of various signaling pathways that finally promote antiviral immune responses through the secretion of pro-inflammatory cytokines, as well as the production of type-I interferons (IFN-I) in infected cells (Iwasaki, 2012). The activation

of the IFN-I pathway ultimately induces the expression of multiple IFN-stimulated genes (ISGs) and boosts the innate immune responses (Schoggins, 2019). HSV-1 has been reported to evade host immunity and facilitate its infection and replication through multiple strategies (Schulz and Mossman, 2016; Christensen and Paludan, 2017).

Although different cytosolic DNA-sensing pathways can be activated, HSV-1 has developed multiple mechanisms to attenuate this host antiviral machinery (Zheng, 2018). In this review, we outline the recent findings with the aim of highlighting antiviral innate immune responses in the battle against the HSV infection. A comprehensive understanding of the interplay between HSV-1 and host antiviral innate immunity could contribute to the development of novel immunotherapies and effective vaccines to counteract this virus over the next few decades.

INTERPLAY BETWEEN THE HOST ANTIVIRAL DNA-SENSING PATHWAYS AND HERPES SIMPLEX VIRUS TYPE I

The newly emerging DNA in the cytoplasm induces robust and rapid innate immune responses through its binding to various DNA sensors, including TLR9, absent in melanoma 2 (AIM2), RNA polymerase III, Interferon-γ inducible protein 16 (IFI16), DEAD-box helicase 41 (DDX41), and some proteins involved in the DNA damage responses, among which the cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS) is the only one that has been identified as a universal cytoplasmic DNA sensor in various cell types (Lund et al., 2003; Chiu et al., 2009a; Zhang et al., 2011; Sun et al., 2013; Zheng, 2018; Stempel et al., 2019). TLRs have been described to mediate antiviral activities against HSV during infection. If the animals lacked both TLR2 and TLR9, all animals were more susceptible to infection than single knockout animals pointing out the relevance of these receptors during HSV infection (Lima et al., 2010; Uyangaa et al., 2018). Furthermore, HSV-1 infection in human neurons was shown to be suppressed by type-III IFN (IFN-λ) through the upregulation of TLR9 expression and subsequent TLR9-mediated antiviral responses involving the transcription factor interferon regulatory factor 7 (IRF7) (Zhou et al., 2011). But this result remains to be determined because IFN-λ has been reported to be secreted during HSV infection in the vaginal mucosa, mainly by dendritic cells (Iversen et al., 2010). Although AIM2 also detects aberrantly localized DNA, it is currently proposed that it cooperates with IFI16 and activates the inflammasome (Lugrin and Martinon, 2018). Other proposed DNA sensors, such as DDX41, also require further investigation to clarify their role during HSV infection and if they act redundantly in a cell-type-dependent manner (Zhang et al., 2011). Furthermore, unlike cGAS and IFI16, these sensors have, thus far, not been shown to restrict the replication of HSV-1 and have been evaded by HSV-1.

In this review, specific attention is given to the cGAS-STING DNA-sensing signal pathways and its downstream IFN-I signal pathway, which plays a central role in innate antiviral immunity (**Figure 1**).

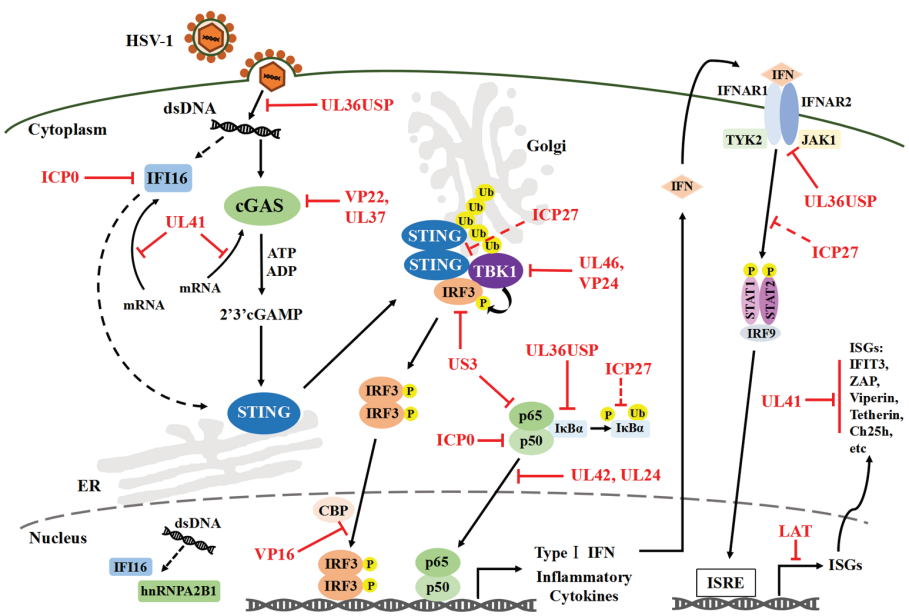

FIGURE 1 | HSV-1 mediated evasion of the DNA-sensing pathway in innate immunity. Cytosolic DNA sensors, such as IFI16 and cGAS, can recognize HSV-1 dsDNA and trigger IRF3 and NF-κB activation, which results in the production of IFN-I and antiviral immune responses. Multiple steps in the DNA-sensor-mediated IFN-I signaling pathway can be targeted by HSV-1 proteins, including cGAS-mediated viral recognition and subsequent signaling pathways. Solid lines indicate confirmed interactions between the host signaling proteins and HSV-1 proteins. Dashed lines indicate uncertain interactions. HSV-1, Herpes simplex virus type I; IFI16, Interferon-γ inducible protein 16; cGAS, Cyclic guanosine monophosphate-adenosine monophosphate synthase; dsDNA, Double-stranded DNA; IRF3, Interferon regulatory factor 3; NF-κB, Nuclear factor κB; IFN-I, Type I interferons; P, Phosphate; Ub, Ubiquitin.

Interferon-γ Inducible Protein 16

IFI16 is a host sensor of nucleic acids that has been reported to recognize cytosolic double-stranded DNA (dsDNA) as well as HSV-1-derived DNA in the nucleus (Unterholzner et al., 2010). HSV-1 recognition by IFI16, which itself results in acetylation and redistribution to the cytoplasm, then induces the activation of the transcription factor interferon regulatory factor 3 (IRF3) and transcription factor's nuclear factor κB (NF-κB) into the nucleus (Pilli et al., 2012; Ansari et al., 2015). These processes are followed by the production of IFN-I and IL-6, which are able to restrict viral replication and initiate an inflammatory response (Conrady et al., 2012; Ansari et al., 2015; Zheng, 2018).

Furthermore, the host sensor IFI16 (or AIM2) that encounters viral determinants could also interact with the inflammasome and hence induce the pro-caspase-1 activation by an apoptosis-associated speck-like protein containing a caspase recruitment domain (Johnson et al., 2013). HSV-1 immediate early protein infected-cell polypeptide 0 (ICP0), which is an E3 ubiquitin ligase, has been shown to successfully inhibit IFI16 activation by guiding it to the proteasome and accelerating its degradation (Johnson et al., 2013). HSV viral protein 22 (VP22), encoded by the UL49 gene, has also been reported to block this pro-caspase-1 activation and inhibit the secretion of pro-inflammatory cytokines such as IL-1β or IL-8 (Maruzuru et al., 2018). Moreover, apart from directly inhibiting the activation of IFI16, a recent study revealed that an HSV-1 UL41 protein contributed to the decrease of IFI16 expression by degrading its mRNA (Orzalli et al., 2016).

In addition, within the nucleus, viral DNA is not only sensed but also loaded with heterochromatin to silence its expression and to restrict viral replication (Orzalli and Knipe, 2014). Several restriction factors, including IFI16 and promyelocytic leukemia (PML) protein, limit viral gene expression and replication (Orzalli et al., 2013; Merkl et al., 2018). Merkal et al. have defined a novel mechanism of epigenetic silencing of HSV-1 DNA, which revealed that an IFI16 filamentous structure could recruit other restriction factors, including PML protein, speckled protein 100 (Sp100), and alpha-thalassemia mental retardation syndrome x-linked (ATRX), to aid in the restriction (Merkl and Knipe, 2019). Notably, an increasing body of evidence suggests that HSV-1 ICP0 protein can promote the degradation of the IFI16, ATRX, Sp100, as well as PML proteins and prevent their restriction activities (Lukashchuk and Everett, 2010; Jurak et al., 2012; Orzalli et al., 2012). However, further studies are still needed to identify the full protein composition of this new infected cell nuclear structure and investigate the underlying mechanisms by which HSV-1 regulates the downstream pathways related to IFI16.

Cyclic Guanosine Monophosphate-Adenosine Monophosphate Synthase-Stimulator of Interferon Genes

Several DNA viruses, including adenovirus, cytomegalovirus, hepatitis B virus, and HSV-1, can induce IFN-I in a cGAS/stimulator of interferon genes (STING)-dependent pathway (Gao et al., 2013; Ma et al., 2015; Wu et al., 2015; Dansako et al., 2016; Paijo et al., 2016). As a result, cGAS plays a crucial role in antiviral innate immunity, which triggers cyclic GMP-AMP (cGAMP) production through its enzymatic activity upon binding to cytosolic dsDNA (Cai et al., 2014). Then, cGAMP activates the endoplasmic reticulum (ER)-anchored STING, which then translocates from the ER to the Golgi apparatus and leads to the recruitment and phosphorylation of TANK-binding kinase 1 (TBK1) and IκB kinase (IKK). Notably, the trafficking step from the ER to the Golgi apparatus is crucial for the induction of IFN-I transcription by STING (Saitoh et al., 2009). Finally, the IRF3 and NF-κB are activated to induce the production of IFN-I and inflammatory cytokines (Sun et al., 2013; Wu et al., 2013).

At the upstream of the cGAS/STING signaling pathway, our previous study showed that an HSV-1 UL41 protein could degrade cGAS mRNA during viral infection (Su and Zheng, 2017). This finding showed that the ectopic expression of UL41 remarkably reduced the accumulation of cGAS *via* its RNase activity and downregulated the cGAS/STING-mediated activation of the IFN pathway to escape antiviral innate immune responses. Besides, HSV-1VP22, a highly abundant tegument protein, also has an effect on this DNA-sensing pathway. VP22 has been shown to interact directly with cGAS and thus suppress the enzymatic activity of cGAS, and it acts as an important inhibitor of IFN-β production and downstream antiviral genes (Huang et al., 2018).

Interestingly, Deschamps et al. have showed that the stable overexpression of HSV-1 tegument protein UL46 in cells can reduce the expression of STING and inhibit its downstream IFN-I signaling pathway (Deschamps and Kalamvoki, 2017). However, during viral infection, UL46 does not affect the expression and function of STING, which is obviously illogical. Our recent study revealed a novel underlying mechanism – that HSV-1 UL46 downregulated antiviral immune responses by interacting directly with TBK1. UL46 was shown to significantly reduce the dimerization of TBK1 and affect the interaction between TBK1 and IRF3, which resulted in inhibiting the activation of TBK1 and the production of IFN-β (You et al., 2019). Based on this evidence, HSV-1 UL46 disrupts the cGAS-STING signaling pathway and possibly interacts with both STING and TBK1 *via* separate domains.

An increasing amount of evidence has shown that post-translational modifications, such as phosphorylation and ubiquitination, directly or indirectly modulate the cGAS/STING pathway and significantly affect viral infections (Liu et al., 2013, 2016). cGAS can be targeted for deamidation by the HSV-1 tegument protein UL37, which causes cGAS inactivation and facilitates HSV-1 lytic replication (Zhang et al., 2018). Moreover, Sun et al. demonstrated that HSV-1 UL36 ubiquitin-specific protease (UL36USP) could inhibit viral capsid ubiquitination and subsequent degradation through its deubiquitylase activity, thus avoiding the recognition of cGAS instead of affecting the stability of cGAS or STING (Sun et al., 2015).

These findings reveal some novel mechanisms of viral evasion. More importantly, the multifaceted strategy of HSV-1 to

compromise the DNA-sensing pathway highlights how STING is a key restriction factor for HSV-1.

TANK-Binding Kinase 1-Interferon Regulatory Factor 3

At the downstream of the cGAS/STING signaling pathway, studies from our lab demonstrated that VP24, a serine protease of HSV-1, could also block dsDNA-triggered IFN production by abrogating the interaction between TBK1 and IRF3 and inhibiting the activation of IRF3 (Zhang et al., 2016), while HSV-1 VP16 could prevent IRF3 from recruiting the CREB-binding protein (CBP) coactivator, thus blocking IRF3-mediated transcription (Xing et al., 2013). What is more, protein kinase US3 of HSV-1 has been shown to interact with and hyperphosphorylate IRF3 at Ser175 to prevent IRF3 activation and dampen IFN-I production (Wang et al., 2013, 2014). Christensen et al., found that HSV-1 ICP27 protein, a product of viral immediate early genes conserved among all human herpesviruses, could impair the upstream of IRF3 activation (but could not impair TBK1 phosphorylation) by interacting with TBK1 and STING in human macrophages (Christensen et al., 2016). Nevertheless, ICP27, as an immediate early gene, can regulate the production of many viral genes through stimulating transcription and translation of viral early and late genes, indicating that the results from the ICP27 deletion virus does not guarantee that the viral immune evasion is mediated by ICP27 (Sandri-Goldin, 2011). It is plausible that ICP27 might affect the IFN-I signaling pathway through the regulation of the expression of viral early and late genes. Altogether, these findings from our and other labs will be important for understanding the interaction between HSV-1 and the host DNA-sensing signal pathway.

TANK-Binding Kinase 1-Nuclear Factor κB

NF-κB is known for its critical role in innate immune responses and can be strongly induced at the downstream of most PRRs, resulting in the production of IFN-β as well as inflammatory interleukins (Woronicz et al., 1997). During the activation of NF-κB, IκBs are phosphorylated by activated IKK, and then the NF-κB p50/p65 heterodimer is released and transferred to the nucleus, which finally regulates the innate immune responses (Bonizzi and Karin, 2004; Hayden and Ghosh, 2008; Chiu et al., 2009b; Iwai, 2014). New evidence suggests that TBK1 is essential for the activation of the NF-κB signaling pathway mediated by dsDNA and utilizes the IKK activation loop to activate the subunit p65 (Abe and Barber, 2014).

Consequently, HSV-1 has also evolved various elaborate mechanisms to subvert this signaling pathway. For example, the HSV-1 immediate early protein ICP0 interacts with NF-κB subunits p50/p65 and degrades p50 through its E3-ubiquitin ligase activity (Zhang et al., 2013a). Kim et al. also reported that HSV-1 ICP27 could repress NF-κB activity through blocking the phosphorylation and ubiquitination of IκBα and stabilize IκBα to evade immune responses during the very early period of HSV-1 infection (Kim et al., 2008). Meanwhile, our study has showed that HSV-1 UL36USP deubiquitinates IκBα and prevents its degradation, which inhibits p50/p65 transportation and finally abrogates NF-κB activation (Ye et al., 2017).

The production of IFN-I depends on transcription factors of both IRF3 and NF-κB, which bind to distinct regulatory domains in the promoter. HSV-1 US3 has been shown to hyperphosphorylate p65/RelA at serine 75, which significantly inhibited NF-κB activation by blocking its nuclear translocation and decreased the expression of inflammatory chemokine interleukin-8 (Wang et al., 2013, 2014). Similarly, HSV-1 UL42, a DNA polymerase processivity factor, also significantly prevents NF-κB-dependent gene expression by retaining p50/p65 in the cytoplasm (Zhang et al., 2013b). Additionally, HSV-1 UL24, another conserved viral protein that is important for viral replication, selectively blocked activation of the NF-κB, but not IRF3, by binding to Rel homology domains of p50/p65 and abrogating their nuclear translocation (Xu et al., 2017). For the first time, UL42 and UL24 are demonstrated to effectively inhibit cGAS/STING-induced NF-κB activation and dsDNA-mediated IFN-β or IL-6 production during HSV-1 infection. It is worth noting that some HSV-1 proteins may target the cytosolic DNA-sensing pathway through similar mechanisms.

Janus Kinase-Signal Transducer and Activator of Transcription

Although HSV-1 can antagonize the production of IFN-I *via* many mechanisms, a certain amount of IFN-I produced during early infection will induce ISGs through the Janus kinase-signal transducer and activator of transcription (JAK-STAT). HSV-1 also evolves mechanisms to disrupt the JAK-STAT pathway, which is the downstream of the IFN signaling pathway, and further evades the antiviral immunity.

It is known that IFN-I can be initially produced following the detection of viral RNA, DNA, or proteins by intracellular PRRs in host cells (Raftery and Stevenson, 2017). IFN-I include IFN-α as well as IFN-β, IFN-ε, IFN-κ, and IFN-ω (van Pesch et al., 2004; Ivashkiv and Donlin, 2014). After its secretion, IFN-I interacts with the cell-surface receptor known as the type I IFN receptor (IFNAR), which is a heteromeric receptor that contains subunit IFNAR1 and IFNAR2 (Decker et al., 2005). When the receptor is activated, it recruits and phosphorylates the tyrosine kinase 2 (Tyk2) and JAK1, which leads to the formation of a heterodimer of phosphorylated STAT1 and STAT2 (Brooks et al., 2014). Then, STAT1/STAT2 binds to the cytoplasmic IRF9, forming a complex known as IFN-stimulated gene factor 3 (ISGF3) that translocates into the nucleus and binds to a DNA sequence called the IFN-stimulated response element (ISRE), resulting in the transcription of many ISGs, including viperin, zinc-finger antiviral protein (ZAP), Cholesterol 25-hydroxylase (Ch25h), tetherin, ISG15, and some proteins of the *tripartite motif* (TRIM) family, which are responsible for the effector properties of directly antiviral responses (Schoggins and Rice, 2011; Schneider et al., 2014; Raftery and Stevenson, 2017). However, HSV-1 has evolved multiple strategies to evade this process.

Johnson et al. observed that HSV-1 protein ICP27 was sufficient to inhibit IFN-mediated STAT1 phosphorylation and

nuclear accumulation at or before the phosphorylation of JAK (Johnson et al., 2008; Johnson and Knipe, 2010). HSV-1 can also downregulate the protein levels of JAK1 and STAT2 through the virion host shutoff protein at a relatively high multiplicity of infection (Chee and Roizman, 2004). Previous studies from our and other labs have shown that UL41 can degrade the mRNAs of some ISGs, such as IFIT3, ZAP, viperin, tetherin, as well as Ch25h, to attenuate the IFN-mediated antiviral immune responses *via* its RNase activity (Zenner et al., 2013; Shen et al., 2014; Su et al., 2015; Jiang et al., 2016; You et al., 2017; Yuan et al., 2018). Furthermore, we have recently demonstrated that HSV-1 UL36USP also antagonizes the activation of the IFN-JAK-STAT pathway through specifically binding to IFNAR2 and blocking the interaction between JAK1 and IFNAR2, which is independent of its DUB activity (Yuan et al., 2018).

The HSV-1 latency-associated transcript (LAT), which is not known to encode a functional protein but regulate the virus latency and reactivation, has been shown to inhibit apoptosis *via* inhibiting activation of pro-apoptotic caspases and promoting cell survival or immune evasion (Perng et al., 2000; Henderson et al., 2002; da Silva and Jones, 2013; Phelan et al., 2017). However, the mechanism of this process is unknown. Tormanen et al. have recently observed that LAT affected apoptosis by downregulating the expression of JAK1 and JAK2, as well as several downstream ISGs of the JAK-STAT pathway at the level of a transcriptional mechanism during HSV-1 latency (Tormanen et al., 2019).

Overall, a growing amount of evidence suggests that HSV-1 has evolved multiple mechanisms to inhibit IFN signaling not only in infected cells but also in neighboring cells, thereby allowing for increased viral replication and spread. Therefore, the increased understanding of the IFN-JAK-STAT signal pathway is essential for our ambition to develop novel, less toxic, and more effective anti-viral treatments.

VIRAL MANIPULATION OF OTHER ANTIVIRAL PROCESSES *VIA* REGULATING TRIPARTITE MOTIF PROTEINS

The function of autophagy is well known for its regular degradation and recycling of cellular components through isolating certain targeted cytoplasmic proteins within a double-membraned autophagosome (Dong and Levine, 2013). The pathway of autophagy is an essential component of host defense against viral infection and innate immune responses (Lussignol and Esclatine, 2017). Besides, recent studies have demonstrated that autophagy and innate immune signaling, in particular the IFN-I signaling pathway, are intricately interconnected (Tal and Iwasaki, 2009; Levine et al., 2011; Deretic et al., 2013). It is worth noting that several key molecules, such as TBK1, IRF3, and p62, involved in IFN-β induction are also important regulators of autophagy (Sharma et al., 2003; Pilli et al., 2012; Richter et al., 2016).

Interestingly, TRIM proteins, which belong to the larger family of RING E3 ligases and are well known to regulate antiviral cytokine production in DNA-sensing pathways, also play important roles in autophagy as well as autophagy-mediated antiviral defenses (Versteeg et al., 2013; Mandell et al., 2014). Previous study indicated that HSV-1 infection could lead to ER stress-relating signaling networks including many pathways of immune responses and other mechanisms that restrict viral pathogenesis (Li et al., 2015). However, to survive and propagate within the host, many viruses, including HSV-1, have evolved a variety of strategies to evade autophagy for their own benefit.

It has been proved that both TRIM56 and TRIM32 could catalyze K63-linked polyubiquitination on STING when STING had been activated by cGAMP and then translocated from the ER to the Golgi apparatus (Tsuchida et al., 2010; Ishikawa and Barber, 2011; Zhang et al., 2012). Meanwhile, after activation of TBK1 and IRF3, this excess of STING can be degraded by p62/Sequestosome1-dependent autophagy (Prabakaran et al., 2018). Konstantin et al. have revealed that unconventional K27-linked auto-ubiquitination is essential for the GTP hydrolysis activity of TRIM23, which is necessary for the recruitment of TRIM23 to autophagosomal membranes and the activation of TBK1- and p62-mediated selective autophagy (Sparrer et al., 2017). Interestingly, HSV-1 US11 could drastically suppress this autophagy loop by disrupting the TRIM23-TBK1-Hsp90 complex and inhibiting the restriction of HSV-1 infection (Liu et al., 2018). The underlying mechanism is that US11 can block recruitment of TBK1 by targeting the C-terminal ADP-ribosylation factor domain in TRIM23, which results in a negative impact on both pathways of autophagy and the type I IFN response.

Many functions of HSV-1 ICP0 have been directly linked to its E3 ubiquitin ligase activity that is required for efficient infection (Lilley et al., 2010; Orzalli et al., 2012, 2013). Conwell et al. have presented that ICP0 utilized its own RING E3 ligase activity to induce polyubiquitination and degradation of TRIM27, which might play a role in intrinsic resistance to HSV-1 infection (Conwell et al., 2015). Similarly, the Epstein-Barr virus induces the expression of TRIM29, which was reported to modify STING with K48-linked polyubiquitin and negatively regulate innate immune responses to DNA viruses (Xing et al., 2017).

The results revealed some previously undocumented mechanisms of DNA viruses in infected cells and their resistance to innate immunity, which has greatly improved our understanding of the interplay between HSV-1 and host antiviral responses through targeting TRIM family.

CONCLUSION AND PERSPECTIVES

In this review, a growing number of findings have explained the active interactions between HSV-1 and the host antiviral innate immunity, which have revealed some novel mechanisms of viral evasion. Upon infection, HSV-1 has developed sophisticated strategies with viral proteins to counteract IFN-I production in innate immune responses, mainly through interactions with the DNA-sensor-mediated antiviral signal pathways. Through these

achievements, we stand to gain an enriched understanding of viral evasion mechanisms in host cells. Nevertheless, there remain several knowledge gaps to be further investigated.

Firstly, how cGAS senses and binds to the dsDNA of HSV-1 has remained elusive. Secondly, the mechanisms through which the dysregulation of innate immune responses by HSV-1 affect human viral diseases and pathogenesis, such as AD, remain largely elusive. In other words, the clinical models of many observations coming from the overexpression of viral proteins in human cell systems remain to be established in the future. Thirdly, it warrants further investigation whether HSV-1 evades the antiviral potential of the TRIM family, and this will open up a new potential area of viral immune escape mechanisms.

Strikingly, there is still a firestorm of controversy about what is the DNA sensor of a virus in the nucleus really is. Finally, perhaps most challenging and essential issue is what nuclear receptor initiates the innate immune response to DNA viruses, how does it achieve this, and does it include HSV-1? Surprisingly, during preparation of our manuscript, a recent discovery from Cao's group has showed that the nuclear-localized heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) recognizes viral DNA and then translocates to the cytoplasm where it activates the TBK1-IRF3 pathway and amplifies IFN-α/β production (Wang et al., 2019). But, whether hnRNPA2B1 plays an important role in initiating the IFN production and enhancing the cytoplasmic antiviral signaling in HSV-1 infection still needs further investigation. Further understanding of these questions will help us to reveal the detailed and molecular mechanisms of HSV-1 infection or viral diseases, which may accelerate future development of novel antiviral therapeutics and vaccines.

AUTHOR CONTRIBUTIONS

YL wrote the manuscript and designed the figures. CZ reviewed and modified the manuscript.

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The Interaction Mechanism Between Herpes Simplex Virus 1 Glycoprotein D and Host Antiviral Protein Viperin

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Viperin is an interferon-inducible protein that responsible for a variety of antiviral responses to different viruses. Our previous study has shown that the ribonuclease UL41 of herpes simplex virus 1 (HSV-1) can degrade the mRNA of viperin to promote HSV-1 replication. However, it is not clear whether other HSV-1 encoded proteins can regulate the function of viperin. Here, one novel viperin associated protein, glycoprotein D (gD), was identified. To verify the interaction between gD and viperin, gD and viperin expression plasmids were firstly co-transfected into COS-7 cells, and fluorescence microscope showed they co-localized at the perinuclear region, then this potential interaction was confirmed by co-immunoprecipitation (Co-IP) assays. Moreover, confocal microscopy demonstrated that gD and viperin co-localized at the Golgi body and lipid droplets. Furthermore, dual-luciferase reporter and Co-IP assays showed gD and viperin interaction leaded to the increase of IRF7-mediated IFN-β expression through promoting viperin and IRAK1 interaction and facilitating K63-linked IRAK1 polyubiquitination. Nevertheless, gD inhibited TRAF6-induced NF-κB activity by decreasing the interaction of viperin and TRAF6. In addition, gD restrained viperin-mediated interaction between IRAK1 and TRAF6. Eventually, gD and viperin interaction was corroborated to significantly inhibit the proliferation of HSV-1. Taken together, this study would open up new avenues toward delineating the function and physiological significance of gD and viperin during HSV-1 replication cycle.

Keywords: herpes simplex virus 1, viperin, gD, IFN-β, NF-κB

INTRODUCTION

Herpes simplex virus 1 (HSV-1), a widespread human pathogen that can cause lytic infection in mucosal epithelial cells and life-long latent infection in neurons, is a nuclear-replicating DNA virus with a genome encoding ~80 different proteins, among which at least 44 proteins are the structural components of the virions. According to their known or putative localizations in the virions, the proteins can be classified into five groups, namely envelope, tegument, capsid, unclassified, and non-structural proteins (1, 2).

HSV-1 glycoproteins are found in the virion envelope as well as membrane of the infected cell, and gD is a multifunctional protein that can interact with three cellular receptors for entry (3), including nectins (nectin 1 and 2) (4, 5), a modified heparin sulfate (6) and herpesvirus entry mediator (HVEM, also named herpesvirus entry protein A) (7), hence it defines the viral tropism. Once binding to the receptor, an ensuing change in gD conformation exposes to profusion domains, which enables fusogenic glycoprotein gB, gH, and gL to complete fusion of the envelope with the plasma membrane (8). Therefore, binding of gD to a cell surface receptor is an essential step of virus entry (8, 9). gD also plays a key role in multiple events during HSV-1 infection, including cell-to-cell spread and virus-induced syncytia formation. However, packaging of gD into virions is almost completely blocked in the absence of tegument protein UL16 (10).

It is well-known that innate immune response is the first line for host defense. When viral infection, virus can activate the host innate antiviral response and result in the expression of series cellular protective genes, e.g., proinflammatory cytokines and type I interferon (IFN-I, including IFN-α, and IFN-β), which then induces a subset of interferon-stimulated genes (ISGs) to reinforce IFN-I signaling and prime cells with enhanced antiviral activity to inhibit viral replication (11, 12).

Viperin is an evolutionarily conserved iron-sulfur (Fe-S) cluster-binding protein (13–15), which can be induced in various cell types by distinct stimuli of IFN-I and IFN-II, viral DNA, dsRNA, polyI:C, LPS, and by infection with diverse viruses, such as human cytomegalovirus (HCMV) (16), pseudorabies virus (17), Japanese encephalitis virus (18), West Nile virus (19), hepatitis C virus (HCV) (15), Chikungunya virus (20), rhinovirus (21), yellow fever virus (22), lymphocytic choriomeningitis virus (23), and dengue virus (DENV) (24). Nevertheless, viperin shows antiviral ability to many types of viruses. For example, viperin can reduce cholesterol/sphingomyelin on the membranes that are the main components of lipid rafts, which are essential for the entry, assembly, and budding of rabies virus in RAW264.7 cells (25). Viperin also can inhibit the release of influenza A virus (IAV) by down-regulating cholesterol synthesis and perturbing lipid rafts, which are required for the stability and infectivity of IAV (26, 27). In addition, viperin can associate with some host and viral proteins, such as mitochondrial antiviral signaling protein (MAVS) (28), signal mediators interleukin-1 receptor-associated kinase 1 (IRAK1), TNF receptor-associated factor 6 (TRAF6) (29), DENV-2 NS3 (30), HCV NS5A (15), and HCMV vMIA (13), and its function is therefore regulated.

Since IFN-I and nuclear factor B (NF-κB) play key roles in regulating the antiviral response (31), HSV-1 has evolved multiple strategies to escape these two innate systems (11, 32). Specifically, US3 protein kinase inhibits the IFN-β-signaling pathway by interacting with and hyperphosphorylating IFN regulatory factors 3 (IRF3) (33), UL36 ubiquitin specific protease deubiquitinates TRAF3 and then blocks IFN-β production (34), VP16 abrogates the interferon antiviral response by suppressing NF-κB and preventing IRF3 to recruit its co-activator, CREB binding protein (35). Our previous study has demonstrated that the ribonuclease UL41 can degrade the mRNA of viperin to restrain its antiviral function (36). However, it is still not clear whether other HSV-1 encoded proteins can interact with viperin, and what is the effect or mechanism of their interaction? Therefore, given viperin plays a very important role in the regulation of host antiviral response, a screening of fluorescence microscope was firstly carried out to find which HSV-1 protein can co-localize with viperin or alter its normal subcellular localization, then their interaction was tested by co-immunoprecipitation (Co-IP) assays, and other experiments such as confocal microscopy, dual-luciferase reporter (DLR) assays and real-time quantitative PCR (RT-qPCR), were performed to explore how this interaction regulates the signaling pathways of IFN-β and NF-κB in the host innate immune system.

MATERIALS AND METHODS

Cells

COS-7 and HEK293T cells were cultured at 37°C in Dulbecco's modified MEM (DMEM. Gibco-BRL) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco-BRL).

Antibodies

Mouse anti-Flag, anti-Myc, anti-hemagglutinin (HA), and anti-β-actin monoclonal antibodies (mAbs) were purchased from ABmart. Rabbit anti-Flag polyclonal antibody (pAb) was purchased from Proteintech. Mouse non-specific control IgG antibody was purchased from eBioscience Inc. Rabbit anti-gD pAb was gifted by Dr. Roselyn J. Eisenberg (School of Veterinary Medicine, University of Pennsylvania), and mouse anti-gD mAb was purchased from Santa Cruz Biotechnology.

Plasmids Construction

The ORF of viperin was amplified by PCR using pViperin-Flag expression plasmid (provided by Dr. Yi-Ling Lin, Genomics Research Center, Academia Sinica, Taiwan) (18) as the template, which was then cloned into pEGFP-N1 (Clontech) to yield pEGFP-viperin. The US6 ORF of HSV-1 (F strain) glycoprotein D (gD) was also amplified from HSV-1 DNA pYEbac102 (37, 38), with forward primer 5′-AGG AAT TCA TGG GGG GGG CTG CCG CCA GG-3′ and reverse primer 5′-CGG GAT CCT TGT AAA ACA AGG GCT GGT G-3′. The purified PCR product was digested with *Eco*RI and *Bam*HI and then inserted into the corresponding digested pEYFP-N1 (Clontech) to yield plasmid pgD-EYFP, as described previously (39–43). Reporter plasmids pNF-κB-Luc, pIFN-β-Luc and pRL-TK were offered by Dr. Chunfu Zheng (School of Basic Medical Sciences, Fujian Medical University) (40–43). Ubiquitin expression plasmids pEFIRES-HA-Ub, pEFIRES-HA-Ub (K48) and pEFIRES-HA-Ub (K63) were provided by Dr. Jun Cui (School of Life Sciences, Sun Yat-sen University) (44). Other expression plasmids including IRAK1-HA (Dr. Hongyan Wang, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) (45), pCMV-Flag-IRAK1 (Dr. Hongbin Shu, School of Life Sciences, Wuhan University), TRAF6-myc (Dr. Jiahuai Han, School of Life Sciences, Xiamen University) (46), Flag-tagged IRF3/5D (Dr. Rongtuan Lin, Department of Medicine, McGill

University) (47), Flag-tagged IRF7/6D (Dr. John Hiscott, Lady Davis Institute, Jewish General Hospital) (48), pcDNA3.1-gD (Dr. Gary H. Cohen, University of Pennsylvania), pECFP-Golgi (Dr. Suzanne R. Pfeffer, Department of Biochemistry, Stanford University School of Medicine) (49), mCherry-KDEL (Dr. Lee H. Wong, Department of Biochemistry and Molecular Biology, Monash University; Dr. Philippe Collas, Institute of Basic Medical Sciences, University of Oslo) (50) and TOM70-CFP (Dr. Frits Kamp, Adolf-Butenandt-Institute, Ludwig-Maximilians-University) (51) were gifts from the providers shown as indicated.

Plasmid Transfection, Indirect Immunofluorescence Assays (IFA), and Confocal Microscopy

COS-7 cells were grown overnight to 80% confluence on microscopy cover glass (NEST) placed in six well plate (Corning), then plasmid transfection and fluorescence microscopy experiments were carried out as described previously (40–43, 52). Briefly, COS-7 cells were transfected with the indicated plasmids DNA mixed with polyethylenimine (PEI) transfection reagent (Polysciences) according to the manufacturer's instructions. Twenty-four hours post-transfection, cells were fixed with 4% (v/v) paraformaldehyde (Beyotime Biotechnology) for 20 min at room temperature, washed for 3 times with PBS, and incubated with 0.2% Triton X-100 (Beyotime Biotechnology) for 30 min. Subsequently, cells were incubated with rabbit anti-gD pAb or mouse anti-Flag mAb, followed by incubation with tetramethyl rhodamine isocyanate (TRITC)-conjugated goat anti-rabbit IgG (Pierce) or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma-Aldrich), then stained with or without Nile Red (Sigma) for lipid droplets for 30 min, and finally stained with DAPI (4′6-diamidino-2-phe-nylindole) (Cell Signaling Technology) for 5 min when needed. Images were obtained with a confocal microscope (Axio-Imager-LSM-800, ZEISS, Germany) using a 600× oil-immersion objective. Each image represents a vast majority of the cells with similar subcellular distribution, and white color shows the co-localization of colors merged with green, blue and red, yellow color shows the co-localization of colors merged with green and red. All scale bars indicate 10 um.

DLR Assays

The DLR assays were performed as described previously (38, 40–42, 53). In short, HEK293T cells were plated on 24 well dish (Corning) at a density of 1×10^5 cells per well-overnight before transfection. Cells were then co-transfected with 100 ng of the indicated expression plasmid, 100 ng of IFN-β or NF-κB promoter reporter and 10 ng of pRL-TK (internal control) to normalize transfection efficiency. Twenty-four hours post-transfection, the luciferase activity was detected with a luciferase assay kit (Promega).

Viral Proliferation

HSV-1 bacterial artificial chromosome (BAC) Luc (F strain, synchronally expressing firefly luciferase, and GFP fluorescent protein) was offered by Dr. Chunfu Zheng (54), which was reproduced and reposited in our lab. HEK293T cells were

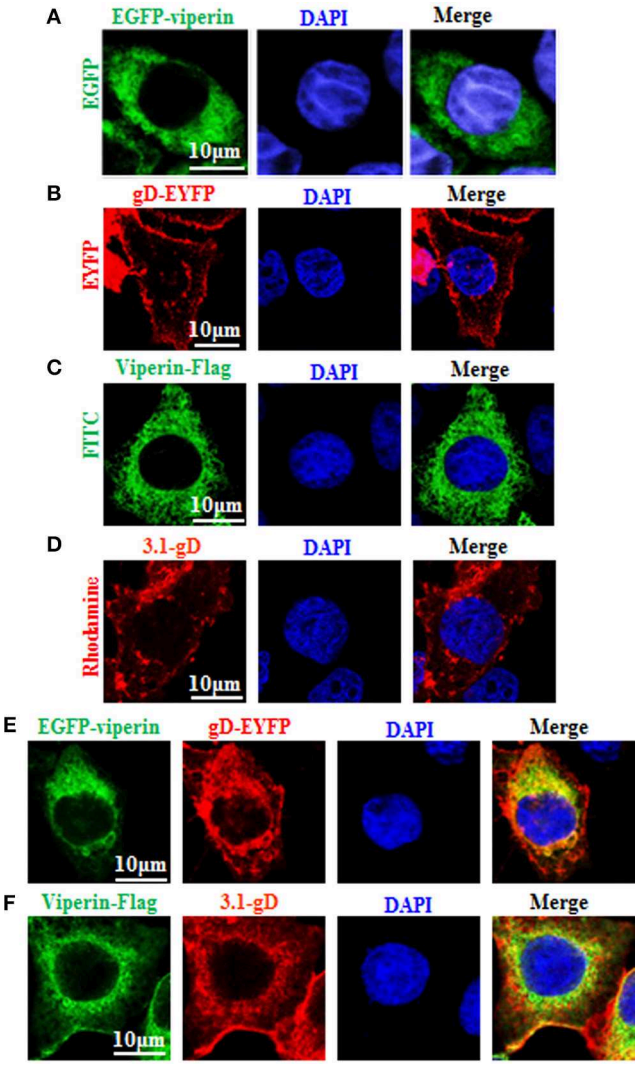

FIGURE 1 | Co-localization of gD with viperin. **(A,B)** Subcellular localization of viperin and gD in live cells. COS-7 cells were transiently transfected with EGFP-viperin **(A)** or gD-EYFP **(B)** expression plasmid. Fluorescence image of EGFP-viperin fusion protein was presented in its original color green, and gD-EYFP fusion protein was presented in pseudo-color red. **(C,D)** Subcellular localization of viperin and gD in chemically fixation cells. Viperin-Flag **(C)** or 3.1-gD **(D)** expression plasmid was transfected into COS-7 cells, then IFA was performed with primary antibody mouse anti-Flag mAb or rabbit anti-gD pAb, and secondary antibody FITC-conjugated goat anti-mouse IgG or TRITC-conjugated goat anti-rabbit IgG, respectively. Fluorescence images of FITC-conjugated protein and TRITC-conjugated protein were presented in their original colors green and red, respectively. **(E)** Co-expression of EGFP-viperin and gD-EYFP in live cells. COS-7 cells were co-transfected with EGFP-viperin and gD-EYFP expression plasmids. Fluorescence images of fusion proteins were presented as indicated in **(A)**, and yellow color shows the co-localization of colors merged with green and red. **(F)** IFA analysis of COS-7 cells co-expressed with Viperin-Flag and 3.1-gD, with primary antibodies mouse anti-Flag mAb and rabbit anti-gD pAb, and secondary antibodies FITC-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG. Twenty-four hours post-transfection, all the cells were stained with DAPI (blue) for 5 min, and analyzed with confocal microscopy. All of the photomicrographs were taken at a magnification of 600×. Each fluorescence image was representative of the vast majority of the cells observed. All scale bars indicate 10 um.

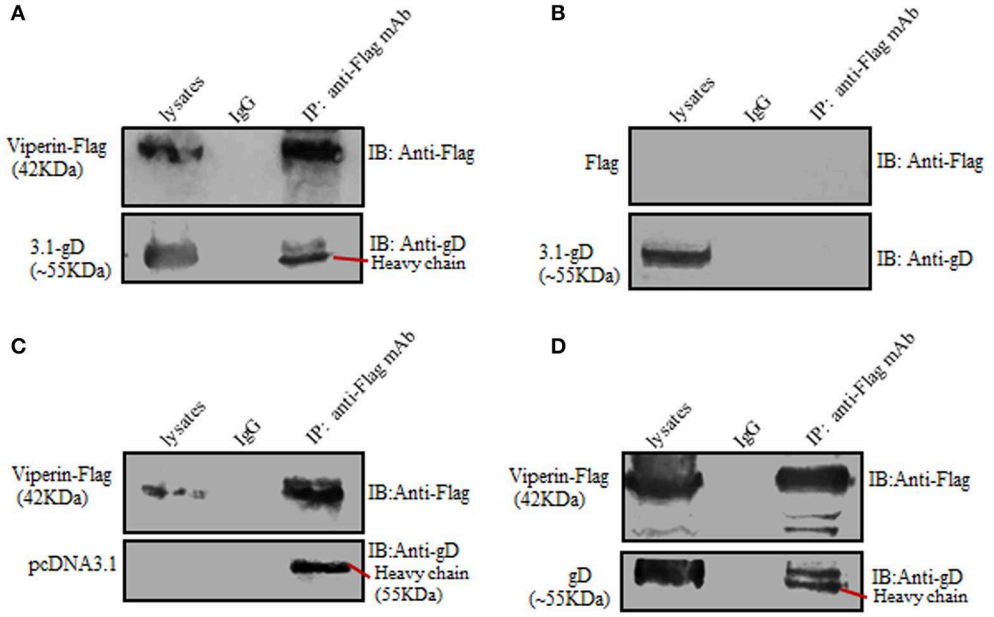

FIGURE 2 | Verification of the interaction between gD and viperin. **(A–C)** Co-IP of viperin and gD from the lysates of transfected cells. HEK293T cells were co-transfected with plasmids combination pViperin-Flag/pcDNA3.1-gD **(A)**, Flag vector/pcDNA3.1-gD **(B)**, or pViperin-Flag/pcDNA3.1 **(C)**. Twenty-four hours post-transfection, cells were lysed and immunoprecipitated with mouse anti-Flag mAb or mouse IgG control. Immunoprecipitated proteins, as well as the cell lysates, were separated in denaturing 10% SDS-PAGE, and analyzed by IB with mouse anti-Flag mAb or mouse anti-gD mAb. **(D)** Co-IP of viperin and gD from the lysates of HSV-1 infected cells. HEK293T cells transfected with pViperin-Flag for 24 h were infected with HSV-1 BAC Luc at an MOI of 1 for 16 h. Then, cells were lysed and Co-IP assays were carried out, and analyzed by IB with mouse anti-Flag mAb or anti-gD mAb.

plated on 12 well-plate (Corning) overnight before infection, then HSV-1 BAC Luc was dissolved in DMEM medium and added to the cells at an MOI (multiplicity of infection) of 1. The virus was incubated for 1.5–2 h at 37°C in a 5% CO_2 incubator and replaced with medium supplemented with 2% FBS to continue culture for the indicated times, then cells were harvested for luciferase reporter assays to determine the replication kinetics of HSV-1 (33, 34). Here, all experiments related to HSV-1 infection were carried out in the Biosafety Level II laboratory, and all operations were strictly performed in accordance with the biosafety operation requirements of Guangzhou Medical University.

RNA Isolation and RT-qPCR

HEK293T cells cultured in 6 well plate were transfected with indicated amounts of expression plasmid. Twenty-four hours post-transfection, total RNA was extracted with TRIzol reagent (Invitrogen). Samples were then subjected to reverse transcription to cDNA with RT reagent (TSINGKE). The acquired cDNA was taken as a template for qPCR, to detect the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (internal control) and IFN-β, using a qPCR instrument (BIO-RAD, CFX96). Primers used for GAPDH (forward primer 5′-AGG TCG GTG TGA ACG GAT TTG-3′ and reverse primer 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′) and IFN-β (forward primer 5′-ATGACCAACAAGTGTCTCCTCC-3′ and reverse primer 5′-GGAATCCAAGCAAGTTGTAGCTC-3′) were referred to Bing Tian's report (55).

Co-IP Assays

Co-IP assays were performed as previously described (40–42, 56–58). In brief, HEK293T cells were co-transfected with expression plasmids combination bearing EYFP, Flag, Myc or HA tag. Twenty-four hours post-transfection, transfected cells were infected with or without HSV-1 BAC Luc for 16 h, then cells were collected and lysed on ice with RIPA lysis buffer (Beyotime Biotechnology). For each immunoprecipitation (IP), an equivalent of lysate was incubated with mouse anti-Flag, anti-Myc or anti-HA mAb or non-specific control mouse antibody (IgG) and a 1:1 slurry of protein A/G PLUS-Agarose (Santa Cruz Biotechnology) at 4°C overnight. The Sepharose beads were then washed at least three times with lysis buffer added with 500 mM NaCl. Finally, immunoprecipitated proteins and cell lysates were subjected to immunoblotting (IB) assays with the indicated antibodies. The original IB results are shown in the **Supplementary Material**.

Statistical Analysis

Statistical analyses were performed using Graphpad Prism 6 software. All data were normally distributed, and the homogeneity of variances was examined with Levene's test. As the samples were normally distributed and displayed homogenous variance, statistical analyses were performed using one-way

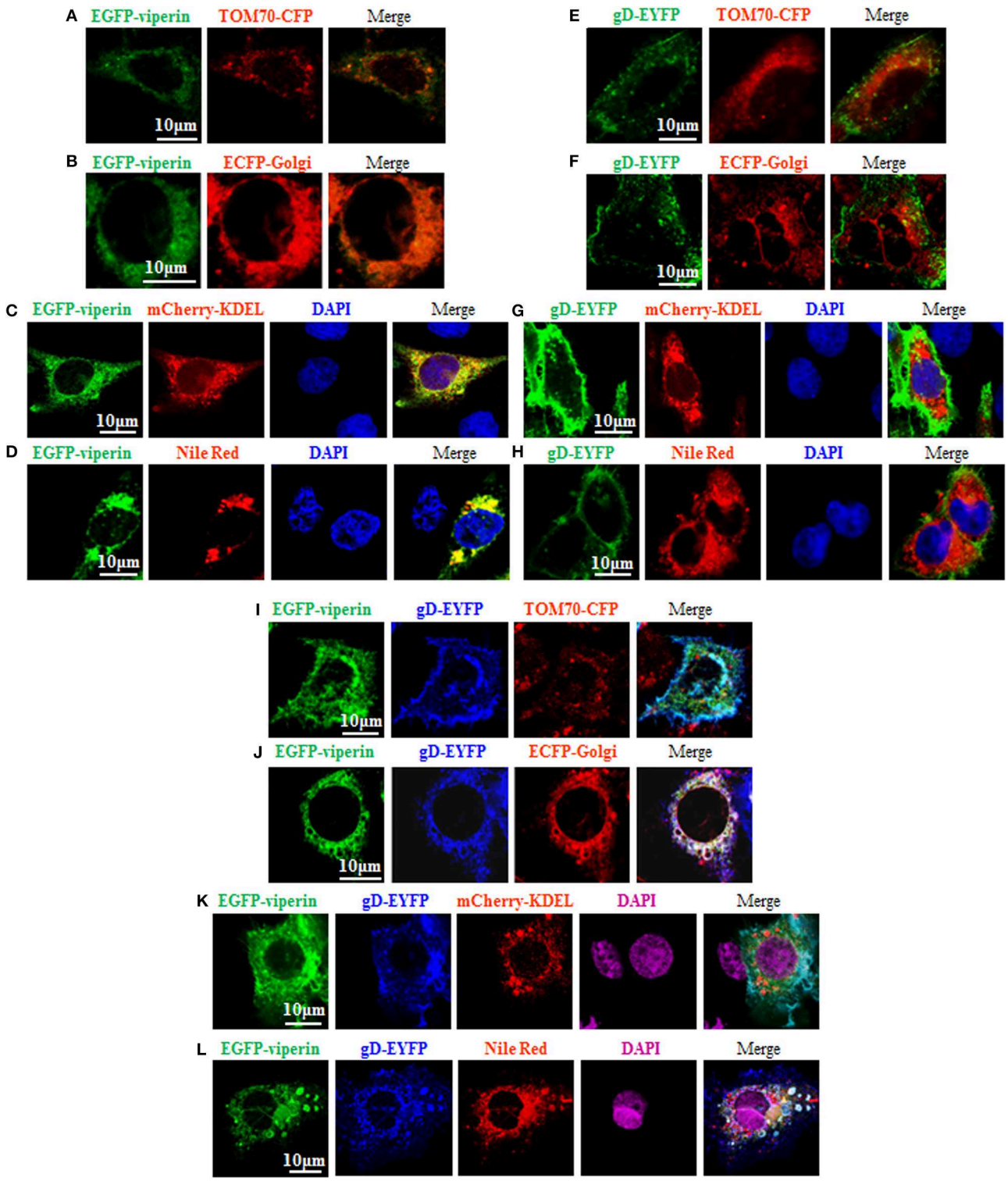

FIGURE 3 | Viperin is accumulated at the Golgi body and lipid droplets in the presence of gD. **(A–H)** Expression plasmid of GFP-Viperin or gD-EYFP was transiently co-transfected with the subcellular marker expression plasmid of TOM70-CFP (Mitochondrial marker) **(A,E)**, ECFP-Glogi (Golgi marker) **(B,F)** or mCherry-KDEL (ER marker) **(C,G)** into COS-7 cells seeded on the coverslip in six well-plate. Twenty-four hours post-transfection, cells were stained with or without Nile Red (lipid droplets marker) **(D,H)** for 30 min and/or DAPI (blue) for 5 min when needed (only GFP-Viperin/mCherry-KDEL and GFP-Viperin/Nile Red panels can stained for DAPI, since the emission wavelength of CFP is similar with that of DAPI), then fixed and visualized with a confocal microscope using a 600× oil-immersion objective. Fluorescence

(Continued)

FIGURE 3 | image of fusion protein EGFP-viperin was presented in its original color green, subcellular organelle makers TOM70-CFP **(A,E)** and ECFP-Golgi **(B,F)** were presented in pseudo-color red, mCherry-KDEL **(C,G)** and Nile Red **(D,H)** were presented in their original color red, and gD-EYFP fusion protein was presented in pseudo-color green. Yellow color shows the co-localization of colors merged with green and red **(C,D)**. **(I–K)** Plasmids combination of gD-EYFP/EGFP-viperin were transiently co-transfected with the subcellular maker TOM70-CFP **(I)**, ECFP-Golgi **(J)**, or mCherry-KDEL **(K)** into COS-7 cells seeded on the coverslip in six well plate. Twenty-four hours post-transfection, cells were stained with or without Nile Red **(L)** for 30 min and/or DAPI (purple) for 5 min when needed. Then, confocal experiments were performed as described for **(A–H)**. Fluorescence image of fusion protein EGFP-viperin was presented in its original color green, subcellular makers TOM70-CFP **(I)** and ECFP-Golgi **(J)** were presented in pseudo-color red, mCherry-KDEL **(K)** and Nile Red **(L)** were presented in their original color red, and gD-EYFP fusion protein was presented in pseudo-color blue. White color shows the co-localization of colors merged with green, blue and red **(J,L)**. All scale bars indicate 10 um.

ANOVA. In the event of a difference being present, Bonferroni-adjusted *post hoc* tests were performed to identify specific effect. Moreover, Student *t* test (unpaired two-tailed *t*-test) was used when needed. Data were expressed as means and standard deviations (mean ± SD) from three independent experiments, with significant differences marked on the figures. Significance levels were defined as ns, not significant, $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$.

RESULTS

gD Co-localizes With Viperin

To find out which HSV-1 protein may interact with viperin, some HSV-1 encoded cytoplasmic localization proteins (2) were firstly screened, by co-transfection of viperin and HSV-1 protein expression plasmids and analyzing which HSV-1 protein can co-localize with viperin or alter its subcellular localization, and gD (US6), US4 (gG), and UL1 (gL) were identified. Our preliminary experiments found that there were significant differences in the interaction mechanisms among viperin-gD, viperin-US4 and viperin-UL1 (unpublished data). Therefore, the in-depth study of the interaction mechanisms between viperin and each protein of gD, US4, or UL1 would be an independent big project, and they need to be investigated separately. In addition, gD, US4 or UL1 encode glycoproteins, they (especially gD) play a very important role in the invasion of HSV-1. Accordingly, gD was firstly selected to investigate the potential interaction mechanism with viperin. To this end, pEGFP-viperin, pViperin-Flag, pgD-EYFP, or pcDNA3.1-gD expression plasmid was individually transfected into COS-7 cells to characterize their subcellular localizations in live cells or chemically fixed cells. As shown in **Figure 1**, viperin was absolutely distributed in the cytoplasm in cells transfected with EGFP-Viperin (**Figure 1A**) or Viperin-flag (**Figure 1C**) expression plasmid, and gD mainly exhibited nuclear membrane or cytoplasmic membrane localization in cells transfected with gD-EYFP (**Figure 1B**) or 3.1-gD (**Figure 1D**) expression plasmid, which are consistent with previous studies (59–61). In an attempt to pursue whether gD binds to viperin, EGFP-Viperin, and gD-EYFP expression plasmids were co-transfected into COS-7 cells to detect whether gD co-localizes with viperin, since co-localization experiment is one of the important and popular methods to detect the potential interaction between different proteins. As results, gD co-localized with viperin and predominantly accumulated at the perinuclear region (**Figure 1E**, yellow signal). Furthermore, IFA also proved the co-localization of gD and viperin at the perinuclear region (**Figure 1F**, yellow signal), confirming the potential interaction between gD and viperin.

gD Interacts With Viperin

To further prove the interaction between gD and viperin, Co-IP assays were carried out. HEK293T cells were co-transfected with pcDNA3.1-gD and pViperin-Flag expression plasmids, then cell lysates were immunoprecipitated with anti-Flag mAb or non-specific control mouse IgG. As a result, gD was immunoprecipitated by Viperin-Flag with anti-Flag mAb (**Figure 2A**), whereas no such protein was immunoprecipitated with the control mouse IgG (**Figure 2A**). As negative controls, HEK293T cells were co-transfected with plasmids combination pcDNA3.1-gD/Flag vector (**Figure 2B**) or pViperin-Flag/pcDNA3.1 vector (**Figure 2C**). Then, cell lysates were immunoprecipitated with anti-Flag mAb or mouse IgG. Similarly, no target protein was immunoprecipitated by Flag vector (**Figure 2B**) or Viperin-Flag (**Figure 2C**), indicating gD could interact with viperin.

To continue determine the interaction between gD and viperin in the context of viral infection, HEK293T cells were transfected with pViperin-Flag expression plasmid, then infected with HSV-1 at an MOI of 1. Subsequently, cells were collected and Co-IP assays were performed. As shown in **Figure 2D**, gD again was immunoprecipitated by Viperin-Flag with anti-Flag mAb, whereas no such protein was immunoprecipitated with control mouse IgG, confirming gD could interact with viperin under physiological condition.

Viperin Accumulates at Golgi Body and Lipid Droplets in the Presence of gD

It is known that the N-terminal amphipathic a-helix is important for viperin to target to ER (60) and lipid droplets (62), and this subcellular localization is essential for suppressing viral replication (63). However, the vMIA-mediated mitochondria localization of viperin is favorable for HCMV replication (13). In order to probe the underlying mechanism of gD and viperin interaction, we continued to analyze whether gD can alter the normal localization of viperin. As control, GFP-Viperin or gD-EYFP was transiently co-transfected with the subcellular marker expression plasmid of TOM70-CFP (Mitochondrial marker) (**Figures 3A,E**), ECFP-Glogi (Golgi marker) (**Figures 3B,F**) or mCherry-KDEL (ER marker) (**Figures 3C,G**) into COS-7 cells, or cells were stained with Nile Red for lipid droplets (**Figures 3D,H**), and the cells were subsequently examined by confocal microscopy, to test the normal subcellular localizations of viperin and gD. As expected, viperin could co-localize with mCherry-KDEL (**Figure 3C**, yellow signal) and lipid droplets (**Figure 3D**, yellow signal), but not TOM70-CFP (**Figure 3A**) or ECFP-Glogi (**Figure 3B**) (13, 60, 62). However, gD-EYFP could not co-localize with all of the mentioned subcellular markers

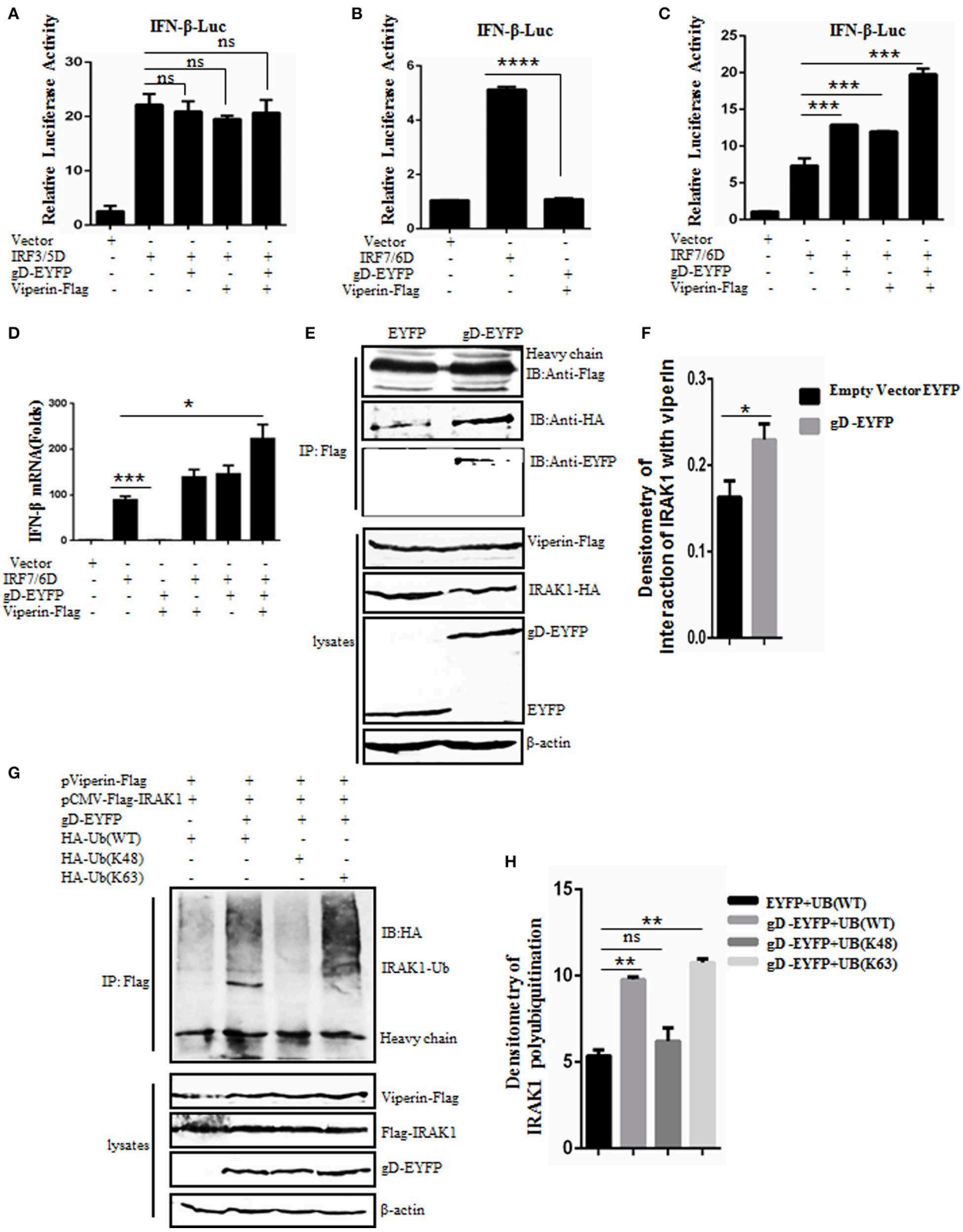

FIGURE 4 | gD facilitates IRF7-mediated IFN-β promoter activity through enhancing the interaction of viperin with IRAK1 and increasing K63-linked polyubiquitination of IRAK1. **(A–C)** HEK293T cells were co-transfected with IFN-β-Luc reporter, pRL-TK and gD-EYFP or pViperin-Flag or plasmids combination of gD-EYFP and pViperin-Flag, with or without IRF3/5D **(A)** or IRF7/6D **(B,C)** expression plasmid. Twenty-four hours post-transfection, luciferase activity was analyzed. **(D)** HEK293T

(Continued)

FIGURE 4 | cells were co-transfected with the indicated plasmids as described in **(C)**, except for the reporter plasmids. Twenty-four hours post-transfection, RT-qPCR was performed to analyze the relative mRNA expression level of IFN-β. Data were expressed as means ± SD from three independent experiments. **(E,F)** HEK293T cells co-transfected with expression plasmids pViperin-Flag, IRAK1-HA and gD-EYFP, or EYFP control construct were harvested and immunoprecipitated with mouse anti-Flag mAb or non-specific mouse IgG, and IB analysis was probed with the indicated antibodies. Densitometry of the IRAK1 and viperin interaction bands were normalized to the loading control β-actin. **(G,H)** HA-tagged Ub (WT), Ub (K48), or Ub (K63) expression plasmid was co-transfected with plasmids combination of pCMV-Flag-IRAK1 and pViperin-Flag into HEK293T cells, with or without the presence of gD-EYFP. Twenty-four hours post-transfection, cells were collected, followed by Co-IP with mouse anti-Flag mAb and IB analysis with mouse anti-HA mAb. Densitometry of IRAK1 polyubiquitination bands were normalized to the loading control β-actin. Data were expressed as means ± SD from three independent experiments. Statistical analyses were performed using one-way ANOVA, except **(F)** using student *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$.

(**Figures 3E–H**). Then, expression plasmids combination of gD-EYFP/EGFP-viperin were transiently co-transfected with TOM70-CFP (**Figure 3I**), ECFP-Golgi (**Figure 3J**), or mCherry-KDEL (**Figure 3K**) into COS-7 cells, or cells were stained with Nile Red for lipid droplets. As results, co-expression of gD and viperin resulted in a pronounced co-localization with Golgi and lipid droplets markers (**Figures 3J,L**, white signal). Nevertheless, no obvious overlap area could be detected when gD and viperin were co-transfected with expression plasmid TOM70-CFP (**Figure 3I**) or mCherry-KDEL (**Figure 3K**). Therefore, viperin could accumulate at the Golgi apparatus and lipid droplets in the presence of gD.

gD Facilitates IFN-β Activity in the Presence of Viperin

It is reported that viperin can enhance TLR7/9-dependent production of IFN-I (29). To examine whether gD and viperin interaction is involved in the regulation of IFN-β expression, expression plasmid of gD-EYFP or pViperin-Flag or plasmids combination of gD-EYFP and pViperin-Flag were co-transfected with or without expression plasmid IRF3/5D or IRF7/6D into HEK293T cells, along with pIFN-β-Luc and pRL-TK reporter plasmids. As shown in **Figure 4**, both IRF3/5D and IRF7/6D alone could activate IFN-β expression, but no IFN-β activity was detected when HEK293T cells were only co-transfected with gD-EYFP and pViperin-Flag (**Figures 4A–C**). gD or viperin alone or combination of gD and viperin did not affect IRF3/5D-induced IFN-β activity (**Figure 4A**), however, gD or viperin alone could enhance IRF7/6D-induced IFN-β activity (**Figure 4C**). More importantly, the co-existence of gD and viperin activated a higher IFN-β promoter activity than that of gD or viperin (∼2-fold) (**Figure 4C**). To further explore whether gD facilitates IFN-β activity through IRF7 in the presence of viperin, experiments were carried out as described in **Figure 4C** except for the reporter plasmids, and IFN-β mRNA accumulation was measured by RT-qPCR. As a result (**Figure 4D**), the change tendency of IFN-β mRNA was consistent with the DLR result shown in **Figure 4C**, suggesting the gD and viperin interaction could promote IRF7 mediated interferon expression.

gD Enhances the Interaction Between Viperin and IRAK1

Saitoh's study shows that viperin can interact with the signal mediators IRAK1 and TRAF6, so as to recruit them to the lipid bodies, which can regulate TLR7- and TLR9-IRAK1 signaling axis to mediate the expression of functionally important immune molecules in plasmacytoid dendritic cell (29). To determine whether the gD and viperin interaction can affect the interaction between viperin and IRAK1, pViperin-Flag, and IRAK-HA expression plasmids were co-transfected with gD-EYFP or EYFP control vector into HEK293T cells, then cells were harvested and analyzed by Co-IP assays. In constrast to the EYFP control, the association of viperin and IRAK1 was enhanced in the presence of gD (**Figures 4E,F**).

gD Increases the K63-Linked Polyubiquitination of IRAK1

It is shown that the viperin-related K63-linked polyubiquitination of IRAK1 is crucial for the TLR7- and TLR9-dependent IFN-β production (29). To probe whether gD and viperin interaction participates in the polyubiquitination of IRAK1, HA-tagged Ub (WT), Ub (K48), or Ub (K63) expression plasmid was co-transfected with plasmids pCMV-Flag-IRAK1 and pViperin-Flag into HEK293T cells, with or without the presence of gD-EYFP. Then, Co-IP assays were performed. As results, polyubiquitinated forms of IRAK1 were detected when cells were co-expressed with viperin and Ub (WT) (**Figure 4G**, lane 1, **Figure 4H**), and this polyubiquitination was reinforced in the presence of gD (**Figure 4G**, lane 2, **Figure 4H**). Notably, gD catalyzed IRAK1 polyubiquitination with the expression of Ub (K63) (**Figure 4G**, lane 4, **Figure 4H**), but not Ub (K48) (**Figure 4G**, lane 3, **Figure 4H**), indicating gD interacted with viperin to promote K63-linked IRAK1 polyubiquitination. In short, we demonstrated that gD could facilitate IFN-β production through enhancing the interaction of viperin with IRAK1 and increasing K63-linked polyubiquitination of IRAK1.

gD Attenuates NF-κB Activity in the Presence of Viperin

Viperin is proved to be involved in the activation of NF-κB and AP-1 in T cells (64). To detect whether gD and viperin interaction also can modulate the NF-κB activity mediated by the key regulatory component of NF-κB signaling pathway, TRAF6 (11), expression plasmid gD-EYFP or pViperin-Flag or plasmids combination of gD-EYFP and pViperin-Flag were co-transfected with or without TRAF6-myc expression plasmid into HEK293T cells, along with pNF-κB-Luc and pRL-TK reporter plasmids. As results, overexpression of TRAF6 efficiently activated the NF-κB reporter, but no NF-κB activity was tested when HEK293T cells were only co-transfected with plasmids gD-EYFP and pViperin-Flag (**Figure 5A**). The expression of gD or viperin alone did not affect TRAF6-induced NF-κB reporter activity, however, gD and

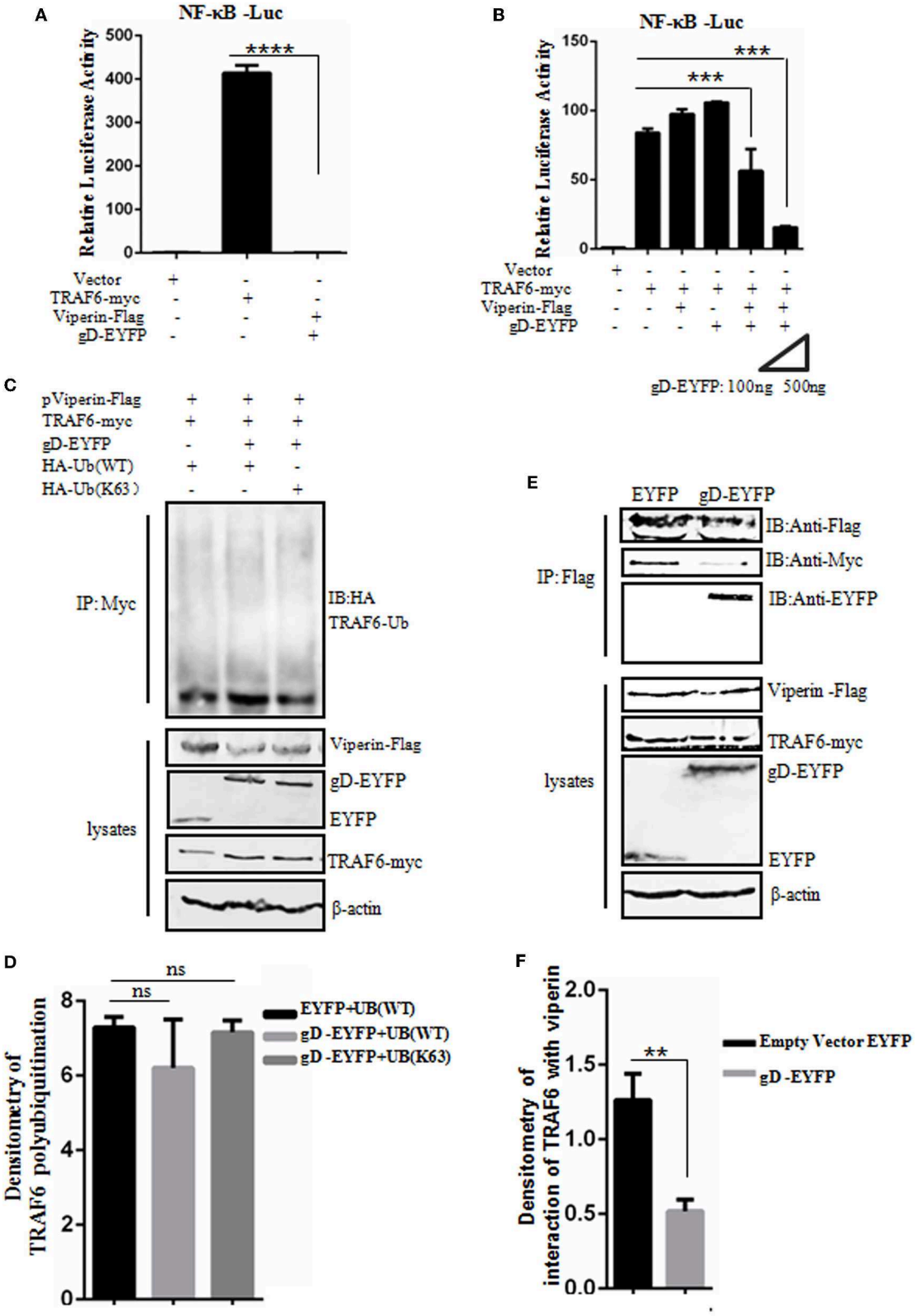

FIGURE 5 | gD attenuates NF-κB activity by reducing the interaction between TRAF6 and viperin, but does not affect the polyubiquitination of TRAF6. **(A,B)** HEK293T cells were co-transfected with NF-κB-Luc reporter, pRL-TK and gD-EYFP or pViperin-Flag or plasmids combination of pViperin-Flag and gD-EYFP (with different amounts), with or without the presence of TRAF6-myc construct. Twenty-four hours post-transfection, luciferase activity was analyzed. Data were expressed as

(Continued)

FIGURE 5 | means ± SD from three independent experiments. **(C,D)** HEK293T cells were co-transfected with expression plasmids of TRAF6-myc, pViperin-Flag and HA-Ub (WT) or HA-Ub (K63), along with or without gD-EYFP expression plasmid. Twenty-four hours post-transfection, cells were collected, followed by Co-IP with mouse anti-Myc mAb and IB analysis with mouse anti-HA mAb. Densitometry of TRAF6 polyubiquitination bands were normalized to the loading control β-actin. **(E,F)** HEK293T cells co-transfected with expression plasmids of pViperin-Flag, TRAF6-myc and gD-EYFP or the control EYFP construct were harvested and Co-IPed with mouse anti-Flag mAb. IB analysis was probed with the indicated antibodies. Densitometry of the TRAF6 and viperin interaction bands were normalized to the loading control β-actin. Data were expressed as means ± SD from three independent experiments. Statistical analyses were performed using one-way ANOVA, except **(F)** using student *t* test. $^{**}P < 0.01$; $^{***}P < 0.001$; and $^{****}P < 0.0001$.

viperin combination significantly inhibited TRAF6-induced NF-κB reporter activity. Additionally, gD and viperin interaction constrained TRAF6-induced NF-κB promoter activity in a gD dose-dependent manner (**Figure 5B**), suggesting gD could modulate TRAF6-mediated NF-κB activity through viperin.

gD Does Not Affect the Polyubiquitination of TRAF6

Polyubiquitination has emerged as an important regulatory mechanism in NF-κB signaling, and TRAF6 acts as a key substrate of K63-linked polyubiquitin chains in TNFR pathway, which serves as a mechanism to recruit TAK1 and IKK kinases and finally stimulate downstream NF-κB activation (65). To investigate whether gD and viperin interaction can affect the polyubiquitination of TRAF6, HEK293T cells were co-transfected with TRAF6-myc, pViperin-Flag and HA-Ub (WT) or HA-Ub (K63) constructs, along with or without gD-EYFP expression plasmid. Then, Co-IP assays were performed. As shown in **Figure 5**, no apparent difference of the TRAF6 polyubiquitination in the presence of gD or gD and viperin combination (**Figures 5C,D**), indicating gD and viperin interaction could not inhibit the polyubiquitination of TRAF6.

gD Reduces the Interaction Between TRAF6 and Viperin

To further elucidate a clear molecular mechanism of how gD and viperin interaction inhibits NF-κB activity, pViperin-Flag, and TRAF6-myc expression plasmids were co-transfected into HEK293T cells, along with gD-EYFP or EYFP control plasmid. Then, Co-IP assays were carried out. In contrast to the EYFP control, gD significantly reduced the interaction between TRAF6 and viperin, suggesting gD could inhibit TRAF6-mediated NF-κB activity through competive binding viperin with TRAF6 (**Figures 5E,F**). Taken together, these results support that gD downregulated NF-κB activity by reducing the interaction between TRAF6 and viperin, but not affecting the polyubiquitination of TRAF6.

gD Inhibits the Interaction Between IRAK1 and TRAF6 in the Presence of Viperin

It is documented that the signal mediators IRAK1 and TRAF6 can interact with each other at the lipid bodies (29). To test whether gD and viperin interaction affects the interaction between IRAK1 and TRAF6, IRAK1-HA and TRAF6-myc expression plasmids were co-transfected into HEK293T cells, along with pViperin-Flag or expression plasmids combination of gD-EYFP and pViperin-Flag. Then, Co-IP assays were performed. As results, overexpression of viperin alone indeed induced significantly stronger interaction of IRAK1 and TRAF6 (**Figure 6A**, lane 2, **Figure 6B**), since viperin is critical for the recruitment of IRAK1 and TRAF6 to lipid bodies, which are the transfer points of TLR7 and TLR9 signaling pathways (29). However, the IRAK1 and TRAF6 interaction became weaker in the presence of gD and viperin (**Figure 6A**, lane 3, **Figure 6B**), which was similar to that of the negative control (**Figure 6A**, lane 1, **Figure 6B**), indicating gD and viperin interaction could impede the interaction between IRAK1 and TRAF6.

gD and Viperin Interaction Inhibits HSV-1 Replication

In order to investigate the physiological significance of gD and viperin interaction during HSV-1 infection, HEK293T cells were mock-transfected or transfected with plasmid pViperin-Flag or plasmids combination of 3.1-gD and pViperin-Flag. Twelve hours post-transfection, cells were infected with HSV-1 BAC Luc at an MOI of 1 for 6, 12, or 24 h. Then, luciferase activity assays were performed to determine the replication kinetics of HSV-1. As shown in **Figure 7**, the luciferase activity gradually increased with the time extension of HSV-1 infection, and transfection with plasmid pViperin-Flag alone had no inhibitory effect on the HSV-1 propagation, which is consistent with our previous study (36). However, the HSV-1 proliferation was remarkably impaired when cells were co-transfected with 3.1-gD and pViperin-Flag expression plasmids. More importantly, this inhibitory trend was consistent at different time points of the infection (**Figures 7A–C**). Accordingly, these results indicated that the interaction between gD and viperin indeed could obstruct the reproduction of HSV-1.

DISCUSSION

Many viruses can induce the up-regulation of viperin during infection, and viperin is shown to have critical roles in inhibiting viral replication and facilitating TLR7- and TLR9-mediated production of IFN-I (29), yet its function can be dampened by some viruses (66). Our previous study has demonstrated that HSV-1 infection can not induce the up-regulation of viperin, since UL41 blocks the expression of viperin by reducing its mRNA accumulation (36). However, a small amount of viperin mRNA and its corresponding protein is not degraded (36), which is surprising and promotes us to probe whether other HSV-1 encoded proteins can interact with viperin to facilitate or inhibit the propagation of HSV-1. Accordingly, we utilized a simple and quick method at the beginning of screening, to analyze whether there are HSV-1 proteins (fused with EYFP) can co-localize with EGFP-viperin or change its normal subcellular

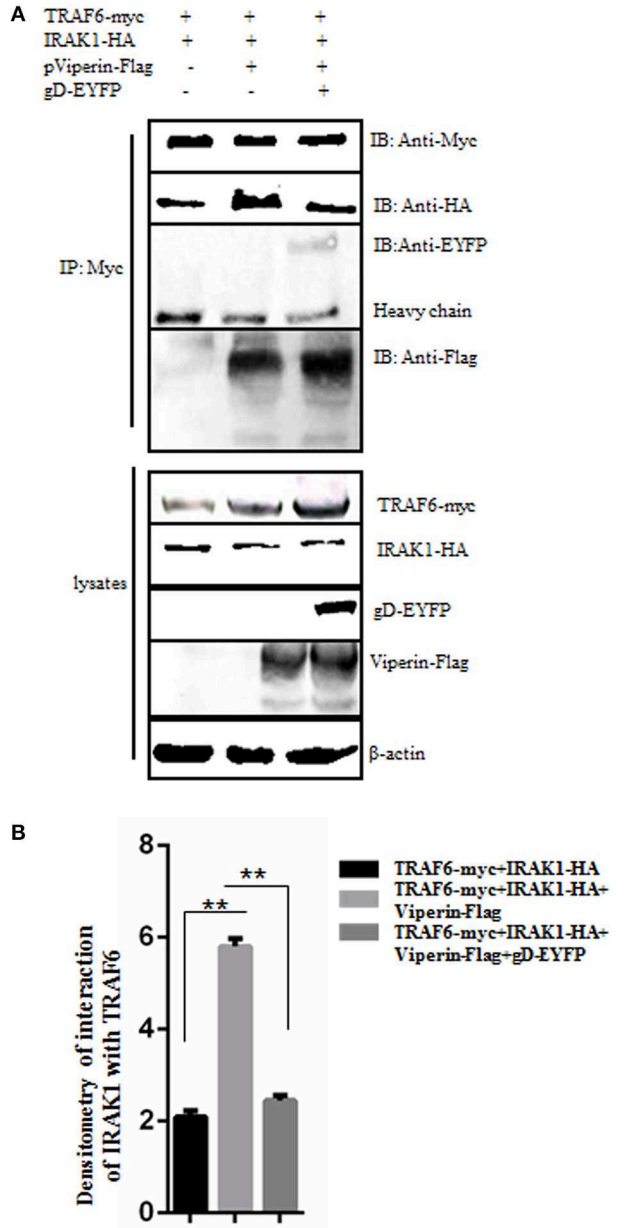

FIGURE 6 | gD suppresses IRAK1 and TRAF6 interaction in the presence of viperin. **(A,B)** HEK293T cells were co-transfected with the expression plasmids of IRAK1-HA and TRAF6-myc, along with pViperin-Flag or plasmids combination of gD-EYFP and pViperin-Flag. Twenty-four hours post-transfection, cells were collected, and followed by Co-IP with mouse anti-Myc mAb. IB analysis was developed with the indicated antibodies. Densitometry of the IRAK1 and TRAF6 interaction bands were normalized to the loading control β-actin. Data were expressed as means ± SD from three independent experiments, and statistical analyses were performed using one-way ANOVA. $^{**}P < 0.01$.

localization. In the above cases, other methods were used to verify the potential interaction and interaction mechanism. In fact, we found that not only gD could co-localize with viperin, but also other HSV-1 proteins (unpublished data), indicating there are other potential interactions exist between HSV-1 proteins and viperin, which needed to be verified by further deep exploration. Certainly, there is bound to be a missed screening of interactions between HSV-1 proteins and viperin, although the method of fluorescence co-localization or localization change can help us to screen for potential interactions. Therefore, it is difficult for us to say when there is no interaction exist between HSV-1 proteins and viperin. Perhaps new interactions will be discovered by using other experimental methods.

In our previous experiment design, we had considered to detect the co-localization of endogenous gD and viperin during HSV-1 infection, but most of the endogenous viperin mRNA will be degraded by UL41 when HSV-1 infection. Western blot analysis showed that viperin was almost degraded, with very low remaining protein amount, hence in our previous experiment the expression of viperin could not be detected by IFA using endogenous viperin antibody (unpublished data), we therefore would not be able to study the interaction mechanism between gD and viperin without viperin overexpression (36). Actually, the interaction experiments were carried out under some physiological conditions, which were performed when HSV-1 infection using gD antibody (**Figure 2**). Furthermore, gD can not be deleted in the viral genome, since it is an essential protein for HSV-1 replication. Once deleted, HSV-1 can not proliferate. Thus, we can not study the interaction mechanism between gD and viperin during HSV-1 mutant (gD deletion or knockdown) infection (3, 8, 9), since it is difficult for us to determine whether the effect of gD-viperin interaction on the proliferation of HSV-1 (after gD knockdown or deletion) is caused by the decrease of gD and viperin interaction or the reduce of gD directly affects the propagation of HSV-1. Besides, various literatures have shown that the combination of plasmid transfection and viral infection (using virus protein specific antibody) is sufficient to validate the interaction between cellular protein and viral protein (35, 41, 67–69).

After screening, our confocal results found that gD could co-localize with viperin at the Golgi body and lipid droplets, and Co-IP results demonstrated that gD interacted with viperin. It is shown that MAVS can interact with viperin to act as a negative regulator of the interferon response (28). IRAK1 and TRAF6 are two other target proteins that can interplay with viperin and be recruited to the lipid bodies to induce the nuclear translocation of transcription factor IRF7 (29). Viperin also can interact with DENV-2 NS3 protein to restrict early DENV-2 RNA production/accumulation (30), and viperin can inhibit HCV replication via its interaction with NS5A (15). Surprisingly, viperin can enhance HCMV infection by interacting with the mitochondrial trifunctional protein, which mediates fatty acids β-oxidation to generate adenosine triphosphate (ATP) (13). Therefore, we wondered what is the effect of gD and viperin interaction.

It is well-known that toll-like receptors can recognize various pathogens (including viruses and bacteria) when they stimulate the innate immunity defense system (70), subsequently myeloid differentiation factor 88 (MyD88) is induced to recruit IRAK1 and form a complex through their respective death domains (71). Then, IRAK1 is phosphorylated and rapidly degraded in a proteasome-dependent manner, resulting in the down-regulation

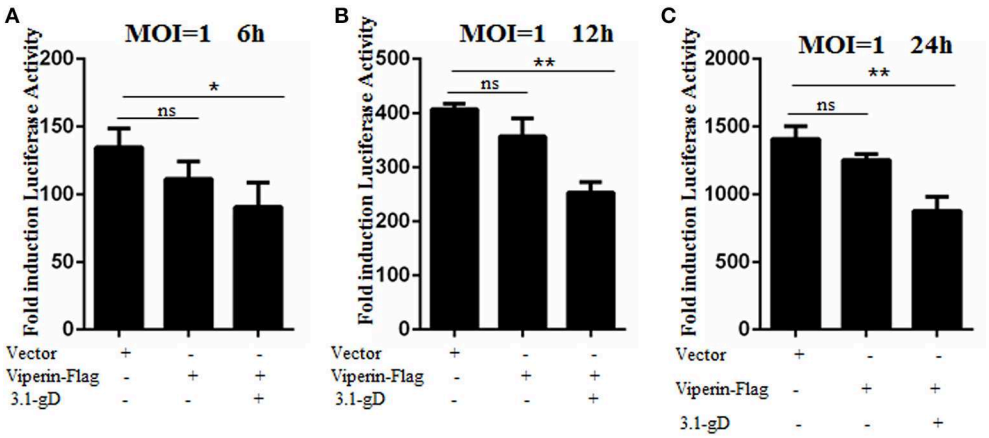

FIGURE 7 | gD impairs HSV-1 replication in the presence of viperin. HEK293T cells were infected with HSV-1 BAC Luc at an MOI of 1 for 6 h **(A)**, 12 h **(B)**, or 24 h **(C)**, after transfection with plasmid pViperin-Flag or plasmids combination of 3.1-gD and pViperin-Flag for 12 h. Then, cells were harvested for luciferase reporter assays. Data were expressed as means ± SD from three independent experiments, and statistical analyses were carried out using one-way ANOVA. $^{*}P < 0.05$; $^{**}P < 0.01$.

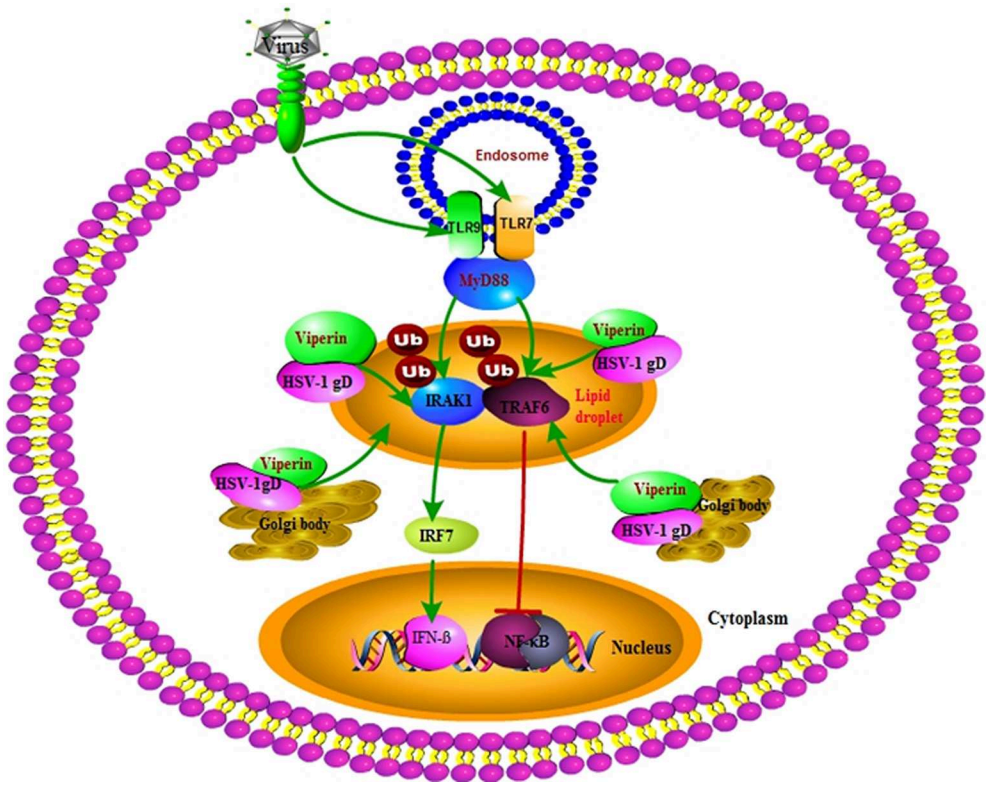

FIGURE 8 | Overview of the molecular mechanism of HSV-1 gD and viperin interaction. HSV-1 gD can interact with viperin, and co-localize with it at the lipid droplets and Golgi body. The gD and viperin interaction facilitates IRF7-mediated IFN-β activity by promoting viperin and IRAK1 interaction and facilitating K63-linked IRAK1 ubiquitination, whereas gD attenuates TRAF6-induced NF-κB activity by inhibiting the viperin and TRAF6 interaction, but not affecting the polyubiquitination of TRAF6. Viperin alone promotes the interaction of IRAK1 and TRAF6, which is inhibited in the presence of gD and viperin. Eventually, gD and viperin interaction is corroborated to significantly inhibit the proliferation of HSV-1.

of IFN-I signaling and inflammatory responses (72). Upon receptor recognition, TLR2 dimerizes with either TLR1 or TLR6 and recruits MyD88. The next, TRAF6, which is an E3 ubiquitin ligase, catalyzes the synthesis of polyubiquitin chains bound to itself and TAK1, thereby activates TAK1 and leads to downstream NF-κB activation (73). Undoubtedly, IRAK1 and TRAF6 both are

key regulatory components of the signaling pathway to mediate IFN-I production and canonical NF-κB-initiated cytokines. Accordingly, the expression of IFN and activation of NF-κB can be regulated through IRAK1 and TRAF6. For instance, newcastle disease virus facilitates K63-linked ubiquitination of IRAK1 to increase TLR7/9-dependent IFN-I production and subsequent expression of viperin (29). The nucleotide-binding domain and leucine-rich-repeat-containing (NLR) protein attenuates NF-κB activation through its interaction with the component of TRAF6 pathway (74). Therefore, we wanted to test whether gD and viperin interaction had any effect on the IFN-β or NF-κB pathway.

Our DLR assays showed that gD and viperin interaction could up-regulate IRF7 (but not IRF3) mediated IFN-β activity. Further Co-IP assays demonstrated that gD strengthened the interaction of viperin with IRAK1 and improved K63-linked IRAK1 polyubiquitination, suggesting the co-localization of gD with viperin at the Golgi body and lipid droplets can improve the antiviral ability of viperin. However, the presence of gD and viperin significantly inhibited TRAF6-mediated NF-κB activity in a gD dose-dependent manner. Co-IP results further showed gD reduced the interaction of TRAF6 with viperin, but not affected the ubiquitination of TRAF6 (**Figure 8**). Moreover, gD bound to viperin constrained the interaction between IRAK1 and TRAF6, which can interact with each other at the lipid bodies (29). Consequently, gD and viperin interaction was proved to restrain HSV-1 replication in physiological significance.

At the level of plasmid transfection, we had elucidated the specific interaction mechanism between gD and viperin, and we also analyzed and verified the effect of gD and viperin co-expression (overexpression) on HSV-1 proliferation. As mentioned above, the mRNA of viperin will be degraded by UL41 when HSV-1 infection, and the specific interaction mechanism between gD and viperin can not be studied after viperin is degraded. Therefore, we considered it was not necessary to carry out the viperin knockdown experiment, since viperin is actually degraded during HSV-1 infection.

Generally, a modest level of IFN-I expression is driven by the activation of NF-κB (75), however, excessive IFN-I expression can be restrained after NF-κB activation (76), this means that NF-κB can be activated but IFN-I is suppressed, and *vice versa*. Hence, gD interacts with viperin to inhibit NF-κB activity while stimulates IRF7-mediated IFN-I transcriptional activity is reasonable. This interaction is supposed to occur in HSV-1 lytic cycle rather than latent infection, since most of the HSV-1 proteins are silent when HSV-1 is latent in neurons, and only latency associated transcript (LAT) and a small amount of proteins are expressed (77).

When virus invades the cell, it activates a series of signaling pathways and then induces the expression of hundreds of ISGs to perform antiviral effects. Among them, viperin is an IFN-induced protein, which plays an important role in this process (11, 12). We supposes that in order to successfully infect the cells and establish effective replication, the HSV-1 encoded tegument protein UL41 can be released into the cytoplasm when HSV-1 invades cells, and the mRNAs of some ISGs (such as viperin) are degraded by UL41, so as to inhibit the host innate immunity and promote the proliferation of HSV-1 (36). However, HSV-1 has evolved very delicate mechanisms that if the functions of most of the ISGs are prohibited or HSV-1 continues to replicate strongly, it is bound to quickly cause the death of HSV-1 infected and adjacent cells, thereby HSV-1 does not have enough time to replicate, which is certainly not favorable for the survival of HSV-1. Accordingly, HSV-1 may take advantage of other encoded proteins (such as gD) to enhance the host's IFN response, by interacting with the pre-existing viperin or HSV-1-induced viperin, to compensate for the mRNA degradation of viperin, since the up-regulation of IFN will in turn promote the expression of viperin. Consequently, HSV-1 may balance the amount of viperin in a very sophisticated way, to regulate the relationship between host's innate immune status and its self-replication, but the specific mechanism needs to be further explored.

In conclusion, here we identified HSV-1 gD could interact with antiviral protein viperin, and co-localize with it at the Golgi body and lipid droplets. Our further results proved that gD and viperin interaction improved IRF7-mediated IFN-β activity to strengthen the antiviral ability of viperin, through enhancing the interaction between viperin and IRAK1, and increasing the K63-linked polyubiquitination of IRAK1. However, gD and viperin interaction could not affect the polyubiquitination of TRAF6, but decrease the interactions of TRAF6 with viperin and IRAK1, which finally inhibited the proliferation of HSV-1. It is noteworthy that the gD and viperin interaction may help us to explore and elucidate the roles of viperin and gD during HSV-1 infection.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MC and ML designed the research. ML, ZL, ZX, XZ, YW, HP, YL, XO, YD, YG, and WG performed the research. MC, ML, and ZL analyzed the data. DC and TP consulted and advised on the research. MC, ML, and ZL wrote and review the manuscript. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02810/full#supplementary-material

Supplementary Material 1 | Original immunoblotting results of **Figure 2**.

Supplementary Material 2 | Original immunoblotting results of **Figure 4E**.

Supplementary Material 3 | Original immunoblotting results of **Figure 4G**.

Supplementary Material 4 | Original immunoblotting results of **Figure 5C**.

Supplementary Material 5 | Original immunoblotting results of **Figure 5E**.

Supplementary Material 6 | Original immunoblotting results of **Figure 6A**.

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Interferon-Independent Innate Responses to Cytomegalovirus

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The critical role of interferons (IFNs) in mediating the innate immune response to cytomegalovirus (CMV) infection is well established. However, in recent years the functional importance of the IFN-independent antiviral response has become clearer. IFN-independent, IFN regulatory factor 3 (IRF3)-dependent interferon-stimulated gene (ISG) regulation in the context of CMV infection was first documented 20 years ago. Since then several IFN-independent, IRF3-dependent ISGs have been characterized and found to be among the most influential in the innate response to CMV. These include virus inhibitory protein, endoplasmic reticulum-associated IFN-inducible (viperin), ISG15, members of the interferon inducible protein with tetratricopeptide repeats (IFIT) family, interferon-inducible transmembrane (IFITM) proteins and myxovirus resistance proteins A and B (MxA, MxB). IRF3-independent, IFN-independent activation of canonically IFN-dependent signaling pathways has also been documented, such as IFN-independent biphasic activation of signal transducer and activator of transcription 1 (STAT1) during infection of monocytes, differential roles of mitochondrial and peroxisomal mitochondrial antiviral-signaling protein (MAVS), and the ability of human CMV (HCMV) immediate early protein 1 (IE1) protein to reroute IL-6 signaling and activation of STAT1 and its associated ISGs. This review examines the role of identified IFN-independent ISGs in the antiviral response to CMV and describes pathways of IFN-independent innate immune response induction by CMV.

Keywords: interferon, cytomegalovirus, IFN-independent, ISG, herpes, innate immunity

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INTRODUCTION

HCMV has a 236 kbp double stranded DNA (dsDNA) genome, 165 genes (1) encoding up to 751 protein products (2), a 45–100% seroprevalence in the adult population (3–7), and remains a significant human pathogen particularly in those with an underdeveloped or suppressed immune system. Just as HCMV infection can profoundly alter the overall adaptive immune response (8–13), it also generates a powerful innate response. Key mediators of this innate response are IFNs. There are three types of IFN: type I (α, β, κ, ω, τ, and ε), type II (γ), and type III (λ1, λ2, λ3, λ4). Type I and II IFNs are the best characterized in the context of HCMV and their induction, antiviral roles as well as the viral antagonism of these processes have been extensively reviewed (14–19). A role for type III IFNs, in the innate response to HCMV and murine CMV (MCMV), whose pathogenesis closely parallels that of HCMV (20), has recently been elucidated (21–27).

The innate response to both HCMV and MCMV infection is initiated when virus is detected by pattern recognition receptors (PRRs) including toll-like receptors (TLRs) TLR2 (28–31) and TLR9 (32–34). Once virus has bound and entered cells, HCMV and MCMV can be detected by cytosolic DNA sensors such as IFI16 (35, 36), ZBP1/DAI (37–39) and cGAS (32, 40) that signal through the stimulator of IFN genes (STING). Each of these pathways culminates in activation and dimerization of IRF3 resulting in production of type I IFN (41–44). Type I IFN production is subsequently enhanced by upregulation of IRF7, an ISG that is also capable of dimerizing and activating the type I IFN promoter (45). HCMV and MCMV infection both trigger production of type II IFN from $CD8^+$ T cells, $CD4^+$ T cells and natural killer (NK) cells (46–48). HCMV even remodels the IFNγ locus (IFNG) for sustained IFNγ expression in $NKG2C^{hi}$ NK cells (49, 50). IFNλ production is induced by HCMV and MCMV infection (22) and these type III IFNs are themselves ISGs with production stimulated by IFNα and IFNβ treatment (51).

Key antiviral mediators of all IFN types are ISGs (52). Interferome, a database dedicated to chronicling all genes significantly regulated by IFN (changes ≥ 2-fold), identifies 12614 ISGs (53). Type I IFNs alone can trigger expression of more than 2,000 genes in humans, many of which are antiviral (54). Canonical induction of ISGs by type I, II, and III IFNs occurs by JAK/STAT signaling downstream of the type I IFN receptor (IFNAR1 + IFNAR2), the IFNγ receptor (IFNGR1 + IFNGR2) and the IFNλ receptor (IFNLR1 + IL10R2), respectively. The type I and II IFN receptors are widely expressed but type III IFN receptor expression is limited to epithelial cells (55, 56). ISGs stimulated by type I and III IFN contain an IFN stimulated response element (ISRE) in their promoter region that is bound by the activated transcription factor IFN stimulated gene factor 3 (ISGF3), comprised of phosphorylated STAT1 and STAT2 with IRF9 (55, 57–62), or by STAT2 homodimers associated with IRF9 (63–65). IFNγ induced ISG promoters contain γ-activated sequences (GAS) that are bound by STAT1 homodimers (66–70). However, upregulation of some ISG mRNAs in the early stages of HCMV infection (prior to DNA replication) are not inhibited by IFN neutralization (71, 72). Since this discovery, the body of literature demonstrating ISG induction independent of canonical IFN signaling pathways has been steadily expanding and those discussed in this review are summarized in **Figure 1**.

IFN-INDEPENDENT ISG PRODUCTION

Initial differential display analyses compared the susceptibility of genes upregulated early vs. late in infection to inhibition by IFN neutralizing antibodies and/or protein synthesis inhibitor cyclohexamide (CHX) (72). Three of these genes: IFIT2/ISG54/p54/cig42, IFIT3/ISG60/p60/cig49 and viperin/cig6, were upregulated by HCMV at 8 h post infection (hpi) and even accumulated following exposure to replication-incompetent ultraviolent-irradiated HCMV (UV-HCMV) (72). Blocking type I IFN with neutralizing antibodies failed to inhibit IFIT2, and IFIT3 induction, demonstrating that their upregulation was both IFN-independent and could be triggered by viral binding entry alone (72). A subsequent, broad mRNA analysis using oligonucleotide arrays found that levels of 258 mRNAs were altered more than 4-fold prior to initiation of HCMV DNA replication (71). IFIT2 and IFIT3 were among these quickly detected ISGs as were MxA, MxB, and ISG15 (71). The immediacy of this induction suggests a direct mechanism requiring few intermediary steps, indeed IFIT2, IFIT3, ISG15 (73) and viperin (72) upregulation can be detected 6 hpi with HCMV in the absence of *de novo* host and viral protein synthesis (cyclohexamide (CHX) treatment). This is also the case for IFIT1/ISG56/p56 (73) and indicates that this subset of ISGs may be induced/upregulated independently of IFN during HCMV infection.

IFN-Independent, IRF3-Dependent ISG Production

When searching for a mechanism underpinning IFN-independent ISG induction during CMV infection, initial studies turned to the powerful transcriptional regulator involved in IFN production, IRF3. Expression of constitutively active IRF3 in the absence of any viral stimulus could induce transcription of a subset of ISGs including IFIT1, IFIT2, IFIT3, ISG15, and viperin (74). IRF3-independent expression of these same ISGs was also observed during infection with other viruses: single stranded RNA (ssRNA) Newcastle disease virus (NDV) upregulated IFIT1, IFIT2 and ISG15 in cells that could respond to but were unable to produce type I IFN (75) and IFIT1 expression could be induced during ssRNA Sendai virus (SeV) infection by IRF3 nuclear translocation in cells unable to respond to type I IFN (76).

Studies using herpes simplex virus type 1 (HSV-1) demonstrated that IFIT1 expression could be driven by infection even in the presence of CHX in human fibroblasts (HFs) but could not be detected in the human epithelial osteosarcoma cell line U2OS (77). U2OS cells can respond to IFN but have defects in the STING signaling pathway (78) involved in IRF3 activation and dimerization in response to DNA sensing by IFI16, ZBP1/DAI, and cGAS (79–82). Furthermore, HSV-1 infection of $IRF3^{-/-}$, $IRF3^{-/-}IRF9^{-/-}$, and $IRF1^{-/-}$ murine fibroblasts revealed that IRF3 was essential for generation of an antiviral state and IFIT2 expression in response to UV-HSV-1 (83). In the case of IFIT1, expression was directly induced by an IRF3-containing complex binding to its promoter region (77, 84).

In the context of HCMV infection, initiation of IFIT2 transcription was found to occur independently of STAT1 nuclear localization (85) and in the presence of CHX (86). Soon it emerged that expression of IFIT1, IFIT2, IFIT3 and ISG15 during HCMV could be IFN-independent but always required IRF3 activation (42, 73, 87). Subsequent studies revealed that viperin expression could be driven directly by HCMV glycoprotein B (gB), in an IFN-independent, IRF3/IRF1 dependent manner (88, 89). This aligns with data demonstrating that IRF3 translocation to the nucleus is a requirement for the IFN-independent induction of an antiviral state in response to UV-HCMV (87). In contrast, another transcription factor implicated in type I IFN production NFκB (90), remains cytosolic (91).

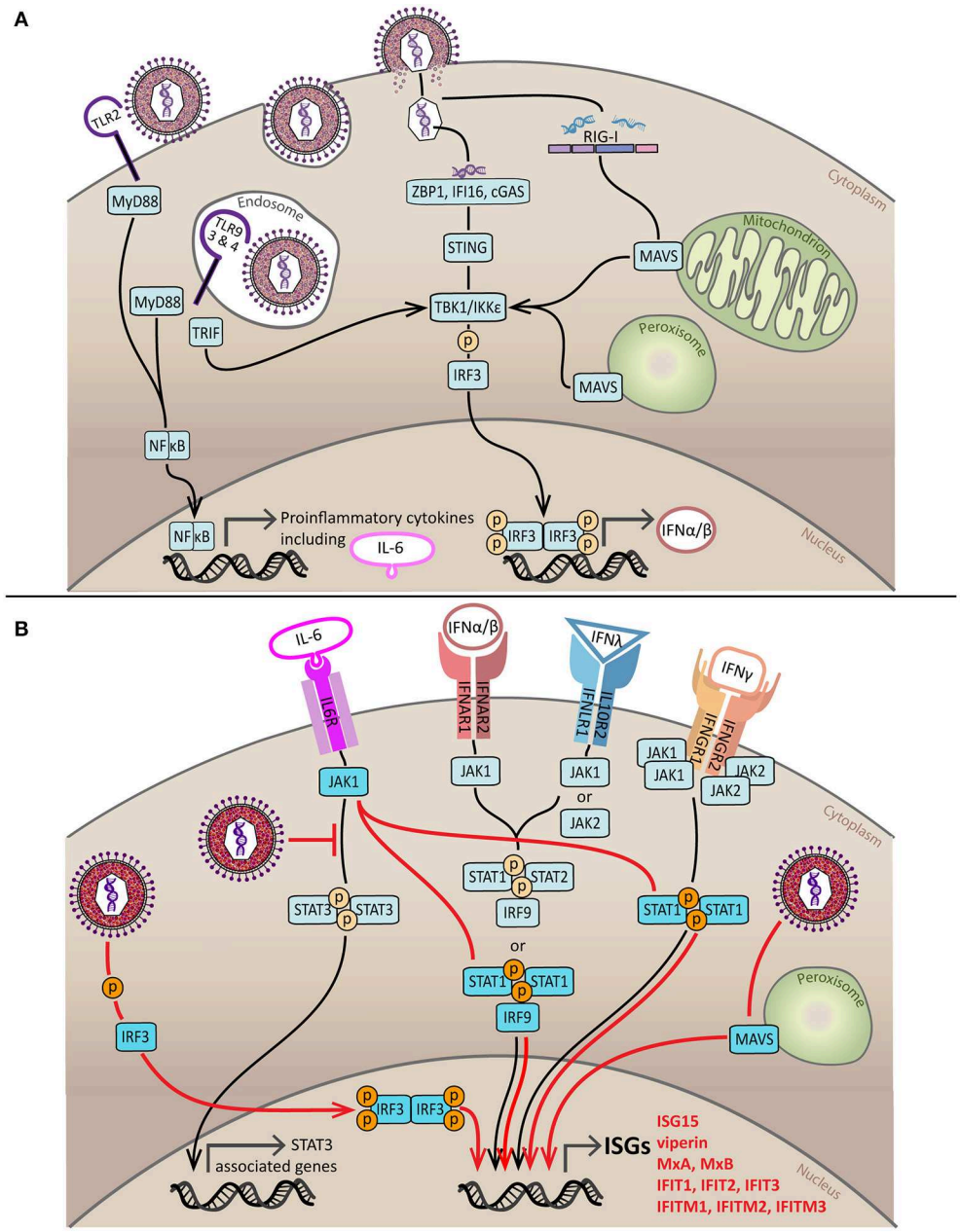

FIGURE 1 | Induction and subversion of the innate IFN response by HCMV. **(A)** Sensing of HCMV by components of the innate immune response initiates production of IFNs and proinflammatory cytokines. HCMV is sensed by PRRs on the cell surface (TLR2) and in endosomes (TLR3, TLR4, and TLR9). Signaling from TLR2, TLR3, and TLR4 is through MyD88 and results in the activation and nuclear translocation NFκB, a transcription factor that stimulates expression of proinflammatory cytokines such as TNF, IL-8, IL-12, and IL-6. TLR9 and TLR4 signal through TRIF which causes activation by phosphorylation of IRF3 via TBK1/IKKε, activated IRF3 dimerizes and enters the nucleus to stimulate production of type I IFNs. HCMV infection can also be recognized by viral nucleic acid detectors in the cytoplasm; DNA sensors ZBP1, IFI16 and cGAS signal through ER-resident STING to activate TBK1/IKKε whilst the viral RNA sensor RIG-I activates TBK1/IKKε by signaling via MAVS located on the mitochondria or peroxisomes. The end result of both of these pathways is IRF3 phosphorylation, dimerization, nuclear translocation and production of type I IFNs. **(B)** IFN-dependent and IFN-independent pathways of ISG induction during HCMV infection. For IFN-dependent induction of ISGs to occur type I, type II and type III IFNs must bind to their cell surface receptors. Type I and III IFN receptors signal through various combinations of JAK proteins to phosphorylate STAT1 or STAT1 and STAT2 which form a complex referred to as ISGF3 with IRF9. ISGF3 then translocates to the nucleus where it binds to the ISRE to induce ISG production. The type II IFN receptor utilizes both JAK1 and JAK2 to phosphorylate STAT1, leading to its dimerization and nuclear translocation. Once in the nucleus, activated STAT1 dimers bind to GAS and stimulate ISG production. The three key pathways of HCMV-mediated IFN-independent ISG induction are indicated in red. Firstly, HCMV can directly activate IRF3; additionally, HCMV can sequester STAT3 and redirect the activated JAK1, created by IL-6 receptor binding, to phosphorylate STAT1; and finally peroxisomal MAVS may be able to trigger IFN-independent ISG expression at early times following infection. Black line = canonical IFN-dependent ISG induction pathway, red line = HCMV-induced, IFN-independent ISG induction pathway.

To interrogate the IFN-independent, IRF3-dependent response to HCMV HFs have been engineered (92, 93) to lack either IRF3 through expression of the nPro protein of bovine viral diarrhea virus (BVDV) (nPro/HFs) which binds and degrades IRF3 (94) or STAT1, by expression of the parainfluenza virus type 5 (PIV-5) V protein (V/HFs) which targets STAT1 for proteasomal degradation (95). These nPro/HFs and V/HFs were recently utilized, alongside IRF3 KO CRISPR/Cas9 HFs, to demonstrate that expression of viperin, ISG15, IFIT1, IFIT2, IFIT3, Mx1, and Mx2 mRNA during infection with HCMV can be induced in an IRF3-dependent, STAT1-independent manner (96). In fact, mRNA levels of IFIT1, IFIT2, and IFIT3 were as highly elevated in the absence of STAT1-mediated IFNAR signaling as in the parental HFs (96) underlining the capacity of such IFN-independent mechanisms to profoundly regulate ISG expression. Many of these IFN-independent, IRF3-dependent ISGs are among the most potently induced by CMV infection and examining the roles these genes play in the innate response to CMV is essential to understanding the ramifications of this non-canonical regulation.

Viperin

Viperin inhibits the egress and replication of many viruses (97–102). However, in the context of HCMV, viperin upregulation is proviral, initiated by infection to manipulate cellular metabolism and cause the accumulation of cytosolic lipids for use in production of the viral envelope (103). In trophoblasts, a cell type of particular clinical relevance due to their role in the transmission of congenital HCMV (104–106), viperin is required for efficient expression of immediate early viral genes (107). Viperin is also known to enhance type I IFN production in plasmacytoid dendritic cells (pDCs) by localizing to lipid rafts and acting as a scaffold for recruitment of interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor associated factor 6 (TRAF6) (108).

In addition, viperin has been identified to act in its capacity as a member of the radical *S*-adenosyl-L-methionine (SAM) superfamily of enzymes to facilitate conversion of cytidine triphosphate (CTP) to 3′-deoxy-3′,4′-didehydro-CTP (ddhCTP) (109). Thus far, ddhCTP is known to act as a terminator of RNA synthesis by viral (Dengue and Zika) RNA-dependent RNA polymerases (109) and so investigations into its interaction with the HCMV encoded viral DNA polymerase are warranted. The viperin gene (RSAD2) lies in close proximity to the gene encoding cytidylate monophosphate kinase 2 (CMPK2) in the genome, suggesting a potential functional link to this pathway (109). Expression of CMPK2 is so closely linked to viperin that, following stimulation by IFN, viperin, CMPK2 and a long non-coding RNA (lncRNA) called lncRNA-CMPK2 are all co-transcribed (110). Interestingly, lncRNA-CMPK2 acts as a negative regulator of ISG expression (including ISG15, IFIT3 and IFITM1) (110). If IFN-independent, CMV-induced viperin upregulation also enhances expression of lncRNA-CMPK2, this could be a novel mechanism utilized by the virus to dampen the antiviral ISG response.

Furthermore, viperin has been demonstrated to be important for replication of Kaposi's sarcoma-associated herpesvirus (KSHV), a function attributed to the ability of viperin to catalyze oxidation of methionine in the viral DNA helicase, enhancing its expression and function (111). In this context, IFN-independent viperin upregulation by HCMV may be a way to ensure viral replication proceeds with maximum efficiency and thus the potential of viperin to modify the HCMV viral helicase-primase complex should be considered for further study. Overall, IFN-independent upregulation of viperin by HCMV seems to be a process initiated by the virus very early in infection to prepare the cell for its role as a virus-producing factory.

ISG15

ISG15 is a small ubiquitin-like protein that exists in three forms: (1) unconjugated within the cell, (2) conjugated within the cell (112, 113), and (3) secreted into serum (mainly by granulocytes) where it promotes NK maturation and IFNγ production (114). During HCMV infection accumulation of both free and conjugated ISG15 can be partially inhibited by interfering with the canonical IFNAR signaling pathway with a JAK inhibitor (115) but some IFN-independent, IRF3-dependent expression remains (96). Whilst the mechanisms by which ISG15 regulates CMV infection are currently unknown, it appears to possess antiviral activity as blocking ISG15 accumulation enhances viral replication (115) and HCMV antagonizes both the production of unconjugated ISG15 and ISGylation (115–118).

On the other hand, it is interesting to note that whilst in murine studies ISG15$^{-/-}$ mice are generally more sensitive to disseminated viral infections (119) human patients presenting with primary immunodeficiencies associated with defects in ISG15 expression are not (120). In fact, ISG15$^{-/-}$ fibroblasts isolated from such patients and primed with type I IFN were less susceptible to infection with HCMV than controls. This was attributed to the elevated levels of antiviral ISGs in these cells, a result of ISG15s ability to bind and stabilize the E3 ubiquitin ligase-like protein USP18, which acts as a negative regulator of the type I IFN response (120, 121).

It is also possible that HCMV manipulates levels of ISG15 to shift monocytes toward the mixed M1/M2 macrophage phenotype that is observed during infection (122) and hypothesized to enhance viral dissemination and persistence (123, 124). This is because in the absence of infection, ISG15 plays a role in the maintenance of mitochondrial homeostasis (125). Specifically, ISGylation of mitochondrial components can control mitochondrial function: reducing the rate of oxidative phosphorylation (OXPHOS) and causing a corresponding decrease in mitochondrial reactive oxygen species (ROS) (126). A reduction in levels of mitochondrial ROS alters macrophage polarization, shifting these cells toward a mixed M1/M2 phenotype (126).

IFITs

IFITs are ISGs with antiviral capabilities against flaviviruses, poxviruses, coronaviruses and papillomaviruses (127–130). A pan-viral mechanism of host defense mediated by IFITs is the sequestration of eukaryotic initiation factor (eIF3) by IFIT1 which slows the overall rate of cellular protein synthesis (76, 84, 131). A more specific strategy depends on the recognition

and binding of viral RNA lacking 2′-O methylation of the 5′ RNA cap by IFIT1 (132). This binding ability is enhanced by association with IFIT2 and IFIT3 (133). Despite the fact that CMV replication takes place wholly within the nucleus, export of viral mRNAs does occur (134) and these may be sensed by IFITs. Another possibility is that IFITs may directly bind essential CMV proteins in the cytoplasm, as IFIT1 does to inhibit human papilloma virus (HPV) infection (135, 136). Although the mechanisms of the IFIT-mediated antiviral response to HCMV are still unclear, a significant reduction in titer has been reported when the virus is grown in IFIT1 overexpressing fetal astrocytes (137).

IFITMs

IFITM proteins are also implicated in the antiviral response against a wide range of viruses: orthomyxoviruses, flaviviruses, filoviruses, and coronaviruses often by blocking membrane fusion (127, 138–140). However, overexpression of IFITM1, IFITM2 and IFITM3 does not inhibit HCMV infection but rather results in a modest increase in the percentage of infected cells (141, 142). Short hairpin RNA (shRNA) knockdown of IFITM1 alone or in combination with IFITM2 and IFITM3 inhibits HCMV infection as they are required for successful formation of the HCMV virion assembly complex (vAC) and production of infectious progeny virions (142). It is interesting to note that despite this proviral role, IFITM proteins are noticeably downregulated at later stages of infection (48–72 hpi) (142).

Direct induction of IFITMs by HCMV may also contribute to the severe consequences of congenital infection as IFITM expression can inhibit the fusion of cytotrophoblast cells into the multinucleated syncytiotrophoblast, a structure at the interface between maternal and fetal tissue, essential for placental development (143).

MxA and MxB

The Mx proteins MxA and MxB are a family of dynamin-like GTPases first reported for their antiviral activity against influenza and are now well characterized in response to other viruses (144, 145). MxA is found in the cytosol and inhibits influenza virus infection through retention of the viral genome (146). On the other hand, MxB localizes to the cytoplasmic face of nuclear pores (147) and is able to inhibit HIV-1 replication by blocking nuclear viral genome accumulation (148, 149). Both MxA and MxB are highly upregulated by HCMV infection (73, 150) and it has recently been discovered that MxB overexpression inhibits replication of HSV-1, HSV-2, Kaposi's sarcoma-associated herpesvirus (KSHV), MCMV, and HCMV (151, 152). HSV-1 and MCMV inhibition manifested in a similar way to that of HIV-1, a block in the delivery of viral genome to the nucleus (151). However, in terms of the regions of protein at play, this mechanism was found to differ substantially with a requirement for GTP binding but not GTP hydrolysis (152, 153). Knockdown of MxB has also been implicated in stalling cell cycle progression (147) and it has been suggested that the HCMV virion protein pUL69 that contributes to the cell cycle arrest (154) does so via an interaction with MxB (155).

ALTERNATE IFN-INDEPENDENT PATHWAYS OF INNATE RESPONSE INDUCTION

Direct ISG induction by IRF3 is not the only pathway associated with the IFN-independent response to CMV. In human monocytes, IFN-independent, biphasic activation of STAT1 with differential phosphorylation at early (30 min) compared to late (24 h) time points post-HCMV infection appears to influence motility, migration, differentiation and polarization (156).

Regulation of mitochondrial activity is emerging as another IFN-independent innate response mediator. A number of years ago it was discovered that HCMV DNA could induce ISG expression in an IRF3-dependent, TLR-independent manner that involved TANK-binding kinase 1 (TBK1), IκB kinase epsilon [IKKε; originally called IKK-inducible (IKKi)], and mitochondrial antiviral-signaling protein (MAVS) (157). More recently, peroxisomal MAVS has been implicated in rapid type-I IFN-independent ISG (viperin, Mx2, IFIT3, IFIT2) expression (158). Conversely, mitochondrial MAVS appears to be involved in IFN-dependent ISG production (158). HCMV actively impairs mitochondrial MAVS signaling through the viral mitochondria-localized inhibitor of apoptosis (vMIA) and reduces type I IFN production (159). vMIA has also been found to localize to peroxisomes and induce their fragmentation by interaction with the cytoplasmic chaperone protein Pex19, hijacking the transport machinery of peroxisomal membrane proteins (160). This suggests that disabling IFN-independent ISG transcription induced by peroxisomal MAVS contributes to efficient CMV infection.

The HCMV immediate early gene 1 (IE1) is also capable of inducing expression of ISGs in the absence of IFN production. HCMV IE1 induces expression of IL-6 (161) which usually signals through JAK and STAT3 (162). However, IE1 binds and sequesters STAT3 (163), leaving JAK, already activated by IL-6, free to phosphorylate STAT1. Thus IE1 re-routes IL-6 signaling to activate STAT1 resulting in transcription of ISGs independently of IFN (164).

FUNCTIONAL IMPORTANCE OF IFN-INDEPENDENT INNATE RESPONSES

Early studies examining IFN-independent induction of an antiviral state showed that treatment of human embryonic lung fibroblasts (HELFs) with UV-HCMV rendered these cells resistant to subsequent viral infection in the absence of detectable IFN production (91). Intriguingly, whilst high multiplicity of infection (MOI) UV-HCMV also induced an antiviral state in the HELFs, this required IFN production (91). Paladino et al. (91) proposed a model by which, when cells are exposed to limited numbers of virus particles (low MOI), induction of an internal antiviral state is sufficient to control infection, however, when many virus particles are present (high MOI), cells secrete IFN to protect neighboring

cells too. The ability to induce an antiviral state in the absence of IFN production may be important in cells such as neurons, where inflammation is undesirable. In this respect, neurotropic arboviruses have been shown to induce protective type I IFN-independent, IRF3-dependent responses (165).

Recently, the power of IFN-independent innate responses to CMV has been illustrated by the finding that human macrophages co-cultured with HCMV-infected retinal pigment epithelial cells (RPEs) can limit viral replication and spread in a cell-cell contact dependent manner that could not be blocked by vaccinia-derived type I IFN binding protein B18R, nor by neutralizing antibodies against either IFNγ or TNFα (166). It has also been shown that HCMV virus particles pre-treated with HCMV-specific antibodies that do not replicate, nor express IE antigens, can enter human macrophages and induce an antiviral state that renders these cells less susceptible to subsequent HCMV infection independently of IFN production (167).

IFN-independent ISG induction can also be used to regulate the development of cells key to viral persistence and dissemination. In human monocytes infected with HCMV, ISGs are upregulated independently of IFN (4 hpi) that function to enhance monocyte motility and migration (156). This occurs in a STAT1-dependent manner that also suppresses transcription of anti-inflammatory M2-associated cytokines (IL-10 and CCL18), promoting polarization of macrophages toward a mixed M1/M2 phenotype (156). ISG15 was among the ISGs found to be upregulated in monocytes 4hpi with HCMV (156). ISG15 may contribute both directly and indirectly to the mixed M1/M2 macrophage phenotype, causing monocyte-specific upregulation of IL-10 (168) whilst simultaneously inducing production of M1 macrophage-stimulating cytokine IFNγ by NK and T cells (114).

CONCLUDING REMARKS

When considering the innate response to CMV infection, IFN and the ISG-mediated induction of an antiviral state are important first elements. The intention of this review has been to highlight the substantial body of literature accumulating around IFN-independent innate responses to CMV. IFN-independent induction of ISGs is an important phenomenon and ISGs produced via this pathway appear to play both pro- and anti-viral roles during infection. This complicates direct interrogation of the IFN response during viral infection and necessitates careful consideration of kinetics, as particular ISG may be upregulated directly by the virus, independently of IFN, to play a proviral role early in infection but later on, when IFN-dependent expression dominates, may antagonize infection.

When examining plaque number and size at 7 days post infection, we reported no difference in rates of CMV replication nor spread between cells unable to produce IFN (IRF3 degraded) and those that could not respond to IFN (lacking STAT1) (92), even though the latter cells still allowed viral induction of IFN-independent ISGs (96). Focusing on earlier time points, before loss of IFN production/signaling becomes the overwhelming factor affecting infection efficiency, may reveal more subtle differences conferred by abrogation of either IRF3 or STAT1 signaling.

A deeper understanding of the various functions of IFN-independent ISGs may enable their relative abundance to serve as a predictor of disease progression. For example, high levels of IL-6 have been correlated with CMV reactivation and poor prognosis for transplant patients (169–171); perhaps this is because ISGs produced by IE1 re-routing the IL-6 response to enhance infection. If this were the case, interference with STAT1 homodimer-mediated ISG expression may improve prognosis.

Since CMV infected, polarized macrophages are key mediators of T cell activation and proliferation (172), if IFN-independent ISGylation influences macrophage polarization then levels of ISG15 induced directly by CMV early in infection may provide an indication as to whether or not a robust T cell response will be generated.

It is also important to note that many of these ISGs, including viperin, IFIT2, IFIT3, Mx1 and ISG15 are defined as part of the 28 core mammalian ISGs i.e., produced in all nine mammalian species tested (54). It would therefore be prudent to determine whether their IFN independence is also conserved across species especially since rhesus CMV does not induce IRF3 activation nor the associated ISG expression (173).

Finally, with the IFN-independent nature of these ISGs becoming clear, caution should be exercised when using these ISGs as surrogate readouts for interferon signaling, as it is clear that they are also induced directly by viral infection.

AUTHOR CONTRIBUTIONS

CA generated the initial draft of the manuscript. All other authors (BM, AA and BS) contributed to the subsequent writing and review of the manuscript.

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BICP0 Negatively Regulates TRAF6-Mediated NF-κB and Interferon Activation by Promoting K48-Linked Polyubiquitination of TRAF6

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The infected cell protein 0 (BICP0) is an immediate early protein encoded by BHV-1, and its RING finger domain, which endows BICP0 with intrinsic E3 ubiquitin ligase activity, is common in all ICP0 proteins. Tumor necrosis factor receptor-associated factor 6 (TRAF6) is one of the TRAF family members and is ubiquitously expressed in mammalian tissues. TRAF6 forms the MyD88-TRAF6-IRF7 complex and activates interferon induction in the TLR (Toll-like receptors) and the RLR (RIG-I-like receptor) pathway. Previous studies showed that BICP0 reduced IFN-β promoter activity by interacting with IRF7. In this study, we found that BICP0 promoted the K48-ubiquitination and degradation of TRAF6 through the ubiquitin proteasome system. The interaction between BICP0 and TRAF6 is a prerequisite for ubiquitination modification, and the 346-PAERQY-351 of BICP0 is indispensable. The motif mutation experiments showed that the tyrosine 351 of BICP0 is the key amino acid involved. Further studies demonstrated that BICP0 suppressed the NF-κB pathway via the interference of TRAF6. Moreover, degradation of TRAF6 protein influenced the K63-linked ubiquitination of IRF7 and activation of interferon promoter. Collectively, these findings indicate that the BICP0 protein suppresses the inflammation signaling and IFN production by K48-linked polyubiquitination of TRAF6 and may further clarify the immune evasion function of BICP0.

Keywords: BICP0, TRAF6, interferon, NF-κB, motif, ubiquitin

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INTRODUCTION

Bovine herpesvirus 1 (BHV-1) is an enveloped virus belonging to the *alphaherpesvirus* subfamily, and is a significant bovine pathogen that leads to abortions, genital disorders, pneumonia, conjunctivitis, and "shipping fever," which is an upper respiratory infection (Tikoo et al., 1995). Immunosuppression caused by BHV-1 infection triggers bovine respiratory disease complex (BRDC). As a poly-microbial disease caused by viral infection and stress, BRDC causes significant economic losses to the global cattle industry (Muylkens et al., 2007). The Infected Cell Protein 0 encoded by bovine herpesvirus-1 (BICP0) is important for the regulation of lytic and latent viral

infections (Saira et al., 2008). Like the related proteins expressed by other *alphaherpesvirus* that infect mammalian species, BICP0 has a C3HC4 zinc RING finger domain in the amino-terminus, which is crucial for activating viral transcription and productive infection (Parkinson and Everett, 2000; Saira et al., 2008; Boutell and Everett, 2013). Aside from being one of the important virulence proteins of BHV-1, BICP0 also has an immunosuppressive function. The RING finger domain of BICP0 is essential for E3 ubiquitin ligase activity and leads to the ubiquitination and the subsequent degradation of a number of immune defense proteins. For example, BICP0 can directly catalyze IκBα ubiquitination (Diao et al., 2005). BICP0 also causes a decrease in IRF3 protein levels via the ubiquitin-dependent proteolysis pathway (Saira et al., 2007). PML-NB (promyelocytic leukemia protein-containing nuclear body) is a specific anti-viral organelle which regulates apoptosis and innate immune responses (Scherer and Stamminger, 2016). Many DNA viruses can recombine or split PML-NB, thereby increasing the copy number of the virus. Studies have shown that BICP0 co-localizes with and disrupts PML-NB (Parkinson and Everett, 2000; Inman et al., 2001). On the other hand, it was observed that BICP0 mediates the co-localization of IRF7 with nuclear structures that may be PML-NB in transfected cells, and that the interaction between BICP0 and IRF7 impairs activation of IFN-β promoter activity but does not change IRF7 protein levels (Saira et al., 2009). BICP0 thus reduces the ability of the IFN-β promoter in a manner correlated with IRF3 degradation, IRF7 interaction, and PML-NB dissolution, which has become a strategy used to destroy inherent innate antiviral defenses (Gaudreault and Jones, 2011).

Tumor necrosis factor receptor-associated factor 6 (TRAF6) is one of the TRAF family members, and is one of the most extensively investigated proteins in inflammatory responses (Lalani et al., 2018). TRAF6 is widespread in mammalian tissues and is conserved among species, and it consists of a RING finger domain in the N-terminal, followed by five Zn finger domains, and a C-terminal TRAF domain (containing a coiled-coil TRAF-N domain and a TRAF-C domain) (Cao et al., 1996; Ishida et al., 1996). The RING finger domain of TRAF6 possesses E3 ubiquitin ligase activity, which is essential for TRAF6 in the NF-κB activation downstream of TLRs (Toll-like receptors) (Akira and Takeda, 2004). TRAF6 forms an ubiquitin-binding enzyme complex with Ubc13 (Ubiquitin-conjugating enzyme 13) and Uev1A (ubiquitin-conjugating enzyme E2 variant 1) to promote the synthesis of lysine 63 (K63)–linked polyubiquitin chains (Deng et al., 2000). This K63-linked ubiquitination not only regulates protein functions and the interaction among proteins but also upregulates autophagic degradation. In general, K63-linked ubiquitination mediated by TRAF6 triggers signal transduction through the activation of downstream proteins (Sun and Chen, 2004). The protein kinase TAK1 (TGF beta-Activated Kinase 1) has been identified as one of the targets of TRAF6 and activated TAK1, which then triggers activation of canonical NF-κB by phosphorylating the IκB kinase complex (IKKα, IKKβ, and IKKγ) (Wang et al., 2001; Akira and Takeda, 2004). On the other hand, phosphorylation of MKK6 by TAK1 leads to activation of the JNK-p38 kinase pathway (Wang et al., 2001). TRAF6 also participates in autophagy stimulation by mediating Lys63-linked polyubiquitination of ULK1 (Nazio et al., 2013) and BECLIN-1 (Shi and Kehrl, 2010). Non-degradative ubiquitination by TRAF6 stimulates ULK1 self-association, which is a prerequisite for its kinase activity. Although TRAF6 has a well-established role in the regulation of both TAK1 and JNK signaling (Sakurai, 2012), the question of whether TRAF6 also controls autophagy through these kinases remains largely unexplored (Antonioli et al., 2017). In addition, TRAF6 may direct the activation of phosphoinositide 3-kinase (PI3K) when it binds to the TNFR superfamily, including TRANCE-R (also called RANK) and CD40, which regulate dendritic cell and osteoclast function (Wong et al., 1999; Arron et al., 2001). TRAF6 has also been shown to form the MyD88-TRAF6-IRF7 complex and activate interferon induction in the TLRs/IL-1 pathway (Honda et al., 2004; Kawai et al., 2004; Takaoka et al., 2005) and in the RLR (RIG-I-like receptor) pathway (Konno et al., 2009).

In this study, we demonstrated that BICP0 promotes the K48-linked ubiquitination of TRAF6, which then leads to the TRAF6 degradation by the ubiquitin proteasome system (UPS). The interaction between BICP0 and TRAF6 requires the involvement of a conservative motif, 346-PAERQY-351, of BICP0. By generating amino acid mutants, we found that the tyrosine 351 in the motif of BICP0 is the key amino acid involved. Further research showed that the degradation of TRAF6 mediated by BICP0 inhibited the functioning of TRAF6 on the NF-κB pathway. Moreover, the activation of the interferon pathway by IRF7 and TRAF6 is also affected by BICP0. Taken together, our study may provide new insights for the complex mechanism by which BICP0 regulates the innate antiviral immune response.

MATERIALS AND METHODS

VSV, Baculovirus, and Plasmids

The vesicular stomatitis virus (VSV) was stored in −80°C prior to use. The recombinant baculovirus (RE-BICP0-FLAG), which carries the Flag-tagged BICP0 gene, was constructed by our laboratory (data not shown). The baculovirus strain was propagated and titrated in insect cells determined by 50% tissue culture infective doses ($TCID_{50}$) as described previously (Shao et al., 2015). The plasmid pcDNA3.1-BICP0-Flag expresses Flag-tagged wild type BICP0 (wt BICP0) under the control of the human cytomegalovirus (CMV) promoter. The mutant BICP0-Flag (13A/51A) contains site mutations within two conserved cysteine residues of the RING finger of Flag-tagged wt BICP0. The Myc-tagged N-terminal truncation mutants (ΔBICP0-Myc) were generated by standard PCR. To generate the motif mutants BICP0-P346A (pcDNA3.1-BICP0-Flag-P346A), BICP0-E348A (pcDNA3.1-BICP0-Flag-E348A), BICP0-Y351A (pcDNA3.1-BICP0-Flag-Y351A), ΔBICP0-Y351A (pcDNA3.1-ΔBICP0-Myc-Y351A), and BICP0-123 (pcDNA3.1-BICP0-Flag-123), alanine was substituted for the corresponding amino acids by PCR (**Figure 1**). Bovine IRF7 and TRAF6 genes were amplified from bovine cDNA and inserted into pcDNA3.1 (+) expression vectors; the recombinant plasmids are named

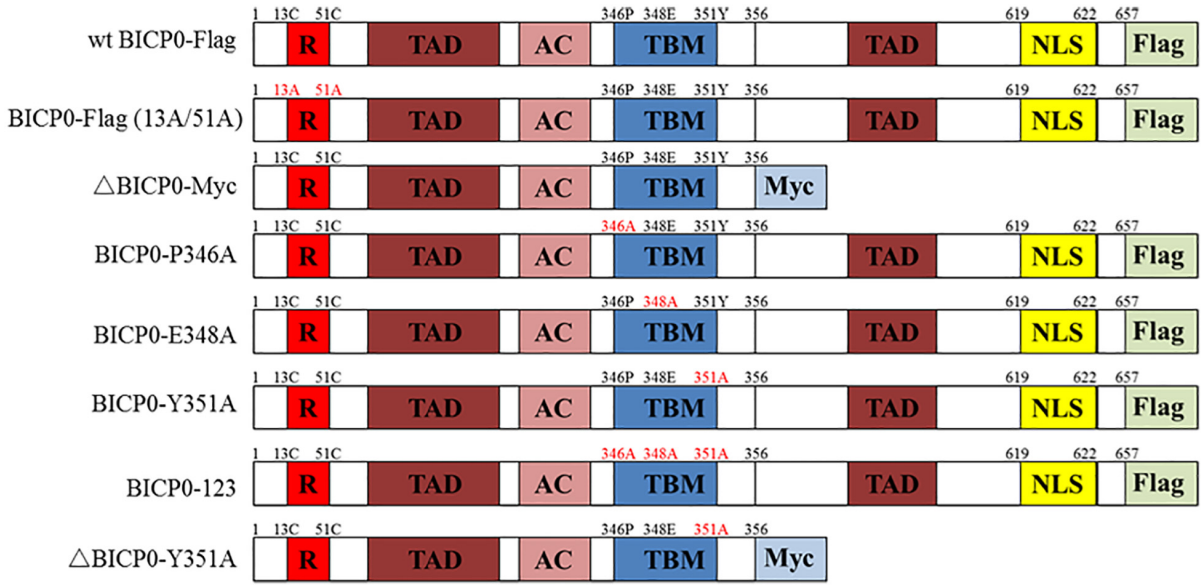

FIGURE 1 | Schematic illustration of BICP0 and its mutants. R, zinc RING finger; TAD, transcriptional-activation domains; AC, acidic domain; TBM, TRAF6-binding motif; NLS, nuclear localization sequence; Flag, Flag-Tag sequence; Myc, Myc-Tag sequence.

pcDNA3.1-IRF7-HA, pcDNA3.1-TRAF6-HA, and pcDNA3.1-TRAF6-Flag, respectively. The plasmid pGL-3κB-luc (NF-κB-luc) was purchased form Promega. The IFN-β promoter construct (IFN-β-luc) was constructed by inserting the promoter region of IFN-β into the appropriate sites of the pGL3 vector.

Cell Lines and Reagents

Madin Darby bovine kidney (MDBK) cells, HEK293T cells, and HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and penicillin (100 U/ml)-streptomycin (0.1 mg/ml) at 37°C in a 5% CO_2 incubator. The antibodies used in this study were obtained from the following manufacturers: rabbit polyclonal antibodies to anti-Flag (GTX115043), anti-HA (GTX115044), anti-Myc (GTX115046), and anti-GAPDH (GTX100118) were purchased from GeneTex, Inc. (United States). Antibody against TRAF6 (A5724) and Anti-Flag magnetic beads (B26102) were purchased from Bimake. Anti-HA magnetic beads (HY-K0201), MG132, and chloroquine were purchased from MedChemExpress (MCE). The rabbit monoclonal antibodies against Ubiquitin (ab134953), K48-Ubiquitin (ab140601), and K63-Ubiquitin (ab179434) were purchased from Abcam. Horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from ZSGB-BIO. The dual-luciferase reporter assay system was obtained from Promega. Sodium butyrate and Lipofectamine 2000 were purchased from Thermo Fisher Scientific, Inc.

Transduction and Transfection

Madin Darby bovine kidney cells (~2 × 10^6) were seeded in 6-well plates 24 h before transduction with either the RE-BICP0-FLAG or an empty control baculovirus. The complete DMEM media contains 3 mM sodium butyrate, which enhances protein expression and transduction efficiency of the virus. After 24 h stimulation, MDBK cells were washed three times in ice-cold Tris-buffered saline (TBS), lysed, and subjected to western blot analysis as described below. For transfection, HEK293T cells (~0.5 × 10^5 and ~1 × 10^7) were seeded in 24-well plates and in 100 mm dishes, respectively. HeLa cells (~2 × 10^6) were seeded in glass-bottom dishes. Lipofectamine 2000 was used according to the manufacturer's instructions.

Western Blot Analysis and Immunoprecipitation

For western blot analysis, cells were lysed with RIPA buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. Lysates were incubated on ice for 30 min and clarified by centrifugation at 10,000 *g* at 4°C for 15 min. Protein concentrations were quantified using the BCA assay. For SDS-PAGE, proteins were mixed with 5 × sample loading buffer and boiled for 5 min. Proteins were separated in a 5–10% polyacrylamide gel and transferred onto a nitrocellulose membrane. Membranes were blocked at room temperature for 1 h in TBST (TBS-containing 0.05% Tween 20) that contained 5% milk. Membranes were then incubated overnight with the indicated primary antibody in TBST at 4°C. Afterward, the membrane was incubated in goat anti-rabbit immunoglobulin G (IgG)-HRP-conjugated secondary antibody for another 1 h at room temperature. At the end of each incubation, membranes were washed three times for 5 min each. Immunodetection was performed using enhanced chemiluminescence Western blotting detection reagents in accordance with the manufacturer's protocol.

For immunoprecipitation assays, HEK293T cell lysates were incubated with Anti-HA/Anti-Flag magnetic beads for 2 h at room temperature according to the manufacturer's instructions. After extensive washing, immunoprecipitated proteins were resolved using 5–10% SDS-PAGE and analyzed using western blotting using the indicated antibodies. Experiments were repeated at least three times and were observed to produce similar results.

Confocal Imaging

The HeLa cells were transfected with the pcDNA3.1-BICP0-Flag, pcDNA3.1-BICP0-Flag-Y351A, or pcDNA3.1-TRAF6-HA (1 g each) for 36 h. Afterward, the cells were stained with the indicated antibodies and the images were acquired using a ZEISS confocal laser scanning system (ZEISS LSM800).

Luciferase Assay

For the luciferase assay, HEK293T cells were transfected using the indicated plasmids. To normalize for transfection efficiency, 50 ng of pRL-TK *Renilla* luciferase plasmid was added to each transfection. At 36 h post-transfection, the HEK293T cells were harvested and whole cell extracts were prepared for the luciferase assay. Luciferase activity was measured using the Luciferase Assay System (Promega) with a GloMax^{TM} 20/20 Luminometer (Promega) and normalized relative to *Renilla* luciferase activities. Data were obtained from three independent transfections and are presented as the fold increase in luciferase activity (means ± SD) relative to the control.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, United States[1]). Data are presented as mean ± SD. One-way analysis of variance (ANOVA) and Student's *t*-tests were performed. *P*-values < 0.05 were considered statistically significant.

RESULTS

BICP0 Reduces TRAF6 Protein Levels

To determine the effect of BICP0 on innate immunity in the absence of other viral proteins, MDBK cells were transduced with different doses of RE-BICP0-FLAG, or with an empty control baculovirus. The results indicate that the presence of TRAF6 protein is significantly reduced in RE-BICP0-FLAG infected MDBK cells in a dose-dependent manner, and that there is no reduction in TRAF6 protein in the control baculovirus-infected MDBK cells (**Figure 2A**). It is known that the UPS and the autophagic lysosomal pathway (ALP) are the two major pathways for protein degradation; as such, we aimed to investigate if BICP0 causes the decrease of TRAF6 through the proteasome pathway or through lysosomal proteolysis. Consequently, we treated MDBK cells with the proteasome inhibitor MG132 or with the lysosome inhibitor chloroquine. The results showed that BICP0-induced TRAF6 degradation was rescued by MG132 but not by chloroquine, and that the decrease of TRAF6 was inhibited by MG132 in a dose-dependent manner (**Figure 2B**). Interestingly, the amount of BICP0 protein also increased with increasing dose of MG132 (**Figure 2B**). To test whether BICP0 can cause the decrease of TRAF6 protein in other cells, pcDNA3.1-BICP0-Flag and pcDNA3.1-TRAF6-HA were co-transfected into HEK293T cells (**Figure 2C**). Western blot results demonstrated that the overexpression of BICP0-Flag reduced TRAF6-HA

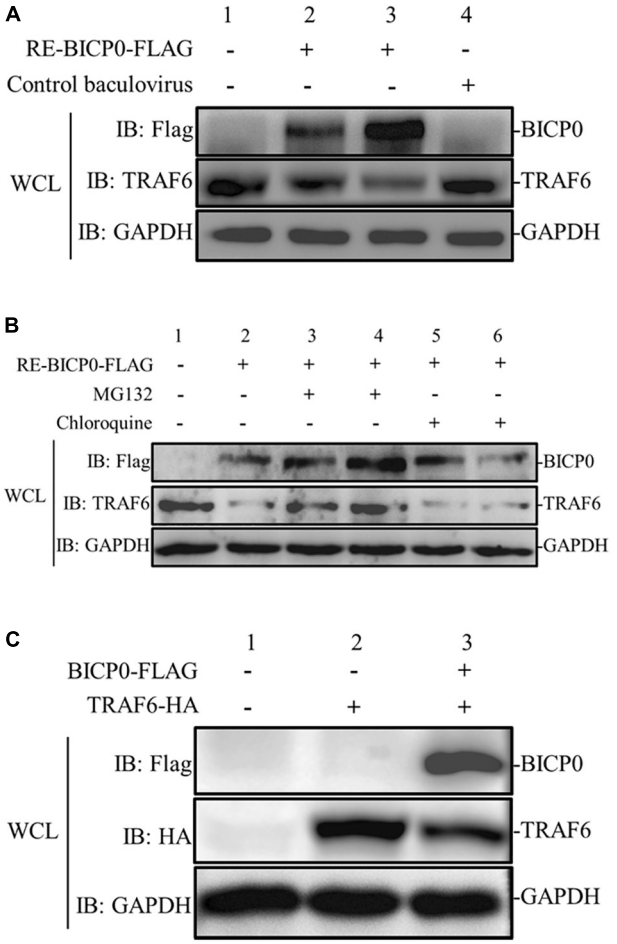

FIGURE 2 | BICP0 leads to the degradation of TRAF6 protein through the proteasome. **(A)** BICP0 reduces TRAF6 protein levels in MDBK cells. The titer of RE-BICP0-FLAG and control baculovirus were 1×10^6 $TCID_{50}$/100 μl and 2×10^6 $TCID_{50}$/100 μl, respectively. MDBK cells were transduced with RE-BICP0-FLAG at 1×10^6 $TCID_{50}$ (lane 2) or 2×10^6 $TCID_{50}$ (lane 3), and the control baculovirus at 2×10^6 $TCID_{50}$ (lane 4). **(B)** TRAF6 was degraded by the 26S proteasome in MDBK cells. MDBK cells were transduced with 2×10^6 $TCID_{50}$ of RE-BICP0-FLAG for 20 h and treated with MG132 (lane 3: 1 μM and lane 4: 4 μM) or chloroquine (lane 5: 35 μM and lane 6: 70 μM) for another 4 h. MG132 reduced the degradation of TRAF6 in a dose-dependent manner. **(C)** BICP0 promoted TRAF6 degradation after transient transfection of HEK293T cells. HEK293T cells ($\sim 0.5 \times 10^5$) were co-transfected with pcDNA3.1-TRAF6-HA (0.5 μg each) and pcDNA3.1-BICP0-Flag (0.5 μg each). The control plasmid pcDNA3.1(+) (0.5 μg each) was used for balanced transfection efficiency. Densitometry analysis to quantify the ratio of TRAF6 to GAPDH is shown below. Experiments were repeated at least three times and produced similar results.

[1]www.graphpad.com

protein expression in HEK293T cells, which was consistent with the conclusion derived from MDBK cells. Collectively, these data indicate that BICP0 can lead to the reduction of TRAF6 protein in MDBK cells and HEK293T cells, and TRAF6 was degraded through the proteasome pathway.

BICP0 Promotes the K48-Linked Ubiquitination of TRAF6

Considering that BICP0 is a RING-type E3 ubiquitin ligase, we hypothesized that TRAF6 degradation is dependent on the RING of BICP0. HEK293T cells were transfected with the indicated plasmids, and results were determined using immunoprecipitation and western blot assays. As can be seen in **Figure 3A**, TRAF6-HA protein was obtained by immunoprecipitation, and its band was detected using anti-HA antibody. Results of the co-transfected group showed that BICP0-Flag causes TRAF6-HA to separate into multiple bands, which were diffused and had a dark background. However, the band was significantly weaker in the TRAF6-HA transfection group and in the co-transfection group of TRAF6-HA with the BICP0-Flag (13A/51A) mutant. It is worth noting that only the wt BICP0-Flag can cause the decrease of TRAF6-HA in the whole cell lysate (WCL) samples. The antibody that specifically recognizes ubiquitin leads to similar results using anti-HA (**Figure 3B**). The results suggest that the ubiquitination of TRAF6-HA induced

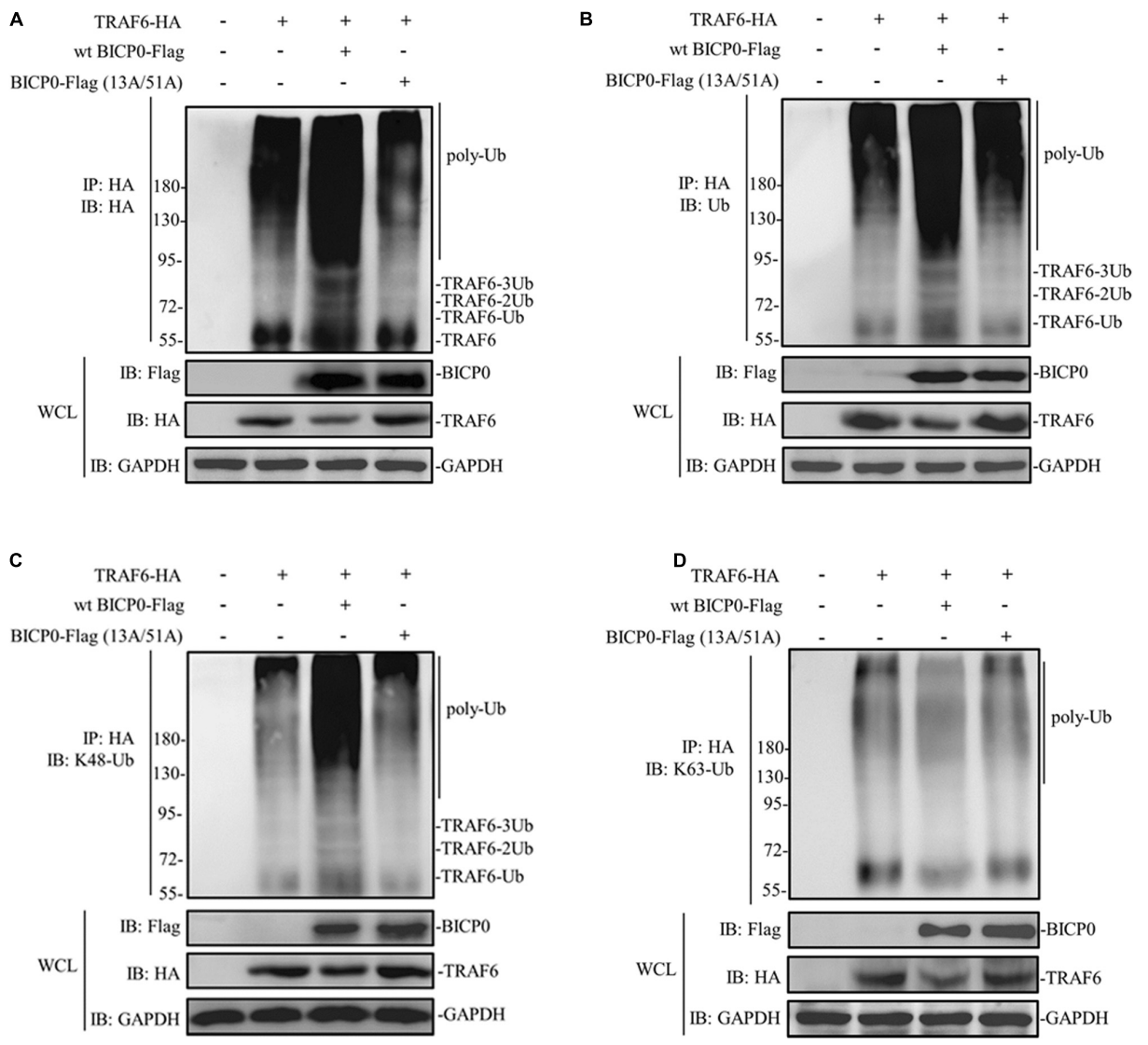

FIGURE 3 | BICP0 promotes the K48-linked ubiquitination of TRAF6. At 36 h post-transfection, 10 μM MG132 was added for another 6 h, and cells were lysed on ice for 10 min. The supernatant was collected by centrifugation, which was immediately immunoprecipitated by anti-HA magnetic beads for 2 h at room temperature. Afterward, western blot was performed using antibodies against HA-tag/ubiquitin to detect TRAF6-HA **(A)** or ubiquitinated TRAF6 **(B–D)**. **(A)** TRAF6-HA protein bands appear diffuse in the presence of BICP0-Flag. **(B)** TRAF6-HA protein is ubiquitinated by BICP0-Flag. **(C,D)** The ubiquitin chains of BICP0-mediated TRAF6 ubiquitination are K48-linked. Experiments were repeated at least three times and produced similar results.

by BICP0-Flag depends on its E3 ligase activity. To confirm the type of ubiquitin chains, antibodies that specifically bind to K48-ubiquitin and K63-ubiquitin were used. The results showed that BICP0-Flag promotes the K48-linked ubiquitination of TRAF6-HA in transfected cells but not K63-linked ubiquitin chains (**Figures 3C,D**). These data suggest that the RING finger of BICP0 is important for its catalytic activity, which promotes the K48-ubiquitination of TRAF6 and leads to a subsequent reduction of TRAF6.

BICP0 Interacts With TRAF6 and the Tyrosine 351 Is the Key Amino Acid

It is known that BICP0 mediates the ubiquitination and degradation of TRAF6; however, we aimed to investigate if the interaction between BICP0 and TRAF6 is necessary for ubiquitination modification, as it seems to have particular importance. To examine protein–protein interactions, Co-IP assay was carried out with the cell lysate prepared from HEK293T cells co-transfected with pcDNA3.1-BICP0-Flag and pcDNA3.1-TRAF6-HA. Immunoblot analysis using anti-HA antibodies revealed that BICP0 directly interacts with TRAF6 in the absence of other viral proteins (**Figure 4A**). In order to further study the domain structures involved in the BICP0-TRAF6 interaction, we analyzed the BICP0 protein sequence and found a conserved motif of BICP0—346-PAERQY-351. PCR was used to create several motif mutations: BICP0-123, BICP0-P346A, BICP0-E348A, and BICP0-Y351A. Co-IP and western blot assays showed that BICP0-123 has a significantly reduced ability to bind TRAF6 and that Y351 is the key amino acid responsible for the interaction between BICP0 and TRAF6 (**Figure 4B**). The result of reverse Co-IP is consistent with Co-IP (**Figure 4C**). To determine the role of the Y351 in the ubiquitination of TRAF6, HEK293T cells were co-transfected with pcDNA3.1-BICP0-Flag, or mutated pcDNA3.1-BICP0-Flag-Y351A and pcDNA3.1-TRAF6-HA. Ubiquitination

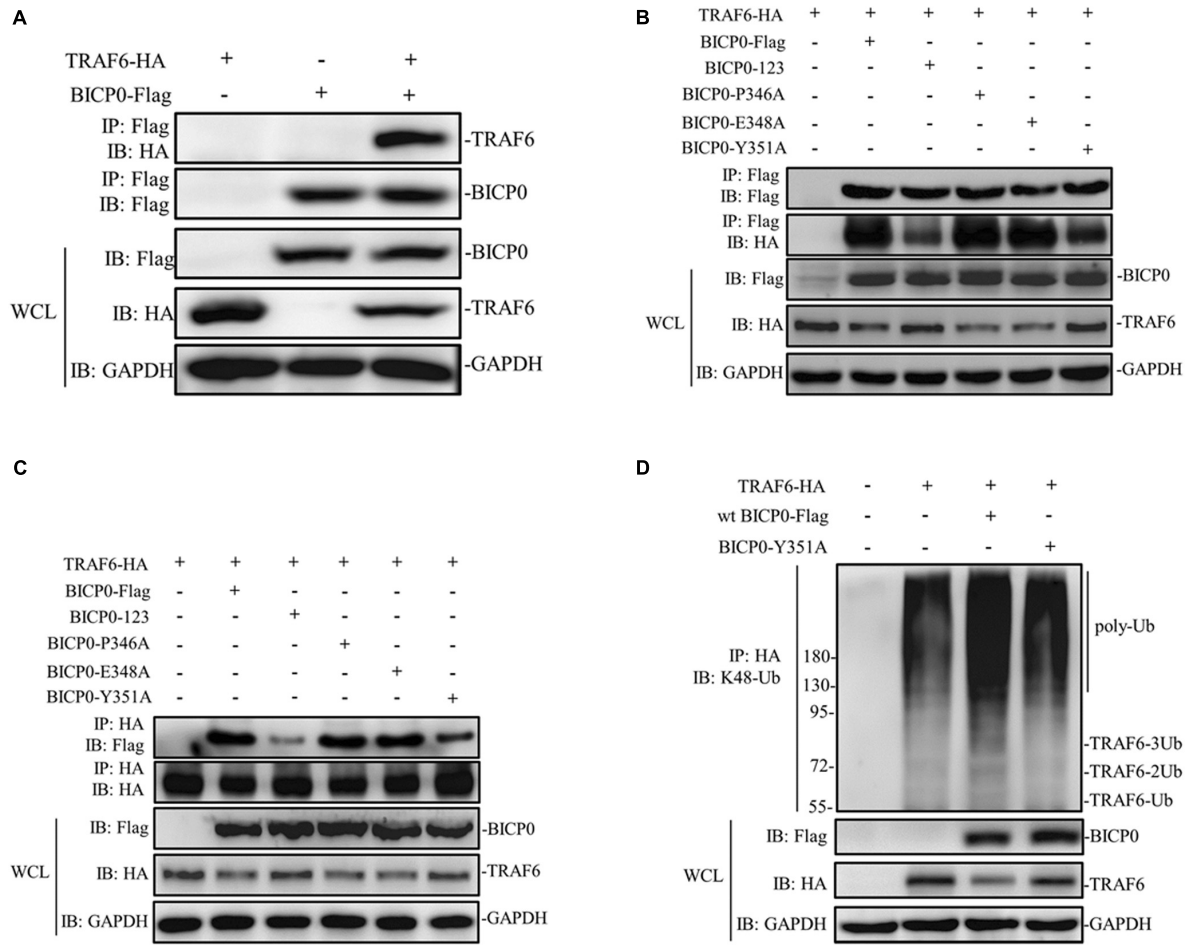

FIGURE 4 | BICP0 binds TRAF6 though "346-PAERQY-351" and Y351 is the key residue. HEK293T cells (~1 × 10^7) were seeded in 100 mm dishes and transfected with the appropriate expression plasmids. At 36 h post-transfection, cell lysates were incubated with Anti-Flag/Anti-HA magnetic beads for 2 h at room temperature according to the manufacturer's instructions. Western blot analysis with the indicated antibodies was performed. **(A)** Co-IP results by anti-Flag magnetic beads showed that BICP0 interacts with TRAF6 in the absence of other viral proteins. Co-IP **(B)** and reversed Co-IP **(C)** showed that the "346-PAERQY-351" motif of BICP0 responds for its interaction with TRAF6 and tyrosine 351 is the key amino acid. **(D)** Ubiquitination analyses revealed that the mutation of tyrosine 351 on BICP0 reduced TRAF6 K48-ubiquitination. Experiments were repeated at least three times and produced similar results.

analyses revealed that the mutation of Y351 on BICP0 reduced TRAF6 K48-ubiquitination (**Figure 4D**). To further identify the interaction between BICP0 and TRAF6, we also evaluated whether the BICP0 protein colocalizes with TRAF6 in HEK293T cells transfected with the indicated plasmids. The results showed the colocalization of BICP0 and TRAF6 in the nucleus; however, the BICP0-Y351A mutant lost its ability to colocalize with TRAF6 (**Figure 5**). It is worth noting that TRAF6 was introduced into the nucleus under increased expression of BICP0. Collectively, these data suggest that the direct binding of BICP0 with TRAF6 requires the involvement of a conservative peptide, and that Y351 is a key amino acid.

BICP0 Negatively Regulates TRAF6-Mediated NF-κB and IFN-β Promoter Activation

Since BICP0 interacts with TRAF6, leading to its degradation by ubiquitination, we aimed to determine which signaling pathways downstream of TRAF6 become affected, as well as the types of changes that will occur. TRAF6 has been most studied in inflammation, so investigating its effect on the NF-κB pathway is a priority. To do this, luciferase tests were performed and results showed that overexpressed TRAF6-HA strongly activated the NF-κB promoter. The wt BICP0-Flag inhibited the activity of TRAF6-HA, but the inhibition effect of BICP0-Flag (13A/51A) was obviously weaker than the wt BICP0-Flag. Moreover, it was seen that the 357–657 aa at the carboxyl terminal of BICP0 is dispensable (**Figure 6A**). On the other hand, the BICP0-P346A and BICP0-E348A mutants exert the same inhibitory effect as the wt BICP0-Flag. However, the inhibitory effects of the BICP0-Y351A and BICP0-123 mutants were significantly weaker than wt BICP0 (**Figure 6B**). Results showed that the interaction between BICP0 and TRAF6 attenuated the relationship between TRAF6 and the NF-κB promoter, and that and the RING and Y351 of BICP0 are essential.

To explore the further impacts of BICP0 binding to TRAF6, we thought to examine the interferon pathways. It is known that IRF7 stimulates alpha/beta IFN (IFN-α/β) expression (Au et al., 1998; Marie et al., 1998) and functions as a significant regulator of the innate immune response. Another study found that TRAF6 binds and activates IRF7, which requires

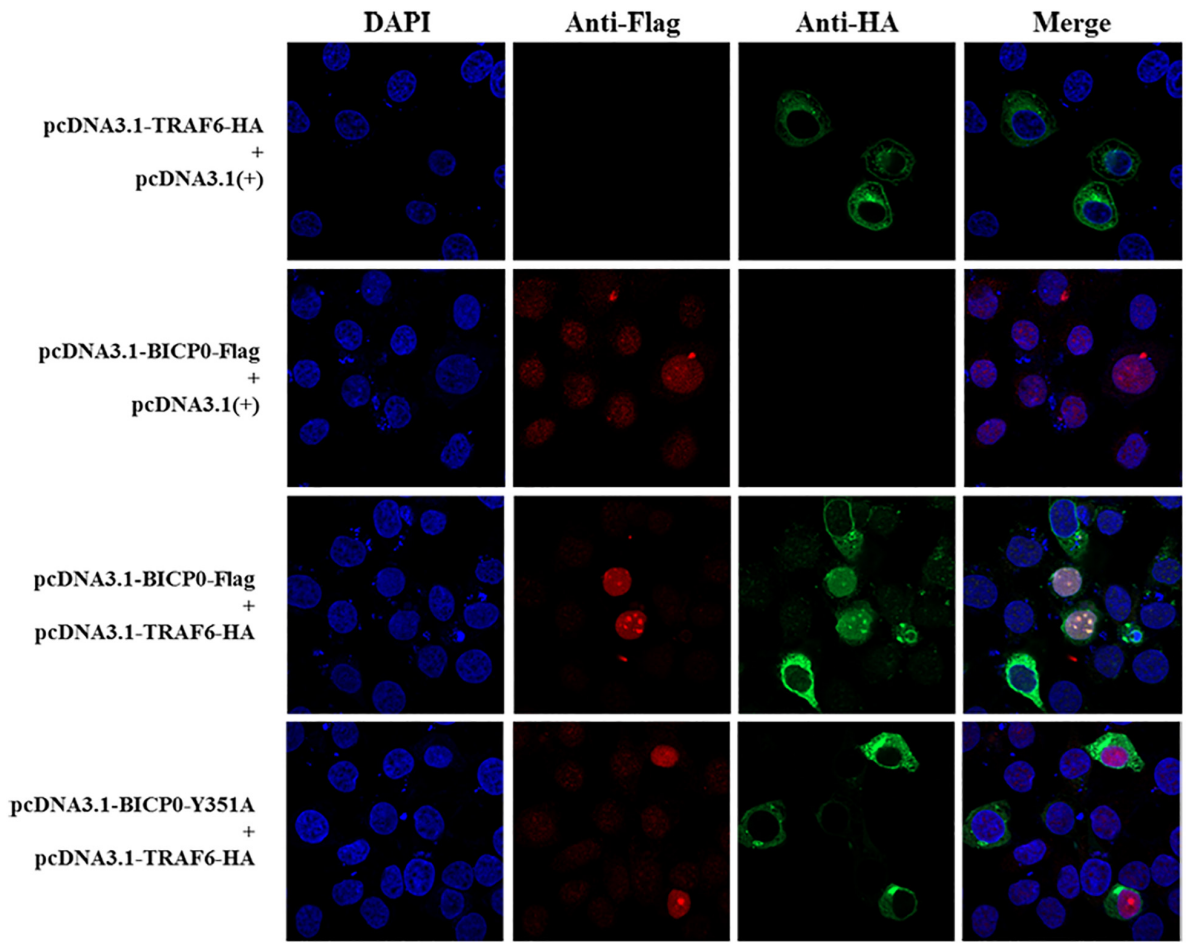

FIGURE 5 | Confocal assay. The Hela cells were transfected with the pcDNA3.1-BICP0-Flag, pcDNA3.1-BICP0-Flag-Y351A, or pcDNA3.1-TRAF6-HA for 36 h. Afterward, the cells were stained with the indicated antibodies and subjected to confocal assay.

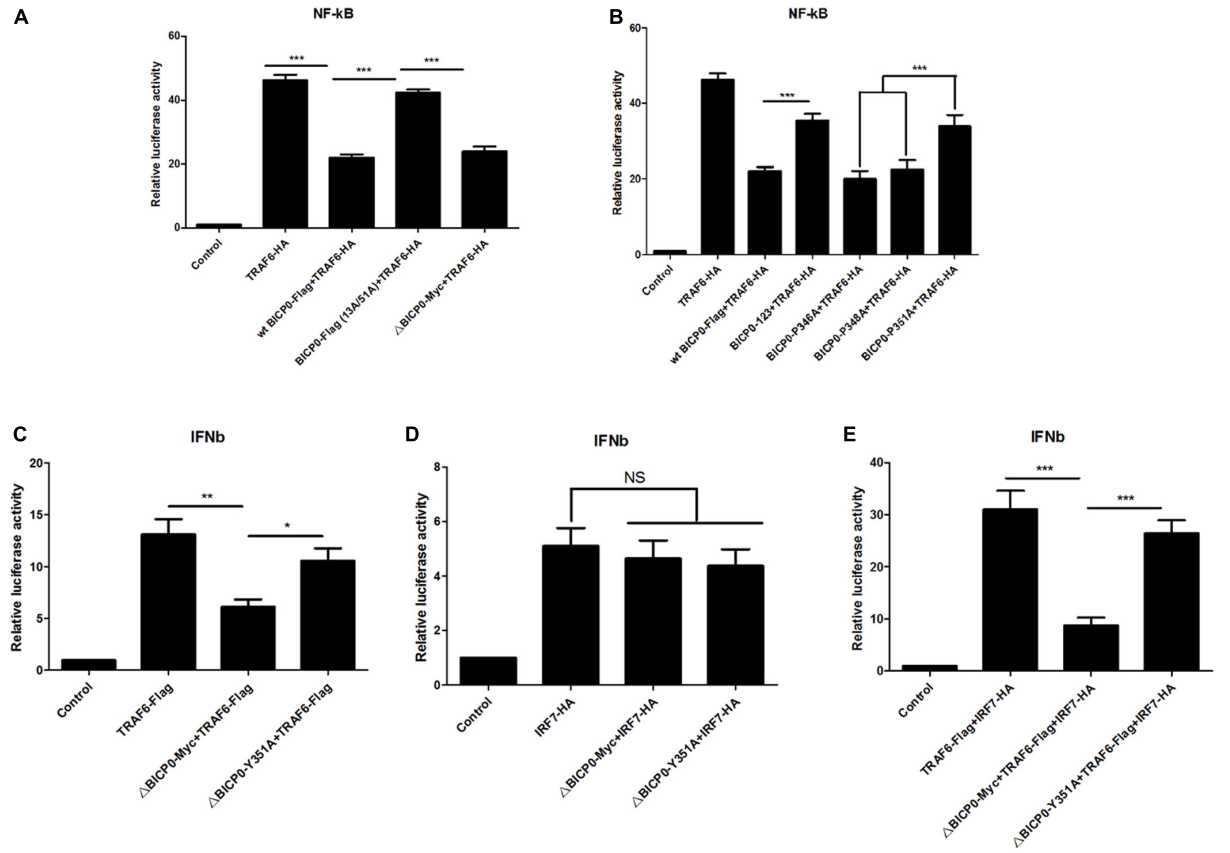

FIGURE 6 | BICP0 inhibited the TRAF6-mediated activation of NF-κB and IFN-β promoter. HEK293T cells were transfected with NF-κB-luc (0.5 μg each) or IFN-β-luc (0.5 μg each) reporter plasmids in the presence of the indicated plasmids. *Renilla* luciferase plasmid (0.05 μg each) was used as an internal control. **(A,B)** At 36 h post-transfection, HEK293T cells were harvested and whole cell extracts were prepared for the luciferase assay. The RING domain **(A)** and Y351 **(B)** of BICP0 are both essential for disturbing the activation of TRAF6 on the NF-κB promoter. **(C,D)** At 24 h after transfection, HEK293T cells were infected with VSV (100 $TCID_{50}$ per well) for 12 h before luciferase assays were performed. ΔBICP0-Myc inhibited TRAF6-mediated activation of IFN-β promoter **(C)** but had no effect on IRF7-HA **(D)**. TRAF6-Flag and IRF7-HA can synergistically activate the IFN-β promoter **(E)**, but the inhibition of ΔBICP0-Y351A was significantly weaker than ΔBICP0-Myc **(C,E)**. Data shown are presented as mean ± SD, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NS, not significant. Experiments were repeated at least three times and produced similar results.

the ubiquitin ligase activity of TRAF6 (Kawai et al., 2004). Combining these findings with our experimental results, we speculated that BICP0 can inhibit the IFN-β pathway by interfering with TRAF6. To test this hypothesis, IFN-β-luciferase activity was measured. Earlier studies have shown that wt BICP0 inhibits the activation of IFN-β promoter by interacting with IRF7 in the nucleus, but a C-terminal deletion BICP0 mutant (4Δ*Nco*I, 1–607 aa) that lacks the nuclear localization signal (NLS) inhibited the IRF7-induced IFN-β promoter activity less efficiently than wt BICP0 (Saira et al., 2009). Therefore, in order to exclude the direct effect and interference of wt BICP0 on IRF7, tests were conducted with the ΔBICP0-Myc, which showed that TRAF6-Flag effectively activated the IFN-β promoter. Co-transfected ΔBICP0-Myc effectively inhibited the activity of TRAF6-Flag, while the inhibition of ΔBICP0-Y351A was significantly weaker than ΔBICP0-Myc (**Figure 6C**). On the other hand, IRF7-HA also activated the IFN-β promoter; however, co-transfected ΔBICP0-Myc or ΔBICP0-Y351A did not inhibit the activity of IRF7-HA (**Figure 6D**). To test whether TRAF6 degradation affected the activation of the IFN-β promoter by IRF7,HEK293T cells were co-transfected with pcDNA3.1-TRAF6-Flag or mutated pcDNA3.1-ΔBICP0-Myc-Y351A and pcDNA3.1-ΔBICP0-Myc and pcDNA3.1-IRF7-HA. Luciferase analyses revealed that TRAF6-Flag and IRF7-HA can synergistically activate the IFN-β promoter, whereas ΔBICP0-Myc effectively inhibited the co-activation; however, the inhibition of ΔBICP0-Y351A was significantly weaker than ΔBICP0-Myc (**Figure 6E**). In conclusion, BICP0 interacted with TRAF6 and promoted its degradation, and then inhibited TRAF6-activated IRF7. Most importantly, Y351 is the key amino acid involved in these interactions.

BICP0 Weakens the Interaction Between TRAF6 and IRF7

The regulatory mechanism of IRF7 in the IFN pathway has been extensively studied. Like other transcriptional regulatory proteins, IRF7 also requires a series of post-translational

modifications (PTMs); for example, in ubiquitination, sumoylation, acetylation, and phosphorylation are most important (Ling et al., 2019). It is worth noting that the activation of IRF7 requires ubiquitination, meanwhile, IRF7 will be ubiquitinated by TRAF6 at multiple sites both *in vitro* and *in vivo* (Ning et al., 2008). Given the interaction between IRF7 and TRAF6 and the effect of BICP0 on the stability of TRAF6 protein, we therefore speculated that BICP0 inhibits ubiquitination of IRF7 by affecting the TRAF6 protein level. To test this hypothesis, HEK293T cells were co-transfected with the indicated plasmids. Co-immunoprecipitation analyses showed that IRF7-HA was ubiquitinated by TRAF6-Flag, and ubiquitination of IRF7-HA was significantly decreased in the presence of BICP0-Flag. In contrast, BICP0-Y351A failed to interfere with the ubiquitination of IRF7-HA mediated by TRAF6-Flag (**Figures 7A,B**). Results showed that the interaction between BICP0 and TRAF6 can inhibit ubiquitination of IRF7 and that the tyrosine 351 in the conserved motif of BICP0 is the essential amino acid. We next sought to determine the ubiquitin chain type of IRF7, and the results showed that TRAF6-Flag promoted K63-linked ubiquitination of the IRF7-HA protein, whereas IRF7-HA was not modified by BICP0-Flag through ubiquitination (**Figures 7C,D**). Taken together, BICP0 interacts with TRAF6 and enhances the K48-linked ubiquitination and degradation of TRAF6, which subsequently leads to the decrease of K63-linked ubiquitination of IRF7.

DISCUSSION

Transient transfection tests are often used to study the function of individual proteins. As it is a protein encoded by bovine virus, the optimal experimental material to study the pathogenic mechanism of BICP0 is bovine cells. MDBK cells are commonly used as experimental materials for BHV-1 infection experiments; however, transfecting nucleic acids into MDBK is very difficult, and this low transfection efficiency (<5.0%) led to the failure in detecting any differences using the gene reporter technology assay (Osorio and Bionaz, 2017). The reasons for low transfection in MDBK cells is unclear. Alternatively, baculovirus mediates the high-efficiency transduction of nucleic acids into mammalian cells such as MDBK cells (Condreay et al., 1999). In this study, the use of baculovirus helped us to successfully deliver BICP0 proteins to MDBK cells; we then found that BICP0 reduced the expression of TRAF6 protein. As an effective foreign gene delivery system, baculovirus is a powerful tool that plays a more important role in the research of BHV-1 immune evasion. Co-transfection experiments showed that BICP0 reduced TRAF6 protein levels in transfected HEK293T cells. The results indicated that BICP0 can function in HEK293T cells; subsequent related studies were thus performed on HEK293T cells.

The use of proteasome inhibitor MG132 and lysosome inhibitor chloroquine demonstrated that a functional proteasome played a role in regulating TRAF6 protein levels, as seen in **Figure 2B**. These results were consistent with previous studies showing that BICP0 can cause protein degradation through the ubiquitin-proteasome pathway (Saira et al., 2007). The amount of BICP0 protein also increased with MG132 in a dose-independent manner, which seems to indicate that BICP0 is also degraded in the MDBK cells via the ubiquitin proteasome pathway. However, we do not know if the degradation of BICP0 is affected by self-ubiquitination or by other proteins in host cells. Further study is required and may lead to new discoveries regarding host antiviral mechanism.

Like other ICP0 proteins encoded by the *alphaherpesvirus* subfamily, the BICP0 of BHV-1 contains a RING finger near its N-terminal, and that its enzymatic activity is essential for its function (Henderson et al., 2005). From the above results, we have found that BICP0 can cause the decrease of TRAF6 through the ubiquitin proteasome pathway. It is known that ubiquitination is one of the most significant and most commonly existing protein PTMs in eukaryotes, which uses ubiquitin molecules to form different types of ubiquitin chains that lead to the modification of protein substrates (Kleiger and Mayor, 2014). Whether they go through the UPS or the ALP pathway, proteins undergo ubiquitination modification as a prerequisite. Therefore, we next studied the E3 ligase activity of BICP0. Ubiquitination analyses suggest that the RING finger of BICP0 is important for its catalytic activity, which promotes the K48-ubiquitination of TRAF6. On the other hand, TRAF6 is a non-conventional E3 ligase that promotes the synthesis of K63-ubiquitination. The K63 ubiquitin chain catalyzed by TRAF6 can not only modify other proteins but also modify itself. Modification of TAK1 leads to its activation (Wang et al., 2001; Akira and Takeda, 2004), and the modification of ULK1 (Nazio et al., 2013) and BECLIN-1 (Shi and Kehrl, 2010) leads to the activation of autophagy. However, no relevant studies have shown that the K63 ubiquitination modification of TRAF6 mediates its degradation. Regardless of whether K63 ubiquitination of TRAF6 worked, the results of this study confirmed that BICP0 modified TRAF6 by K48 ubiquitination and caused its degradation.

Protein ubiquitination involves the cooperation of three families of ubiquitin enzymes: E1, E2, and E3. Briefly, E1 activates ubiquitin with the help of ATP, which then binds ubiquitin for the formation of an E1-ubiquitin thiol ester linkage. Subsequently, ubiquitin is passed from E1 to E2. Finally, the E3 ubiquitin ligase binds to both the E2-ubiquitin complex and the protein substrate, promoting the transfer of ubiquitin onto the protein. Co-IP results showed that BICP0 interacted with TRAF6 without other viral proteins, and that the direct combination between BICP0 and TRAF6 guarantees ubiquitination modification. Apart from the RING finger, sequence analysis indicated BICP0 contains two transcriptional-activation domains (TADs), an acidic domain, and a consensus nuclear localization sequence (NLS; KRRR) (**Figure 1**) (Henderson et al., 2005). However, the mode of interaction of TRAF6 with receptors has been revealed by three available structures of complexes, including TRAF6–CD40 (Ye et al., 2002), TRAF6–TRANCE-R (Ye et al., 2002), and TRAF6–MAVS (Shi et al., 2015). The consensus P-X-E-X-X-Z sequence (x: any amino acid, Z: acidic or aromatic amino acid), which is also known as the TRAF6-binding motif, is in accordance

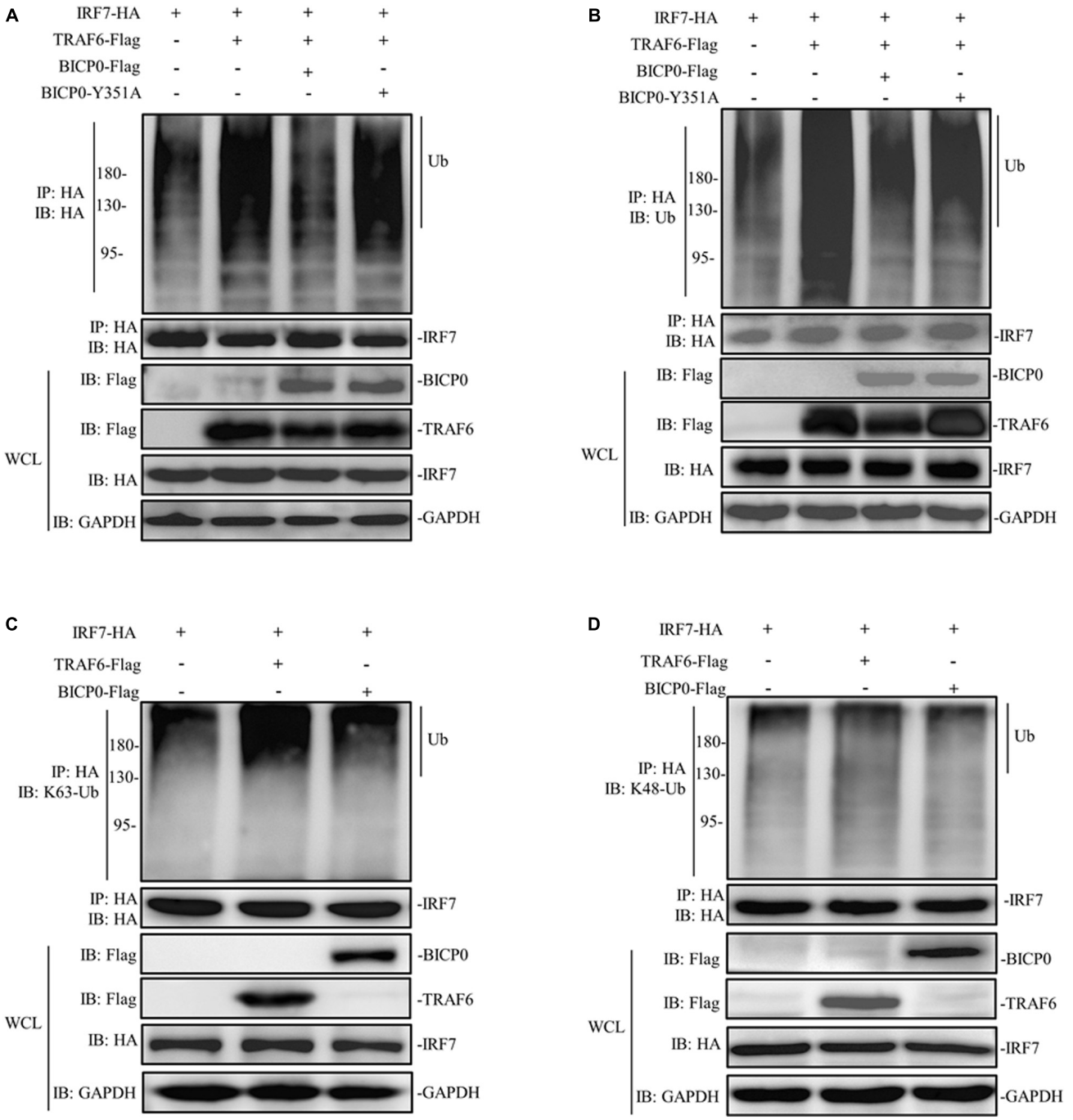

FIGURE 7 | BICP0 eliminates the interaction between TRAF6 and IRF7. HEK293T cells (~1 × 10^7) were seeded in 100 mm dishes and transfected with the indicated plasmids (5 μg each). At 36 h post-transfection, cell lysates were incubated with anti-HA magnetic beads for 2 h at room temperature. Western blot analysis with the indicated antibodies was then performed. **(A,B)** BICP0 suppress IRF7 undergo ubiquitination. Co-immunoprecipitation samples were incubated with anti-HA antibody **(A)** and anti-ubiquitin antibody **(B)**. **(C,D)** TRAF6 mediates K63-linked ubiquitination of IRF7. Co-immunoprecipitation samples were incubated with antibodies against K63-ubiquitin **(C)** and K48-ubiquitin **(D)**.

with the receptor peptide residues of CD40, TRANCE-R, and MAVS directly interacting with TRAF6. Results showed that the TRAF6-binding motif is also in three IRAK adapter kinases (Ye et al., 2002) and in the intracellular domain of IFNλR1 (Xie et al., 2012). In this study, the most valuable finding in the sequence analysis of BICP0 is that the 346-PAERQY-351 peptide of BICP0 is conserved in different subtypes of BHV-1. Further analysis showed that 346-PAERQY-351 peptide is in

accordance with TRAF6-binding motif. The proline 346 (P346), glutamic acid 348 (E348), and tyrosine 351 (Y351) of BICP0 are conserved according to the TRAF6-binding motifs. Mutation experiments showed that 346-PAERQY-351 of BICP0 is the binding domain of BICP0 and TRAF6 interaction, and that the aromatic amino acid (tyrosine 351) is the key interaction site. The residue in CD40 (F238) and TRANCE-R (Y349) is adjacent to several aromatic and basic residues of TRAF6, including R392, forming an amino-aromatic interaction (Park, 2018). This domain configuration of TRAF6 is the same as other mammalian TRAF family members, for example, TRAF2, TRAF3, and TRAF5 (Xie, 2013). TRAF2, TRAF3, and TRAF5 are able to interact with different overlapping motifs, such as P-X-Q-X-T (Chung et al., 2007; Hildebrand et al., 2010); however, the TRAF domain of TRAF6 binds specifically to the consensus TRAF6-binding motif, mainly through its TRAF-C domain (Ye et al., 2002). In this study, we did not test exactly which domain of TRAF6 mediated the interaction with BICP0, and more research needs to be done in the future.

Moreover, the carboxy terminus of BICP0 has a nuclear localization sequence, which can mediate the entry of BICP0 into the nucleus. Although TRAF6 has no nuclear localization sequence, co-immunoprecipitation indicates that BICP0 can bind to TRAF6. It is possible that TRAF6 can break through the nuclear membrane of the nucleus and enter the nucleus when there are enough of the BICP0 binds with TRAF6. On one hand, BICP0 causes K48 ubiquitination and degradation of TRAF6 in the cytoplasm; On the other hand, BICP0 may combines with TRAF6 and mediates its entry into the nucleus, thereby blocking TRAF6 from functioning in the cytoplasm. In subsequent studies, it will be necessary to explore the combined form of TRAF6 and BICP0.

TRAF6 is critical for the induction of many cytokines, such as inflammatory cytokines and interferons. In this study, we found that BICP0 directly binds to TRAF6 and affects its activation of NF-κB. ICP0 is the homolog of BICP0, and there is low similarity between BICP0 and ICP0 except in terms of the RING finger structure. Previous studies have shown that BICP0 and ICP0 could directly catalyze IκBα ubiquitination after transient transfection of HEK293T (Diao et al., 2005). Moreover,ICP0 had been shown to degrade various proteins such as p50/NF-κB1 (Zhang et al., 2013), MyD88, and Mal (also known as TIRAP) (van Lint et al., 2010). Therefore, we hypothesized that BICP0 can affect the function of other proteins in the NF-κB pathway through its E3 ligase. As an upstream molecule of TRAF6 in the NF-κB pathway, MyD88 recruits TRAF6 and forms a signal complex when the cell receives exogenous signal stimulation. We had found that BICP0 can also lead to the degradation of MyD88 (data unpublished); however, we do not know whether TRAF6 is involved in the binding between BICP0 and MyD88, and more work needs to be done.

The type I interferon (IFN-I)-inducing pathway is one of the most commonly stimulated signaling pathways during viral infection. Different pattern recognition receptors (PRRs) stimulated by exogenous stimulation will phosphorylate IRF3 and IRF7. Phosphorylated IRF3/7 then subsequently moves from the cytoplasm to the nucleus, and works together with activated NF-κB and ATF2/c-Jun to induce IFN-I production (Akira et al., 2006). Previous research has shown that TRAF6 also binds to IRF7 and results in IRF7 activation, and for this, the ubiquitin ligase activity of TRAF6 is required (Kawai et al., 2004; Ning et al., 2008). Furthermore, results show that MyD88-TRAF6-IRF7 complex regulates IFN-α production via TLR7, TLR8, and TLR9 (Honda et al., 2004; Kawai et al., 2004). In addition, TRAF6 mediates antiviral responses in RLR signaling that is triggered by viral DNA and RNA in the cytosol; this is different from TLR signaling and is important for the production of IFN-I and activation of NF-κB (Konno et al., 2009). As an immune-evasion gene encoded by BHV-1 that promotes productive infection, BICP0 reduces IFN-β promoter activity by causing the degradation of IRF3 in transient transfection studies (Henderson et al., 2005; Saira et al., 2007). BICP0 also impairs the activation of IFN-β promoter by interacting with the IRF7 protein, but it does not reduce IRF7 protein levels (Saira et al., 2007, 2009). However, it is not clear whether BICP0 interacts directly with IRF7 or with protein complexes containing IRF7 (Saira et al., 2009), and there have been no more developments in this field during the last decade. In this study, we showed that the interaction between BICP0 and TRAF6 promoted the degradation of TRAF6, which in turn caused the decrease of K63-linked ubiquitination of IRF7 and attenuated activation of the IFN-β promoter. Whether the binding of BICP0 and TRAF6 directly destroys the formation of MyD88-TRAF6-IRF7 complex is unknown, and follow-up work is currently being performed. In addition to the NF-κB and IFN pathway, TRAF6 may also direct the activation of mitogen-activated protein kinase (MAPK) (Wang et al., 2001), PI3K (Wong et al., 1999; Arron et al., 2001), and autophagy (Shi and Kehrl, 2010; Nazio et al., 2013). The effect of BICP0 on TRAF6 in these areas is worth investigating for future in-depth research.

In summary, this is the first study to demonstrate that BICP0 suppresses NF-κB signaling and IFN activation via TRAF6 interference. These results regarding BICP0 may help to further understand the interactions between viruses and hosts.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

JW and CC designed the study and wrote the manuscript. RA, YY, and HD carried out the experiments. MG and ZQ analyzed the results. All authors read and approved the final manuscript.

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REVIEW
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Manipulation of the Innate Immune Response by Varicella Zoster Virus

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Varicella zoster virus (VZV) is the causative agent of chickenpox (varicella) and shingles (herpes zoster). VZV and other members of the herpesvirus family are distinguished by their ability to establish a latent infection, with the potential to reactivate and spread virus to other susceptible individuals. This lifelong relationship continually subjects VZV to the host immune system and as such VZV has evolved a plethora of strategies to evade and manipulate the immune response. This review will focus on our current understanding of the innate anti-viral control mechanisms faced by VZV. We will also discuss the diverse array of strategies employed by VZV to regulate these innate immune responses and highlight new knowledge on the interactions between VZV and human innate immune cells.

Keywords: varicella–zoster virus, immune evasion, innate immune response, herpes zoster (HZ), varicella (chickenpox)

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INTRODUCTION

Varicella zoster virus (VZV) is a medically important human herpesvirus and infections are extremely common, with seroprevalence rates >90% in most populations around the world. Primary VZV infection causes chickenpox (varicella). The virus then establishes life-long latency in sensory neurons from where it can reactivate years later to cause shingles (herpes zoster), which is typified by a skin rash with a dermatomal distribution. Following herpes zoster rash resolution, many individuals continue to experience severe neuropathic pain, termed post-herpetic neuralgia (PHN), that can persist for months to years (1).

VZV is a member of the alphaherpesvirus family and is closely related to herpes simplex virus type 1 (HSV-1). VZV is genetically stable, a property which is demonstrated by little nucleotide variation between isolates (2). The VZV virion is composed of a double stranded (ds) deoxyribonucleic acid (DNA) genome, an icosahedral capsid, tegument, and envelope (3). The genome resides within the icosahedral capsid, which is composed of 162 capsomeres. The VZV genome is the smallest of the alphaherpesviruses and is composed of 71 unique open reading frames (ORFs) (4). Once VZV enters a host cell, a temporal cascade of gene expression occurs in which immediate early transactivating genes are expressed (5). This allows for the expression of early genes which are involved in VZV DNA replication. After viral DNA replication, late genes which encode for structural VZV proteins such as glycoproteins are expressed to allow the virus to egress from the host cell. VZV can be distinguished from other members of the alphaherpesvirus family as it exhibits a highly restricted host specificity to human and simian cells (6, 7).

One of the major obstacles in studying VZV pathogenesis and the host immune response is the virus' strict species specificity. Thus, our current knowledge has stemmed from clinical studies and examination of human tissues, experimental models of VZV infection *in vitro* utilizing human

cells and infection of human tissue xenografts implanted in severe combined immunodeficient (SCID-hu) mice, as well as observations from the simian varicella virus (SVV) infection of non-human primates, which has been used to model VZV infection *in vivo* (8). In this review, we draw upon a range of such studies to provide an update on how VZV interacts and manipulates early innate anti-viral responses in cell-types critical to VZV disease, encompassing both immune and non-immune cells.

PATHOGENESIS OF VZV

Pathogenesis of Primary VZV Infection

In order to appreciate the innate anti-viral immune response to VZV it is important to review the pathogenesis of VZV infection (**Figure 1**). Primary infection is initiated through exposure to highly infectious vesicular fluid from cutaneous lesions or through inhalation of infectious respiratory droplets from an individual with varicella. It is presumed that VZV initiates infection in the epithelial mucosa of the upper respiratory tract, from where the virus gains access to immune cells in the tonsils and local lymphoid tissue. It has been postulated that dendritic cells (DCs) are the first immune cell type to become infected in the respiratory mucosa (9, 10). DCs extensively interact with other cells via direct contact, which would provide a mechanism for VZV to be transmitted to other immune cells in the tonsils, especially T cells (11). VZV infection then progresses to a viremia, which may include dissemination of virus to internal organs. During this phase of infection, there is a prolonged incubation period of typically 14–16 days in which there are no detectable symptoms. This is followed by the infection progressing back to the respiratory mucosa and spreading to the skin. It is at this site that symptoms develop, most notably via the infection of keratinocytes which results in a vesiculopustular exanthema, with highly infectious lesions, spread across the body, as well as mucous membranes such as the oral cavity (1, 12–14). During primary infection, VZV dissemination around the body is considered to be facilitated by the migration of infected T cells (15–17). This model of VZV pathogenesis is supported by clinical studies of immunocompetent patients with varicella, where VZV could be cultured from peripheral blood mononuclear cells (PBMCs) isolated during the incubation phase of disease and peaking before the onset of the vesicular cutaneous rash (18, 19).

Primary varicella is resolved by the host immune response typically within 1–2 weeks. However, in the absence of a fully functional immune response, VZV may spread to other sites including the central nervous system (CNS) and lungs. Dissemination of infection may result in a number of serious complications, including VZV encephalitis, cerebellar ataxia, demyelinating neuropathy, myelitis, and pneumonia (20, 21).

During primary infection, despite a robust immune response, VZV is not completely eliminated from the host but rather the virus gains access to neurons in the sensory ganglia and establishes a life-long latent infection (22–24). The virus spreads to the sensory ganglia through retrograde axonal transport from free nerve endings in the skin (25, 26), and potentially via hematogenous spread in immune cells infiltrating the ganglia (24, 27, 28). It has also been proposed that VZV can establish latency in the enteric nervous system, providing a possible explanation for cases linking VZV with gastrointestinal disorders (29, 30).

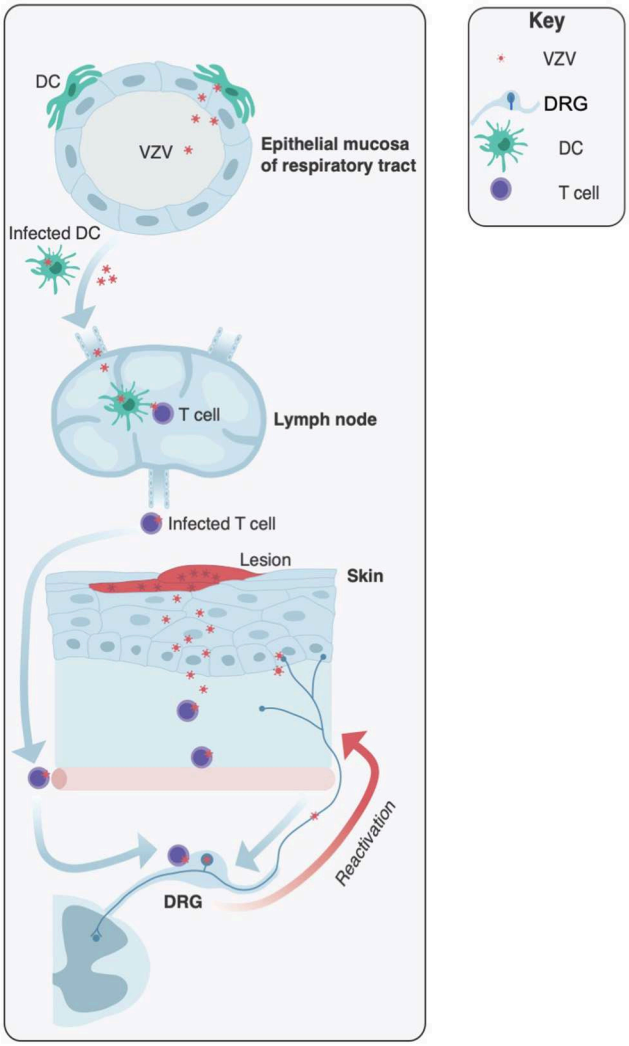

FIGURE 1 | Key sites of infection during varicella zoster virus pathogenesis. Initial infection is usually mediated by inhalation of highly infectious particles from patients undergoing acute varicella infection. It is proposed that VZV initiates infections in the upper respiratory tract, infecting the epithelial mucosa. Local dendritic cells (DCs) become infected and virus is transferred to the lymph nodes (and tonsils) where T cells are infected. Viremia leads to VZV dissemination to the skin and sensory neurons of the dorsal root ganglia (DRG) where the virus establishes a latent infection. Later in life VZV has the potential to reactivate and travel via anterograde spread to the skin, resulting in productive infection and the characteristic herpes zoster rash.

Pathogenesis of VZV Reactivation and Latency

Reactivation from latency causes herpes zoster (shingles), a neurocutaneous disease which occurs in 10–20% of seropositive individuals and involves anterograde axonal transport of virus

from the reactivating ganglia to the innervating dermatome (31–33). The incidence of herpes zoster is thought to correlate with a reduction in VZV-specific T cell mediated immunity (34, 35). Specifically, increasing age is a strong predisposing factor, with ∼68% of herpes zoster cases occurring in individuals over 50 years of age (36). Concomitant infection with other pathogens can also influence VZV reactivation. Adults with disseminated non-tuberculous mycobacterial infections can reactivate latent VZV infection and this is associated with anti-IFNγ autoantibodies (37). Additionally, there has been evidence of concurrent reactivation of HSV-1 and VZV, however this occurs rarely (38). It is unclear whether specific pathogens can increase the likelihood of VZV reactivation or whether VZV reactivation during other infections is due to a weakened VZV specific immune response.

Herpes zoster rash development is often preceded by a prodrome of dermatomal pain and is clinically characterized by a unilateral cluster of lesions typically across a single dermatome, accompanied by localized pain of varying intensity, and neuritis. The cutaneous lesions contain infectious virus and provides another reservoir for virus transmission to other susceptible individuals (39). Occasionally VZV reactivates in individuals experiencing dermatomal restricted neuropathic pain but without cutaneous lesions present; a condition known as zoster sine herpete (pain without rash) (40).

Herpes zoster has the potential to severely impact an individual's quality of life. The most common complication of herpes zoster is PHN which is a pain persisting for months to years after herpes zoster rash resolution (41). PHN occurs in 5–30% of people who experience herpes zoster and the prevalence and severity increases dramatically with advancing age (42). To date the mechanisms underpinning PHN are not yet fully understood. Other complications associated with VZV reactivation include meningitis, vasculopathy (including giant cell arteritis), myelopathy, ocular manifestations including herpes zoster opthalmicus, acute retinal necrosis, and progressive outer retinal necrosis (24, 39).

VZV MODULATES APOPTOSIS IN A CELL TYPE SPECIFIC MANNER

Programmed cell death is a critical component of the intrinsic and innate immune response, as it allows for the rapid elimination of damaged or infected cells (43). Viral infection can trigger programmed cell death via multiple pathways such as sensing of the virus through pattern recognition receptors (PRR), damage to host cell DNA and endoplasmic reticulum stress (44). The main forms of programmed cell death initiated by viral infection include apoptosis, necroptosis, and pyroptosis (44). Apoptosis is a non-inflammatory form of programmed cell death, which can be distinguished by the cleavage of caspase 3 and has been considered to be the main cell death mechanism used (45). Necroptosis is an inflammatory form of cell death which shares some of the apoptosis biochemical pathway. In particular, if components of the apoptosis pathway are inhibited, necroptosis can be initiated, eventually causing the phosphorylation of mixed lineage kinase domain-like (MLKL) and the formation of pores at the cell membrane (46). Pyroptosis is mediated by the inflammasome which contains a PRR from the Nod-like receptor (NLR) family, the adaptor ASC and caspase-1. Inflammasome activation causes cell membrane disruption and is therefore also an inflammatory form of programmed cell death (46).

In the context of VZV, apoptosis has been the most comprehensively investigated programmed cell death pathway. Apoptosis contains distinct biochemical pathways, which are highly complex and involve an energy dependent cascade of molecular events (47, 48). Three apoptosis pathways have been identified: the extrinsic, intrinsic, and perforin/granzyme pathway. All of these pathways converge in the cleavage of caspase 3, the major hallmark of apoptosis induction. This causes DNA fragmentation, nuclear, and cytoskeletal protein degradation, formation of apoptotic bodies, and engulfment by phagocytes (49). Apoptosis can be triggered in viral infection through cellular damage, viral detection through PRRs or through natural killer (NK) cell or cytotoxic T lymphocyte (CTL) recognition of target cells (43). CTLs and NK cells can kill virally infected cells through the expression of FasL which binds to Fas on the target cell and induces the extrinsic apoptotic pathway or through the delivery of perforin and granzyme B (50).

VZV Modulation of Apoptosis in Neuronal and Non-neuronal Cells

Interestingly, VZV has been shown to modulate apoptosis in a cell type specific manner. Specifically, VZV induces apoptosis in multiple skin cell types such as MeWo cells (51) and human fibroblasts (HFs) (52) (**Figure 2**). It was identified in MeWo cells that VZV infection caused a downregulation in Bcl-2 expression, a known anti-apoptotic protein (51). This downregulation of Bcl-2 has also been observed in SVV infection, where apoptosis was induced in infected monkey kidney cells via the intrinsic apoptotic pathway (53). It remains to be determined whether the downregulation of Bcl-2 directly leads to intrinsic apoptosis induction or whether there are other factors involved.

VZV has also been shown to induce apoptosis in immune cells such as T cells, B cells, and monocytes (54–56), however the factors which cause this induction are unclear. Investigating whether the downregulation of Bcl-2 occurs in VZV induced apoptosis in human immune cell types would be pertinent to determine whether VZV apoptosis induction occurs through a similar pathway in all cell types. Overall, it is not clear whether specific VZV gene products cause the induction of apoptosis as a strategy to increase viral dissemination, or rather whether the apoptosis induction is an intrinsic cellular response to limit viral replication and spread.

In contrast to some skin cell types and immune cells, VZV does not induce apoptosis in neurons (**Figure 2**). This was first identified in the context of primary human sensory ganglionic neurons, whereby VZV could productively infect dissociated human fetal dorsal root ganglia (DRG) cultures, but did not induce apoptosis (52). In intact human fetal ganglia, VZV was also shown to infect neurons without apoptosis induction (57). This phenomenon has been demonstrated in various other

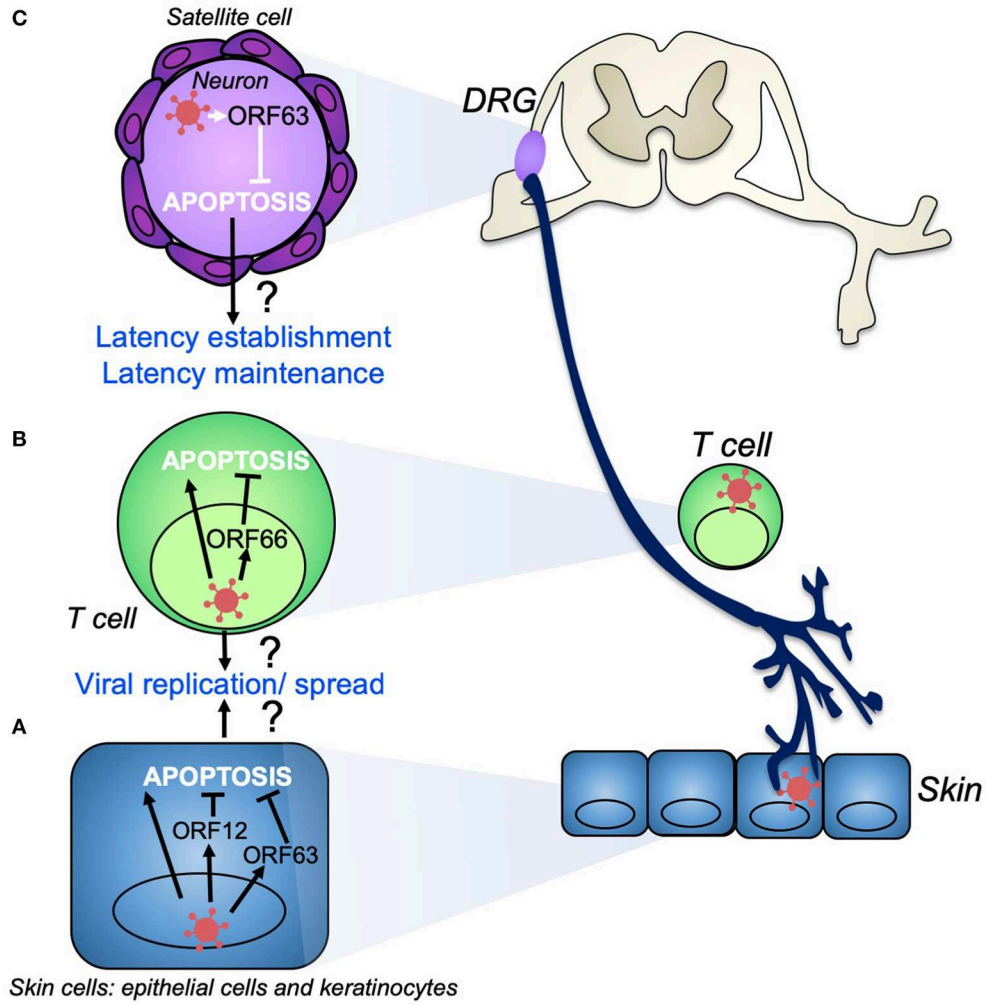

FIGURE 2 | VZV modulation of apoptosis during productive infection and the establishment of latency. During productive infection VZV infects skin cells **(A)** such as keratinocytes, fibroblasts, and epithelial cells. VZV induces apoptosis in skin cell types, despite the production of anti-apoptotic gene products such as VZV ORF12 and ORF63, which may act to delay apoptosis to ensure efficient viral replication and spread. **(B)** T cells are also infected during primary infection and act as a conduit to transport VZV to the skin and dorsal root ganglia (DRG). VZV induces apoptosis in T cells as well as other immune cells. VZV ORF66 may act to delay T cell apoptosis to promote viral dissemination. VZV establishes life-long latency in sensory neurons of the DRG **(C)**. VZV ORF63 is able to inhibit apoptosis in these neurons which may aid in the establishment and maintenance of latency.

neuronal models such as the SCID-hu xenograft DRG mouse model, where explanted human neurons displayed less apoptosis induction than was observed within VZV-infected SCID-hu skin cells (58). VZV also does not induce apoptosis in neurons derived from human neural stem cells (59, 60). Interestingly, in post-mortem ganglia samples from patients with herpes zoster at the time of death, neurons were not identified as being apoptotic, however other cells within the ganglia did display apoptotic markers (61).

Contribution of VZV ORFs in the Inhibition of Apoptosis

The ability of VZV to protect neurons from apoptosis induction was attributed to ORF 63, using a recombinant virus which was able to express only one copy of the diploid ORF63 gene (62). However, as ORF63 is a potent viral transactivator, it was unclear whether its impact was due to an effect on another VZV ORF. More recently, using lentiviral expression of ORF63 in the differentiated SH-SY5Y neuronal cell line model, it was confirmed that VZV ORF63 could protect against intrinsic apoptosis induction (63). Interestingly, this was also observed in a human keratinocyte cell line known as HaCaTs, suggesting that ORF63 when expressed alone can protect multiple cell types from apoptosis induction (63). VZV infection was also shown not to induce apoptosis in HaCaT cells, a finding which has been previously reported in VZV-infected human papillomavirus (HPV)-immortalized keratinocytes (64). It would be interesting to examine VZV apoptosis induction in the context of primary human keratinocytes, as cell lines can have deficiencies in the apoptotic pathway, which makes them less sensitive to apoptosis

induction (65, 66). To date it remains unclear as to why certain cell types are protected from apoptosis during VZV infection and others are not, however there is evidence to suggest that VZV alters the transcriptional profile of apoptotic genes differentially in neuronal cells vs. HFs (67).

Cell type specific modulation of apoptosis is a crucial component of VZV research due to its link to VZV pathogenesis. As VZV establishes life-long latency in neurons of the DRG, the inhibition of apoptosis in neurons is critical for viral maintenance of latency and the establishment of reactivation (68). In contrast, within productive infection in the skin, the induction of apoptosis in HFs may aid in viral dissemination. In the context of VZV ORF63, it will be useful to investigate whether it can protect other cell types when expressed by itself. If this were the case it would suggest that even in productive infection in HFs where apoptosis is induced, the gene product may delay the onset of apoptosis long enough for the virus to replicate. The ORF63 transcript is also one of the major transcripts produced during VZV latency (69), thus it may play a role in apoptosis protection in this context. The mechanism of ORF63 inhibition of apoptosis is still unknown but may be related to its relocalization within the cell during apoptosis induction (63).

Other VZV gene products have also been shown to play a role in apoptosis inhibition. For example, VZV ORF66 inhibits apoptosis in T cells, as evidenced by T cells undergoing apoptosis more readily when infected with a virus in which ORF66 protein expression is impaired (70). Investigation of whether ORF66 can protect against apoptosis when it is expressed alone in immune cells and other cell types would be a potential avenue for future research. VZV ORF12 has been shown to interact with the extracellular-signal-regulated kinases (ERK) signaling pathway in MeWos and fibroblasts (71, 72). This optimizes the capacity for viral replication and causes the inhibition of the apoptosis pathway (71, 72). Specifically, ORF12 enhances the phosphorylation and activation of Akt in a Phosphatidylinositol-4,5-bisphosphate 3-kinase dependent manner (PI3K) (73). This activation was associated with increased levels of cyclin B1, cyclin D3, and the phosphorylation of glycogen synthase 3β (GSK-3β) (73), which are crucial in advancement of the cell-cycle. It has also been reported that the activation of ERK signaling pathway causes the phosphorylation and inhibition of Bim (74). Bim is a pro-apoptotic member of the Bcl-2 family that is usually involved in the propagation of the intrinsic apoptotic pathway (75). Thus, the ability of ORF12 to stimulate cell cycle progression via the ERK signaling pathway can also cause the inhibition of intrinsic apoptosis (74). The effect of ORF12 on apoptosis and cell-cycle progression in neurons is yet to be investigated.

It is clear VZV encodes multiple ORFs with anti-apoptotic mechanisms, demonstrating the importance of modulating apoptosis for viral replication and spread. Interestingly, when expressed alone or deleted from VZV, these genes have an anti-apoptotic effect in cell types where VZV is known to induce apoptosis. It will be important to determine whether these gene products delay the onset of apoptosis in vulnerable cell types during VZV infection as this could be a critical component of VZV pathogenesis in the skin and during reactivation. Furthermore, it would be beneficial to determine whether VZV can protect against other forms of cell death, as when apoptosis is inhibited other cell death forms such as necroptosis can occur to limit viral spread (46). HSV-1 has been shown to inhibit necroptosis (76) and as VZV is closely related to HSV-1, this warrants investigation in the context of VZV.

INNATE IMMUNE RECOGNITION AND VZV INTERFERENCE

The innate immune response to VZV involves the recognition of viral pathogen associated molecular patterns (PAMPs) via PRRs, which triggers inflammatory cytokine secretion and/or cell death. Of the Toll-like receptors (TLRs), Wang and co-workers demonstrated that exposure of monocytes to VZV induced TLR2 and NFκB dependent secretion of interleukin (IL)-6. Furthermore, this report suggested sensing of VZV involved cell-surface TLR2 binding to virion envelope glycoproteins (77). Recently, sensing of VZV through endosomal TLR3, which senses dsRNA has also been proposed (78). The significance of TLR3 sensing initiating anti-VZV responses has been inferred from individuals with defects in genes of the TLR3 pathway suffering from severe varicella resulting in VZV encephalitis (78). Interestingly, there has also been evidence of patients with TLR3 mutations suffering from HSV-1 encephalitis but not VZV encephalitis (79). This may suggest that differing mutations in TLR3 may predispose patients to different susceptibilities to viral infections or that TLR3 sensing is more critical for controlling HSV-1 than VZV. Patients with mutations in downstream components of TLR signaling such as interleukin-1 receptor-associated kinase 4 (IRAK-4) and MyD88 are not susceptible to viral infections such as VZV, highlighting the functional redundancy in the TLR pathogen sensing pathway (80). In particular, it has been shown that IRAK-4 deficient patients can control viral infection through both TLR3 or TLR independent production of type I IFN (81).

TLR3 is known to be expressed in human neurons and peripheral nerve Schwann cells (82, 83), thus implying TLR3 may play a pivotal role in controlling VZV spread in the nervous system. More recently there was a case report describing a 28 year old individual suffering from multiple recurrences of herpes zoster opthalmicus- a disease primarily seen in immunocompromised individuals or elderly individuals following VZV reactivation (84). This study revealed a novel TLR3 variant in an otherwise immunocompetent individual was associated with recurrent herpes zoster opthalmicus. Interestingly, the patient's fibroblasts but not antigen presenting cells (APCs) showed an inability to respond to stimulation with a TLR3 agonist (84). This report further supports the notion of TLR3 in innate activation and control of VZV infection.

Retinoic acid-inducible gene I (RIG-1) is a cytoplasmic PRR which senses both RNA and DNA viruses and can result in the production of the type I IFN response (85). Knockdown of RIG-1 in the context of VZV infection does not affect viral titers in MRC-5 cells, however in human dermal fibroblasts (HDF) RIG-1 overexpression caused a significant suppression of viral replication (86). This suggests that in HDF a RIG-1 induced IFN

response may play a role in controlling VZV infection, however RIG-1 is not essential for the control of VZV replication (86). As of yet there have been no VZV ORFs implicated in the inhibition of RIG-1 sensing, however VZV ORFs do target downstream transcription factors such as NFκB, that are involved in the production of inflammatory cytokines (87).

Monocytes and other myeloid cells are also able to sense virus through NLRs, which trigger a pro-inflammatory response through inflammasome formation (88). Interestingly, it has been demonstrated that VZV induces the formation of an inflammasome through the NLR, NLRP3, leading to secretion of pro-inflammatory IL-1β following infection of the monocytic THP-1 cell line (89). Furthermore, in SCID-hu mice with human skin xenografts, NLRP3 was detected throughout VZV infected skin, indicating a function for NLRP3 inflammasomes in local cutaneous immunity (89). The role of NLRP3 inflammasomes and whether VZV can actively modulate this at other key sites of infection such as human ganglia has yet to be explored.

Another intrinsic defense mechanism limiting VZV infection in human skin is the formation of promyelocytic leukemia (PML) cages in infected epidermal cells, which trap VZV nucleocapsids resulting in inhibition of virion assembly (90). Wang and colleagues demonstrated that the ability of VZV ORF61 to bind small ubiquitin-like modifier (SUMO) is required to counterbalance PML nuclear body-mediated control of VZV replication, and enable the formation of skin lesions during varicella and herpes zoster (91). Recently it has been shown that human skin cells including dermal fibroblasts and HaCaT keratinocytes can sense cytosolic VZV DNA through stimulator of interferon genes (STING), triggering secretion of type I and III interferons, which limited VZV replication (86).

VZV MODULATION OF THE INTERFERON (IFN) RESPONSES

Interferons (IFNs) are key anti-viral cytokines that mediate their activity through the induction or upregulation of a suite of interferon stimulated genes (ISGs), which have a range of anti-viral activities (92). Recognition of incoming pathogens by both cell-surface and intracellular PRRs initiates a signaling cascade driving the production of type I IFNs through the action of key transcription factors including interferon regulatory factor (IRF) 3 and NF-κB. The IFNs produced can then signal through canonical IFN receptors on the cell-surface leading to activation of a JAK-STAT signaling cascade to drive ISG production (92).

Clinical Observations Regarding the Importance of IFN in the Control of VZV Infection

Given the key role of IFNs in controlling many viral infections it is unsurprising that IFNs can also profoundly modulate VZV infection. This is emphasized by a number of *in vivo* observations. More than 30 years ago a clinical trial to evaluate the efficacy of IFNα in inhibiting VZV infection in children suffering from cancer indicated that IFN treatment could limit the dissemination of severe varicella lesions (93). Analogously, in the SCID-hu skin model of VZV infection, blocking the type I IFN receptor by neutralizing antibody led to a 10-fold increase in virus titer compared to control antibody treated mice (16).

Patients presenting with primary immunodeficiencies characterized by defects in interferon signaling pathways are also associated with acute VZV infection. Recently four cases of otherwise healthy children presenting with severe VZV infections in both the lungs and CNS were identified as having missense mutations in individual subunits of RNA polymerase III (94). RNA polymerase III acts as a sensor of AT-rich DNA that can drive IFN production (95). Leukocytes isolated from such patients had significantly reduced capacity to transcribe both type I and type III IFNs following stimulation with AT-rich DNA which is a specific characteristic of the VZV, but not other, herpesvirus genomes (94). In a separate study, it was reported that two adult patients suffering from severe VZV infections of the CNS also had mutations in specific RNA polymerase III subunits (96). Cells isolated from such RNA polymerase III deficient patients also demonstrated enhanced susceptibility to VZV infection *in vitro* (94, 96). Other primary immunodeficiencies associated with VZV infection and defects in IFN signaling and/or production include defects in DOCK2 (97), DOCK8 (98), and the IFNγ receptor (99).

Patients with rare genetic defects in downstream components of the type I IFN signaling pathway such as STAT1, TYK2, and NEMO have been shown to increase susceptibility to viral infections such as varicella (100–103). Susceptibility to viral infection has also been reported in patients with mutations in STAT2 (104). STAT2 helps form the ISGF3 complex which binds to IFN sensitive response elements (ISRE) (105). These patients had VZV infection but did not experience severe complications, which questions the importance of type I IFN in controlling VZV infection (104). Interferon independent pathways have also been shown to play a critical role in the control of viral infection and may be able to compensate for the lack of type I IFN response in these patients (106).

VZV Modulation of IFN Signaling Pathways

The key regulatory role of interferons during VZV infection is underlined by the range of mechanisms encoded by the virus to regulate both the production of and response to IFNs. VZV encodes at least three gene functions that can limit the production of type I IFN with a particular focus on disruption of signaling through IRF3. The serine threonine kinase encoded by the ORF47 gene induces an atypical phosphorylation of IRF3 which inhibits the self-dimerization of IRF3 required for efficient IFNβ induction (107). ORF61 can directly interact with the IRF3 protein promoting IRF3 ubiquitination and subsequent proteasomal degradation (108). The IE62 protein was also demonstrated to block IRF3 phosphorylation at three specific residues on IRF3, inhibiting activation of an IFN stimulated reporter element construct (109). Given the key role of NF-κB in amplifying type I IFN transcription it is likely that the identified role for E3 ubiquitin ligase domain of ORF61 in limiting TNF induced NF-κB activation (87) will also contribute to the inhibitory effect of VZV infection on IFN induction.

More recently, it has been identified that VZV can induce suppressor of cytokine signaling 3 (SOCS3) to modulate type I IFN signaling and viral replication (110). Multiple viruses have been shown to increase SOCS3 expression during infection to suppress signal transduction activated by IFNβ (111). VZV infection of fibroblasts (MRC-5) and macrophages (THP-1) caused an increase in IFNα and IFNβ transcripts in early phases of infection whereas in keratinocytes (HaCaTs) IFNα and IFNβ transcripts persisted until later time-points (110). As these cells were infected at a 1:1 ratio with VZV infected HFFs, it is unclear whether inoculating VZV infected HFFs, could be masking the effect of VZV infection on IFNα and IFNβ transcription in these different cell types. An elevation in SOCS3 protein expression was correlated to a reduction in phosphorylation of STAT3 which is required to drive type I IFN induced gene expression (110). As the effects of mock inoculating HFFs were not addressed in the protein analysis of SOCS3 and pSTAT3, it may be pertinent to perform cell associated infections with the same cell type to exclude effects of using different inoculating cells. Overall, it would be interesting to determine if the induction of SOCS3 by VZV extends to different cell types such as neurons and if so, what is driving the increased expression of SOCS3 in the context of VZV infection. When SOCS3 was knocked down in MRC-5 cells, VZV viral gene expression was inhibited suggesting that the induction of SOCS3 by VZV may be critical for VZV spread and pathogenesis (110).

VZV Modulation of IFN in Immune Cells

VZV infection can also target type I and II IFN production through direct infection of immune subsets that play a vital role in anti-viral immunity. Plasmacytoid dendritic cells (pDCs) have the capacity to secrete significant amounts of IFNα following appropriate stimulation (112). Work from our laboratory first identified the tropism of VZV for pDCs both *in vivo* and *in vitro*, with VZV infected pDCs significantly inhibited in their capacity to produce IFNα after stimulation with a TLR9 agonist (113). More recently our identification of the pronounced tropism of VZV for primary human NK cells (114) (covered in more detail in section on NK cells and VZV) led to the observation that such cells have a greatly diminished capacity to produce the type II IFN, IFNγ, following stimulation with PMA/ionomycin (115). Given the tropism of VZV for potent immune effector cells it will be intriguing to determine if this inhibition of IFNγ production also extends to other immune cells, such as $CD4^+$ T cells, that also have the capacity to produce this key anti-viral cytokine.

VZV also has the capacity to regulate the activity of both type I and type II IFNs through disruption of signaling downstream of IFN receptor binding. Following IFNγ stimulation, STAT1 phosphorylation, a key signaling event in intracellular transduction of IFN, was increased in human tonsillar T cells infected with an ORF66 mutant compared to cells infected with the parental virus (70), implicating this immunomodulatory protein in this response. This mirrors the situation in HFs where IFNγ-induced MHC class II expression was significantly reduced in VZV infected cells through inhibition of STAT1 and Jak2 protein expression (116). Use of the SVV model of VZV infection indicated that SVV can inhibit IFNα and IFNγ induced ISG expression (117, 118) including ISG15 and Mx1 with such phenotypes recapitulated with ectopic expression of SVV ORF63 alone (117). Heterologous expression of the VZV homolog ORF63 in HFs also reduced levels of IRF9 mirroring the simian homolog. STAT2 phosphorylation although reduced during VZV infection was not targeted by ORF63 (117), suggesting additional as of yet unidentified viral gene products are responsible.

Despite the numerous identified mechanisms that VZV employs to regulate the effects of IFN, it is clear that *in vitro* IFNs have the capacity to directly inhibit VZV infection. Comparison of the ability of IFNα and IFNγ to block infection demonstrated that IFNγ has more pronounced effects on VZV replication in human embryonic lung fibroblasts (119). Another recent report indicates there are cell type specific activities in the relative ability of IFNβ and IFNγ to limit virus production. IFNγ could profoundly inhibit VZV production in ARPE-19, A549, MRC-5 but had only very limited capacity to inhibit infection in MeWo cells, where IFNβ retained the capacity to significantly reduce viral yield (120). IFNγ could also promote survival of VZV infected neurons to potentially ensure the efficient establishment of latency (121). More recently, Como and colleagues demonstrated that Type I IFNs had an inhibitory effect on VZV replication and spread in VZV infected human iPSC derived neurons *in vitro* (122). Furthermore, the SCID-hu DRG model revealed VZV infection of DRG resulted in an increase in pro-inflammatory cytokines as well as IFNα and IFNγ (123). Further studies to understand the distinct activities of type I and II IFNs in regulating infection will potentially tease apart the roles played by distinct IFNs in regulating infection during different phases of the viral lifecycle. Similarly the role of type III IFNs in viral infections is becoming clear, particularly at mucosal sites, and a recent report indicated that VZV infection can promote IFNλ1/3 and IFNλ2 production in keratinocytes in a STING dependent manner and IFNλ has direct anti-viral activity *in vitro* (86). Additional study will be required to fully define the role of type III IFN (IFNλ) during VZV infection.

VZV INFECTION OF DENDRITIC CELLS AND MODULATION OF IMMUNE FUNCTIONS

DCs are key immune effectors during viral infection as they are professional APCs instrumental in inducing and modulating anti-viral immune responses. DCs are closely implicated during VZV disease as they are present in lymph nodes and other lymphoid tissues significant to VZV pathogenesis, such as tonsils, as well as residing and migrating through the skin (124). DCs sense invading pathogens and induce innate and initiate adaptive immune responses. DCs have the ability to uptake viral proteins, process, and present antigenic peptides loaded onto major histocompatibility complex (MHC) class I and class II molecules that can be subsequently recognized by $CD8^+$ and $CD4^+$ T cells, respectively. The interaction of DCs and antigen-specific T cells results in T cell activation and culminates in defining the phenotype of T cells, and instructs the overall immune response against a viral pathogen, such as VZV (125). Given the pivotal

role DCs play in the innate and adaptive arms of the immune response to viruses, they have been postulated to be a prime target for viruses, seeking to evade and/or delay the host response by disrupting their immune function (126).

VZV Infection of Human Monocyte Derived Dendritic Cells

There have been a number of studies exploring the interaction between VZV and DCs. Work from our laboratory first identified that VZV could productively infect human monocyte derived dendritic cells (MDDCs) *in vitro* and this led to efficient transmission of virus to T cells (9). These findings supported the hypothesis that DCs may be a major target for VZV infection and facilitate virus transport from the site of VZV entry (mucosal sites) to draining lymph nodes where the virus infects T cells. The importance of T cell tropism and dissemination of virus to the skin was elegantly shown by Ku and co-workers, in which SCID-hu mice with human skin grafts inoculated with VZV infected human T cells, developed VZV skin lesions (16). The importance of the DC/T cell axis during VZV dissemination is further supported from SVV experiments. Ouwendijk and co-workers identified infected DC-like cells in the lungs of African green monkeys infected with a recombinant SVV expressing enhanced green fluorescent protein (SVV-EGFP) virus and during viremia, SVV was observed in memory T cells (28).

VZV infected MDDCs *in vitro* showed no significant decrease in cell viability or evidence of apoptosis (9). These results imply VZV has evolved a strategy to limit or prevent the onset of apoptosis in DCs. As discussed earlier, this may provide a transient advantage to the virus, allowing VZV to successfully disseminate during the first critical days after primary infection. Analogously, others have employed the *in vitro* MDDC infection model to demonstrate that the VZV vaccine strain (V-Oka) and virulent VZV clinical isolates equally infect these immune cells (127). Furthermore, Hu and Cohen utilized viruses unable to express VZV ORF10, ORF32, ORF57, or ORF66 proteins and demonstrated there was no impairment for infection of immature DCs. In contrast, when an ORF47 mutant virus was used to inoculate the MDDCs, there was a reduction in VZV infection, suggesting the ORF, which encodes a serine/threonine protein kinase, was required to promote VZV replication (128). These *in vitro* based MDDC infection studies provided an impetus to study the interaction of VZV with various DC cell subsets *in vivo*.

VZV Infection of Langerhans Cells and Plasmacytoid Dendritic Cells

In the skin, a major site for VZV disease, it has been demonstrated via immunostaining of VZV infected skin lesions that there is a significantly reduced frequency of Langerhans cells (LCs) (113, 127), extending an earlier case report which examined CD1a expression in VZV-infected skin (129). These observations suggest activation and migration of LCs to draining lymph nodes (113, 127). In stark contrast, infiltration of pDCs and other inflammatory DCs was observed in varicella and herpes zoster skin lesions (113, 127, 130). In our assessment of DC subsets in skin during natural VZV infection we explored whether these cells were infected by immunofluorescence (IFA) staining. We identified sporadic VZV antigen-positive LCs in the epidermis and VZV antigen-positive pDCs in regions of cellular infiltrate in the dermis of VZV infected skin (113). Notably the subcellular localization of VZV antigen staining within these DC subsets was consistent with replicating virus, indicating these cells are productively infected *in vivo* (113). We extended these analyses to demonstrate that pDCs and MUTZ-3-derived LC *in vitro* are permissive to productive VZV infection (113). Furthermore, Gutzeit and colleagues demonstrated that human skin LCs and dermal conventional DCs isolated *ex vivo*, when exposed to a virulent VZV strain or v-OKA, were susceptible to VZV infection (127). Together, these *in vitro* and *in vivo* based reports highlight the permissiveness of a range of DC subsets to VZV. The next key question is whether virus infection of these DC subsets impacts their functionality.

VZV Modulation of MDDC Function

VZV infection of human DCs has been shown to result in the modulation of cell-surface receptor phenotype and immune functions. Mature MDDCs, like their immature counterparts, are also susceptible to productive VZV infection (10) which results in the selective downregulation of key cell-surface immune molecules such as MHC I, CD80, CD83, and CD86. The cumulative effect is reduced stimulation of allogeneic T cells, thus indicating VZV actively manipulates the functional capacity of DCs by rendering them as inefficient activators of T cells (10). It has been previously reported that VZV ORF66, a protein kinase, has the ability to retain MHC I molecules in the Golgi of infected fibroblasts and MeWo cells (131, 132). However, the viral gene product(s) and molecular mechanism by which VZV modulates cell-surface immune molecule expression on mature MDDCs has yet to be elucidated.

Moreover, VZV has been reported to reduce cell-surface expression of apoptosis receptor Fas on infected immature and mature MDDCs, whereas surface levels of MHC II remain unchanged (128). However, the mechanism of Fas regulation in MDDCs is currently unknown. VZV infected immature MDDCs are unable to upregulate the functionally important immune molecules CD80, CD83, CD86, MHC I, and CCR7, which are essential for DC maturation and induction of an effective anti-viral responses (9). The NFκB pathway largely regulates the expression of these immune molecules. Interestingly, VZV has been shown in human epidermal and MDDCs to directly interfere with the host cell NFκB pathway by sequestering NFκB proteins within the cell cytoplasm (87, 133). Furthermore, the E3 ubiquitin ligase domain of VZV ORF61 was required to modulate this pathway, downstream of triggering receptors TLR3, TLR8, and TLR9 (87). Use of the SVV model indicated that SVV, like VZV, can prevent ubiquitination of IκBα and additionally prevents the phosphorylation of IκBα (134). This study also revealed that in addition to SVV ORF61, SVV is likely to encode additional modulators of NFκB signaling, as an ORF61 deletion virus retained its capacity to prevent IκBα phosphorylation and degradation. Thus, it remains possible that both VZV and SVV encode additional ORFs that afford evasion of NFκB signaling.

VZV Modulation of pDC Function

VZV infection of pDCs and epidermal cells has been observed to occur in the absence of an increase in the type I cytokine IFNα production (16, 113). This is of particular interest for pDCs, as a distinctive functional characteristic is their potent ability to synthesize IFNα following virus infection. Significantly, VZV infected pDCs remain refractory to IFNα production, even when stimulated with a TLR-9 agonist. In the future, it will be important to further define the mechanistic basis of VZV modulating IFNα production by pDCs and identify any viral gene(s) which encode this function. Additionally, pDC also secrete cytokines and chemokines that stimulate activation of effector cells, including B cells, T cells, NK, NKT cells, and also function to present viral antigen to T lymphocytes (135, 136). Elucidating whether VZV interferes with these other pDC functions during infection will therefore be an important consideration of studies to fully define the functional impact of VZV infection of pDCs.

Interestingly, Gutzeit and colleagues reported the secretion of signature Th1 cytokines (IFNγ and IL-12) was enhanced following infection of MDDCs with (v-OKA) but blocked by a VZV clinical isolate. This impairment of IL-12 secretion was shown to be due to a viral disruption of signaling downstream of TLR2, and proposed to be most likely caused by a VZV glycoprotein within the virion envelope (127). Thus, VZV subversion of the Th1-promoting instruction of human DCs is

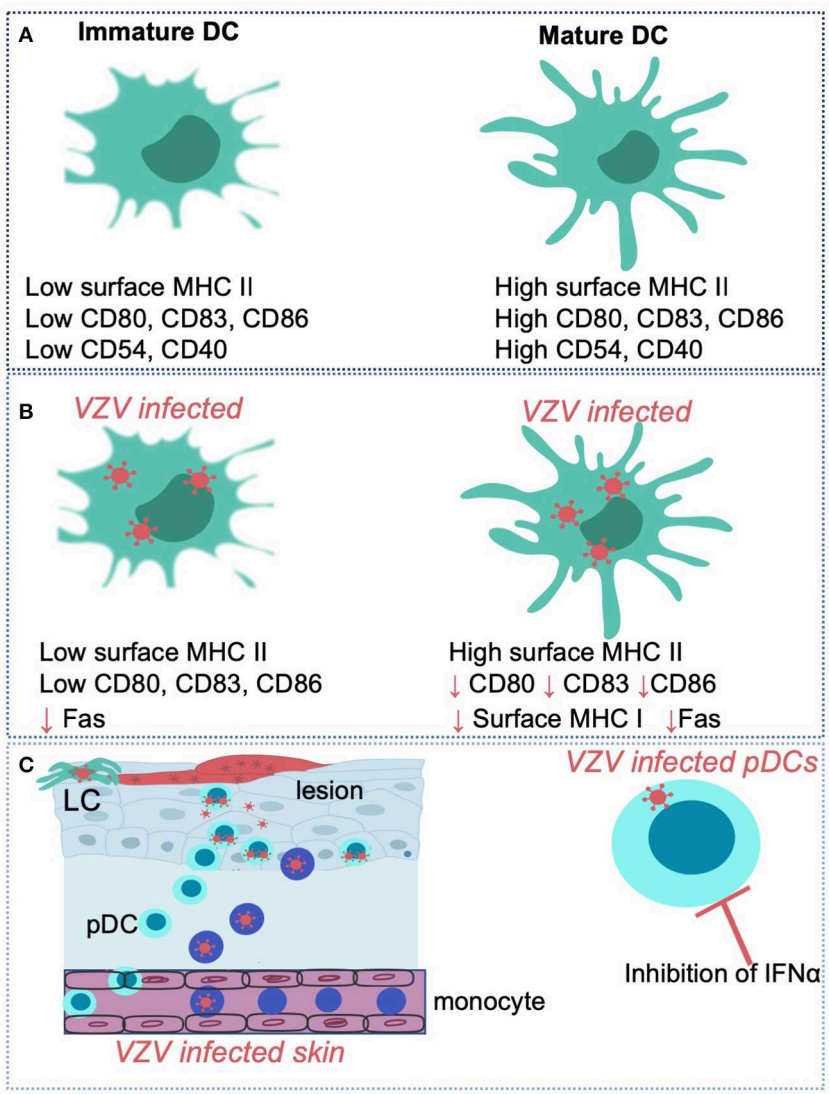

FIGURE 3 | VZV interactions with human dendritic cell subsets and monocytes Immature dendritic cells (DC) are distinguishable from mature DC via differing expression levels of surface markers such as MHC II, CD80, CD83, CD86, CD54, and CD40 **(A)**. VZV has been shown to productively infect human immature and mature monocyte derived dendritic cells and selectively regulate expression of key cell-surface molecules such as CD80, CD83, and CD86 in virus infected cells **(B)**. VZV can also productively infect human Langerhans cells (LCs) an plasmacytoid dendritic cells (pDCs) in the skin **(C)**. VZV infection of pDCs *in vitro* results in the inhibition of IFNα production. VZV also productively infects human monocytes and macrophages in culture.

a novel immune evasion mechanism of clinical VZV isolates. In sum, VZV has encoded a plethora of immune evasion tactics when engaging with various DC subsets (**Figure 3**). It remains important to further elucidate the molecular mechanisms as well as define the viral proteins directly responsible for these immune evasion strategies. VZV like other herpesvirus family members is likely to encode more than one strategy to manipulate DC functions to provide a transient advantage to the virus.

VZV INFECTION AND MANIPULATION OF MONOCYTES AND MACROPHAGES

Monocytes and macrophages play a key role in pathogen sensing, immune defense against infection and are important players in resolving inflammation (137). These cells are capable of potent inflammatory and anti-inflammatory responses that define the activation and suppression of a broad range of immune cells (138). There are several different types of macrophages which can be found at various sites within the host and how they respond to different viruses may vary. Given their location in circulation, migratory capacity and tissue-residency, these cell types are highly likely to interact with VZV during the early innate response.

VZV viremia is associated with primary VZV infection and reactivation, and the interaction between VZV and mononuclear cells during these stages of infection has been well-documented (139–141); reviewed in White and Gilden (142). VZV DNA is observable in many mononuclear cell subsets, although few were extensively characterized (19, 143–146). Previously, little focus was drawn on the susceptibility of individual subsets of mononuclear cells to VZV infection, with monocytes and macrophages being no exceptions. Although magnetically isolated $CD14^+$ cells from varicella patients harbor detectable copies of VZV ORF62 and VZV gB transcripts (147), original studies exposing primary isolated human monocytes to VZV did not corroborate these findings, suggesting that VZV infection in monocytes was abortive (148, 149). Interestingly, further studies went on to detect VZV gE expression on $CD4^-/CD8^-$ populations of mononuclear cells which were presumed to be monocytes (144). This was subsequently substantiated by a series of reports by Köenig and co-workers, who isolated monocytes from fresh PBMCs and identified VZV gE expression by IFA (54).

More recently however, our laboratory performed an investigation into the susceptibility of human monocytes and macrophages to VZV infection. We reported productive VZV infection of both freshly isolated human monocytes and differentiated macrophages (56). Interestingly, macrophages were highly permissive to VZV infection. This report went on to address the influence of VZV infection of these cell types, indicating that VZV infection influences the antigen presentation potential of monocytes, and predicted that VZV infection substantially impacts monocytes longevity and subsequent ability to generate site-specific macrophages. The failure of VZV infected monocytes to differentiate into monocyte derived macrophages is likely due to reduced viability of infected cells and not the inability of macrophages to support a productive infection. The capacity of VZV to productively infect and modulate the function of monocytes may enhance the ability of VZV to establish an infection in the host.

This work was corroborated by a report demonstrating VZV infection of monocytes, NKT cells and B lymphocytes (150) and by productive infection of a THP-1 monocytic cell line (89). Although evidence suggesting monocyte differentiation to macrophage may be influenced by VZV infection *in vivo*, macrophage infection *in vitro* has previously been observed (56, 148). As such, it is likely that although monocytes and macrophages represent a dynamic axis for the induction and maintenance of anti-viral states, VZV is able to counteract this effective branch of the innate immune system through direct infection and immune evasion strategies.

NK CELLS AND VZV: CONTROL AND EVASION

NK cells are innate cytotoxic lymphocytes that play a significant role in the immune response against viral infection (151). In peripheral blood, NK cells represent ∼5–15% of circulating lymphocytes, while also populating additional key sites for anti-viral immunity such as tonsils, lymph nodes, spleen, lungs, and bone marrow. NK cells can rapidly migrate to sites of inflammation where their activity toward infected cells is mediated by the integration of signals from germline-encoded activating and inhibitory receptors. Activated NK cells will release cytotoxic granules containing perforin and granzymes across the immune synapse, triggering lysis of the infected cell. Additionally, NK cells are potent producers of pro-inflammatory cytokines, such as IFNγ and tumor necrosis factor (TNF).

Importance of NK Cells in the Control of VZV Infection

The significance of NK cells in the control of VZV infection is particularly apparent in cases of NK cell deficiency. A common motif in individuals with NK cell deficiencies is increased susceptibility to developing severe, often fatal, herpesvirus infections, especially VZV disease (152–157). These case studies indicate that robust NK cell immunity is required for the control of VZV infection. In immunocompetent hosts, several reports have documented increased frequencies of NK cells (158–161), suggesting an active response to infection. Furthermore, in a study of life-threatening varicella cases it was reported that circulating NK cell numbers were significantly lower compared to cases of mild infection, with counts subsequently normalizing during convalescence (160). Recently it has also been demonstrated that NK cells can be rapidly recruited to sites of VZV antigen challenge in previously exposed hosts (162). *In vitro* experiments have also demonstrated that VZV infected cells are sensitive to granulysin (163)–a cytotoxic protein secreted by NK cells as well as cytotoxic T cells. Together, these observations imply a central role for NK cells in the anti-viral immune response to VZV.

While NK cells constitute a key arm of the early innate immune response, VZV can also infect NK cells, potentially

using them to disseminate virus (114, 150). During primary infection, the spread of VZV to different sites in the body is considered to be facilitated by the migration of infected T cells (15, 16). This has been supported by clinical observations of immunocompetent patients with varicella, where VZV could be cultured from PBMCs with lymphocyte morphology isolated during the early stages of infection (144, 164). Later reports then sought to confirm VZV infection of T cells and B cells in patients with varicella (146, 147, 165), and extensive studies have since elegantly investigated the role of T cells in VZV infection (166). However, reports identifying T cell and B cell infection overlooked the third major lymphocyte population present in peripheral blood–NK cells. It is likely that the delayed development of the NK cell field in comparison to the fields of T cell and B cell immunology accounts for these earlier studies failing to acknowledge a possible role for NK cells in VZV pathogenesis. Work from our laboratory demonstrated that human NK cells, in particular the $CD56^{dim}$ subset which predominates in blood, are highly permissive to productive infection with both clinical and vaccine strains of VZV (114). Moreover, VZV infected NK cells are capable of transmitting infection to epithelial or fibroblast cells in culture and can upregulate skin-homing chemokine receptors, suggesting a potential role in viral dissemination during pathogenesis (114). Jones and co-workers in a later study also demonstrated VZV infection of PBMC derived NK cells (150). A case report of severe, persistent varicella identified VZV DNA in NK cells, amongst other lymphocyte populations (161), however targeted investigation of NK cell infection in additional varicella patients is needed to corroborate the *in vitro* findings.

VZV Manipulation of NK Cell Function

VZV encodes a number of immune modulatory components to interfere with NK cell detection of infected target cells. Like all other herpesviruses, VZV downregulates the expression of MHC I on the surface of infected cells, which would limit effective $CD8^+$ T cell detection of infection (131, 132, 167). However, in response to this common evasion strategy, the immune system counterbalances with NK cell activity which is activated in the absence of cell-surface MHC I. Further modulation of the infected cell-surface is thus required for the virus to reduce detection and clearance by both T cells and NK cells. Specifically, VZV has been shown to reduce cell-surface expression of ULBP2 and ULBP3 (168)–two of eight human ligands detected by the ubiquitously expressed activating NK cell receptor, NKG2D. Interestingly, a third NKG2D ligand, MICA, was found to be upregulated at the total protein level and on the cell-surface of VZV infected cells (168). The differential regulation of NKG2D ligands by VZV is evidence of the dynamic interplay between the virus and NK cell-mediated immune control (**Figure 4**). Additional evasion of NK cell activity is likely to occur through the downregulation of intracellular adhesion molecule 1 (ICAM-1) (129, 169), which is required for NK and T cell adhesion to target cells to form an immune synapse and clear infected cells. *In vitro* assays have demonstrated that NK cell activity is not enhanced when co-cultured with VZV infected target cells (168), suggesting that VZV sufficiently modulates interactions with NK cells to limit detection and activation. Given the pronounced modulation of these NKG2D ligands and ICAM-1 it will be important for future studies to identity the viral gene products responsible and their mechanisms of action.

In addition to lysing target cells through receptor–ligand interactions, NK cells can also mediate target cell death through antibody-dependent cell-mediated cytotoxicity (ADCC). Expression of CD16 (FcγRIII) on NK cells allows engagement of IgG antibodies bound to a target cell, which typically occurs during anti-pathogen immune responses. VZV infected and bystander NK cells, however, potently downregulate cell-surface expression of CD16 (114), which would hinder ADCC function. Notably, this observation has also recently been documented *in vivo* where CD16 expression was significantly reduced on NK cells that had infiltrated the site of VZV antigen challenge (162). A third mechanism of NK cell cytotoxicity is achieved through Fas–Fas ligand (FasL) interactions which stimulate apoptosis of the Fas-expressing cell. VZV has been shown to reduce cell-surface expression of Fas on infected DCs (128), which would limit NK cell induction of apoptosis in these infected cells. Additionally, VZV infected NK cells themselves have been reported to upregulate expression of programmed death ligand-1 (PD-L1) (150), potentially impeding effective immune responses through the inhibitory signal this transmits. Overall, these alterations to the cell-surface landscape of infected cells are likely to protect VZV from effective immune clearance by NK cells.

Not only does VZV regulate detection of infected cells, we have recently shown that it directly impairs NK cell function (**Figure 4**). Both infected NK cells and those merely exposed to VZV in co-culture are rendered unresponsive to subsequent target cell stimulation (115). This potent paralysis of NK cell function was found to be dependent on direct contact between NK cells and VZV infected cells. In support of this finding, a report of patients with herpes zoster observed impaired NK cell activity against target cells (158). More recently, decreased serum levels of granulysin has also been reported in varicella patients (170). As the cell count of circulating NK cells was unchanged in these patients, it was suggested that NK cell activity was decreased during varicella, which supports the *in vitro* characterization of inhibited NK cell function by VZV.

Lastly, an important function of NK cells is the secretion of immune modulating cytokines. In relation to the control of VZV, IFNγ, and TNF are readily secreted by NK cells and have strong inhibitory effects on VZV replication (119, 171, 172). These cytokines are also found to be elevated in the serum of varicella patients (173, 174). Despite this, it has been demonstrated *in vitro* that VZV diminishes NK cell secretion of both IFNγ and TNF (115) (**Figure 4**). This serves as another example of the dichotomy between immune activity necessary for control of VZV and the evasion strategies employed by the virus. As genetically plastic pathogens, viruses only maintain genes of benefit to the survival of the virus, and thus the extent of evasion strategies that subvert NK cell immunity indicates the significance of this cell type in controlling VZV infection. Despite this, our understanding of how VZV interacts with NK cells is only beginning, with many of the most extensive studies on this

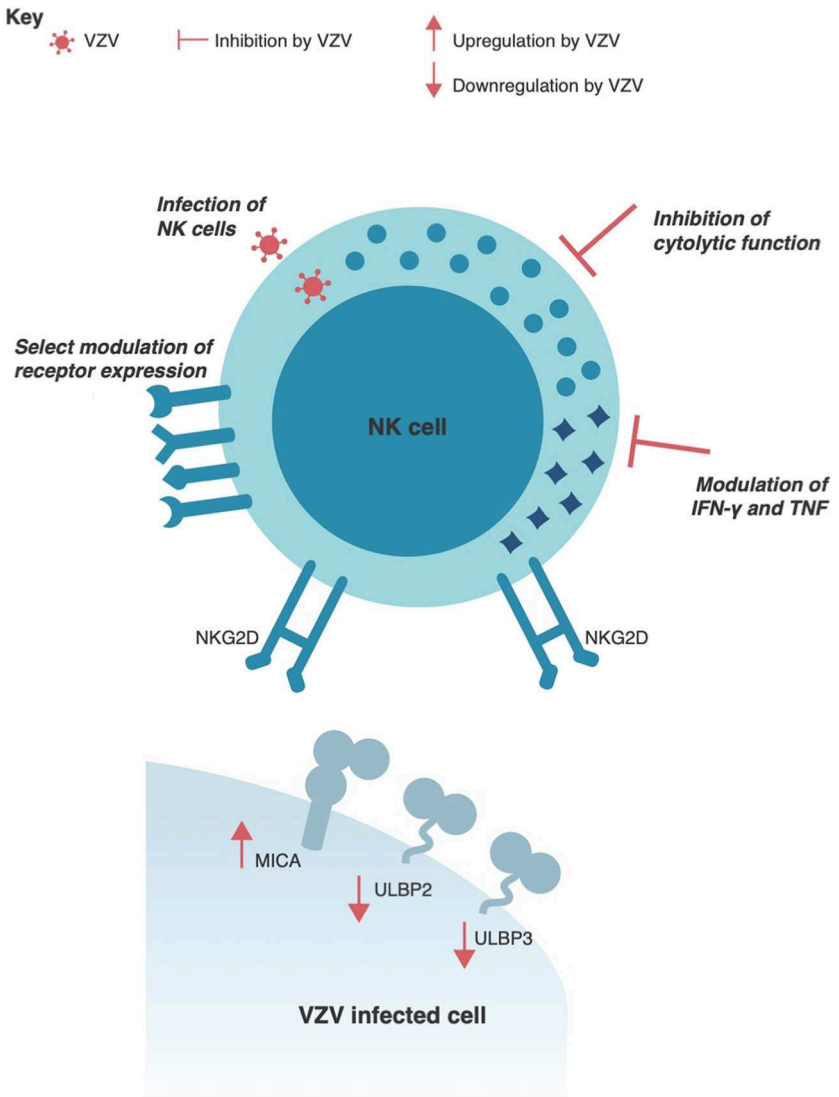

FIGURE 4 | VZV interactions with human Natural Killer cells. VZV has been shown to selectively regulate expression of NKG2D ligands, such as MICA, ULBP2, and ULBP3 in virus infected cells. VZV can also productively infect human NK cells and directly interfere with NK cell function by inhibiting the cytolytic response and modulating IFNγ and TNF cytokine production. Additionally, VZV infection can selectively modulate receptor expression on NK cells.

topic being published in only the last few years. It is likely that we still have much to uncover about the complex interplay between NK cells and VZV.

CONCLUDING REMARKS

VZV has co-evolved with the human host for millions of years (175). In that time there has likely been a dynamic interplay between the emergence of host anti-viral immune responses and subsequently viral mechanisms to evade these defenses. Sensing of viral components and subsequent host cell damage can initiate cell death, the production of type I IFN and pro-inflammatory cytokines to restrict viral spread. VZV produces multiple ORFs such as ORF12, ORF66, and ORF63 to inhibit apoptosis in cells critical for viral dissemination and the establishment of life-long latency. Additionally, VZV can interfere with the type 1 IFN pathway and the production of pro-inflammatory cytokines through the inhibition of pathway components such as IRF3 and NFκB. With the production of pro-inflammatory cytokines and chemokines innate immune cells such as monocytes, macrophages, DCs, and NK cells can target VZV infected cells. VZV has been shown to infect these key immune cells and is able to modulate their function. In this respect, VZV infection modulates expression of key cell-surface immune molecules on DCs, impacts their APC capacity. Furthermore, VZV infection influences the antigen presentation potential of monocytes, and substantially impacts monocytes longevity and ability to

generate site-specific macrophages. Recently, VZV was shown to functionally impair NK cells in both their ability to secrete cytokines and lyse virally infected target cells through NK cell dependent cytotoxicity.

There are still many areas of VZV encoded innate immunity manipulation that warrant further investigation. For example, exploring whether VZV protect against other forms of cell death, as when apoptosis is inhibited other cell death forms can occur to limit viral spread. Additional study will be required to fully define the role of type III IFNs during VZV infection. Specifically it will be of interest to understand the distinct activities of type I, II, and III IFNs in regulating infection as this will potentially dissect the roles played by distinct IFNs in regulating infection during different phases of the viral lifecycle. Despite VZV being shown to modulate immune functions of different DC subsets, the molecular mechanisms and VZV proteins directly responsible for these immune evasion strategies has yet to be elucidated. Finally, recent data showing NK cells and other immune cells within PBMC compartment can be infected with VZV provides an avenue to gain a deeper understanding of the impact VZV infection has on immune cell functions and the importance of these cells in viral pathogenesis.

Modulation of the innate immune response ultimately effects the formation and effectiveness of the adaptive immune response. Therefore, it is clear VZV can modulate components of the intrinsic, innate and adaptative immune response to ensure viral dissemination and the establishment of life-long latency. It is critical to dissect the mechanisms of this immunomodulation to provide important insights into VZV pathogenesis which will likely be of benefit when designing new generation vaccines and anti-virals. Furthermore, the study of herpesvirus modulation of immune responses also enhances our general understanding of the complexity of the human immune system.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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One Step Ahead: Herpesviruses Light the Way to Understanding Interferon-Stimulated Genes (ISGs)

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The host immune system is engaged in a constant battle with microorganisms, with the immediate detection of pathogenic invasion and subsequent signalling acting as crucial deterrents against the establishment of a successful infection. For this purpose, cells are equipped with a variety of sensors called pattern recognition receptors (PRR), which rapidly detect intruders leading to the expression of antiviral type I interferons (IFN). Type I IFN are crucial cytokines which exert their biological effects through the induction of hundreds of IFN-stimulated genes (ISGs). The expression profile of these ISGs varies depending on the virus. For a small subset of ISGs, their anti- or even proviral effects have been revealed, however, the vast majority are uncharacterised. The spotlight is now on herpesviruses, with their large coding capacity and long co-evolution with their hosts, as a key to understanding the impact of ISGs during viral infection. Studies are emerging which have identified multiple herpesviral antagonists specifically targeting ISGs, hinting at the significant role these proteins must play in host defence against viral infection, with the promise of more to come. In this review, we will discuss the current knowledge of the complex interplay between ISGs and human herpesviruses: the antiviral role of selected ISGs during herpesviral infections, how herpesviruses antagonise these ISGs and, in some cases, even exploit them to benefit viral infection.

Keywords: ISG, interferon, herpesvirus, immune evasion, innate immunity, HSV-1, HCMV, KSHV

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INTRODUCTION

The *Herpesviridae* is a family of large, structurally complex viruses with double-stranded DNA genomes. This family is classified into three subfamilies according to biological and genomic similarities: *alphaherpesvirinae*, *betaherpesvirinae*, and *gammaherpesvirinae* (Pellett and Roizman, 2007). Several viruses with significant medical relevance are represented in this family, which cause a series of maladies ranging from cold sores or fever blisters to a variety of human cancers. A distinctive feature of herpesviruses is their ability to establish lifelong latent infections, with infected individuals serving as reservoirs from which period reactivation leads to continual and anew transmission to naive hosts.

Herpesviruses are known for the impressive toolbox they have evolved to circumvent the host's immune response. Throughout the lifelong coexistence with their hosts, herpesviruses antagonise the immune response at every level: the signalling pathways downstream of pattern recognition receptors (PRR) (reviewed in Liu et al., 2019; Stempel et al., 2019) and the IFNα/β receptor

(IFNAR) (Zimmermann et al., 2005), Natural Killer cell responses (reviewed in De Pelsmaeker et al., 2018), the complement system (reviewed in Stoermer and Morrison, 2011) and the adaptive immune response (reviewed in Smith and Khanna, 2013). However, our understanding of the interplay between herpesviruses and the interferon-stimulated gene (ISG) network is only in its infancy. So far, more than 380 human ISGs, with their functions ranging from sensors, cytokines or transcription factors, to proapoptotic proteins or negative regulators, have been tested for their ability to inhibit the replication of a panel of RNA viruses, revealing that different viruses are targeted by unique sets of ISGs (Schoggins et al., 2011). Such a screen has not been performed for the different members of the *Herpesviridae*, however, recent studies have identified multiple herpesviral antagonists which target ISGs, showcasing the importance of ISGs in combating herpesviral infection.

In this review, we will discuss the current knowledge regarding the complex interaction between ISGs and human herpesviruses and highlight how each subfamily of human herpesviruses has evolved unique mechanisms to counteract ISGs or, in some cases, even exploit ISGs to the advantage of the virus (**Figure 1**).

HOW IT ALL STARTS: ISGs ENTER THE GAME

The DNA sensing pathway mediated by the PRR cyclic GMP-AMP synthase (cGAS) and gamma-interferon-inducible protein 16 (IFI16) is crucial for the initial immune response to herpesviral infection in many cell types (Ablasser et al., 2013; Li et al., 2013; Wu et al., 2015; Paijo et al., 2016). The DNA sensors cGAS and IFI16 bind to viral and aberrantly localised cellular DNA. This interaction activates a signalling cascade through the adaptor protein stimulator of interferon genes (STING) and TANK-binding kinase 1 (TBK1), thereby activating the transcription factors interferon regulatory factor 3 (IRF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (reviewed in Chen et al., 2016). This leads to the induction of type I interferons (IFN), which exert their activity in an autocrine and paracrine manner. By binding to the interferon-α/β receptor (IFNAR), these cytokines lead to phosphorylation and activation of the transcription factors signal transducers and activators of transcription, STAT1 and STAT2, recruiting IRF9 into the complex which then translocates to the nucleus, resulting in ISG expression (reviewed in Schneider et al., 2014). Another class of ISGs, known as non-canonical ISGs, are activated directly by IRF3 in the absence of type I IFN (Schoggins et al., 2014). For a third class of proteins classified as ISGs, the presence of IRF3 or type I IFN is not an absolute prerequisite for their expression, since they are already expressed basally or their expression is induced by other pathways, i.e., NF-κB signalling (reviewed in Schoggins, 2019). Thus, due to this complexity and the wide range of functions that ISGs can exert, studying how herpesviruses manipulate ISGs to their advantage serves as a window into a greater understanding of the myriad of ISGs and their role in innate immunity. Similar to the studies on ISGs and RNA viruses (Schoggins et al., 2011), studies identifying how herpesviruses inhibit or exploit the function of ISGs may reveal the essential nature of the role these ISGs play in viral defence.

WHEN HERPESVIRUSES WIN: ESCAPING THE ANTIVIRAL EFFECTS OF ISGs

Herpes Simplex Virus 1

Herpes simplex virus 1 (HSV-1) belongs to the *Alphaherpesvirinae* subfamily. HSV-1 establishes a primary infection in mucosal epithelia and a latent infection in the ganglia of sensory nerves. This infection, as in the case of all herpesviruses, can be asymptomatic, but it may also present as acute gingivostomatitis. Furthermore, HSV-1 can lead to serious illnesses like ophthalmic infections, meningitis or encephalitis (Pellett and Roizman, 2007). Recently, HSV-1 has also been associated as a major risk factor for Alzheimer's disease (Itzhaki, 2018).

Targeting DNA Sensing: ICP0 Degrades the ISG IFI16

Herpesviruses replicate in the nucleus of their host cells. The ISG IFI16 is a cellular DNA sensor localized in the nucleus of many cell types (Unterholzner et al., 2010; Duan et al., 2011; Veeranki and Choubey, 2012; Jonsson et al., 2017). Orzalli et al. (2012) demonstrated that HSV-1 targets the IFI16 protein. During infection, when HSV-1 expresses the immediate-early viral protein ICP0 in the nucleus of human foreskin fibroblasts (HFF), IFI16 undergoes a change in its location and is continuously degraded. However, another study found that the expression of ICP0 alone is neither sufficient nor necessary for degradation of IFI16 in the tumor-derived cell line U-2 OS, since infection with an ICP0-null mutant still resulted in IFI16 degradation (Cuchet-Lourenco et al., 2013). A follow-up study by Orzalli et al. (2016) clarified this discrepancy by showing that IFI16 protein levels decrease upon HSV-1 infection in HFF, keratinocytes (NOK), and HeLa cells, but not in the U-2 OS cell line, and additionally discovered that ICP0 is not the only protein involved in IFI16 degradation (**Table 1**). This suggests that the role of ICP0 for IFI16 degradation is dependent on the cell type and other cellular or viral factors contributing to IFI16 stability (Kalamvoki and Roizman, 2014).

Degrading ISG mRNA: UL41 Counteracts ZAP and IFIT3

The HSV-1 tegument protein UL41, also known as virion host shutoff (vhs) protein, is an endoribonuclease that degrades mRNA (Everly et al., 2002; Page and Read, 2010). It is proposed that viral and cellular mRNAs containing AU-rich elements (ARE) in the 3′-untranslated region (3′-UTR) are the preferred target for UL41 (Esclatine et al., 2004; Taddeo and Roizman, 2006). Since ARE regions are frequently present in transcripts connected to the immune response, including interferons and chemokines (Bakheet et al., 2001), UL41 could potentially target a broad spectrum of transcripts. To date, the zinc finger CCCH-type antiviral protein 1 (ZAP) and the ISG interferon-induced protein with tetratricopeptide repeats 3 (IFIT3) are two ISGs

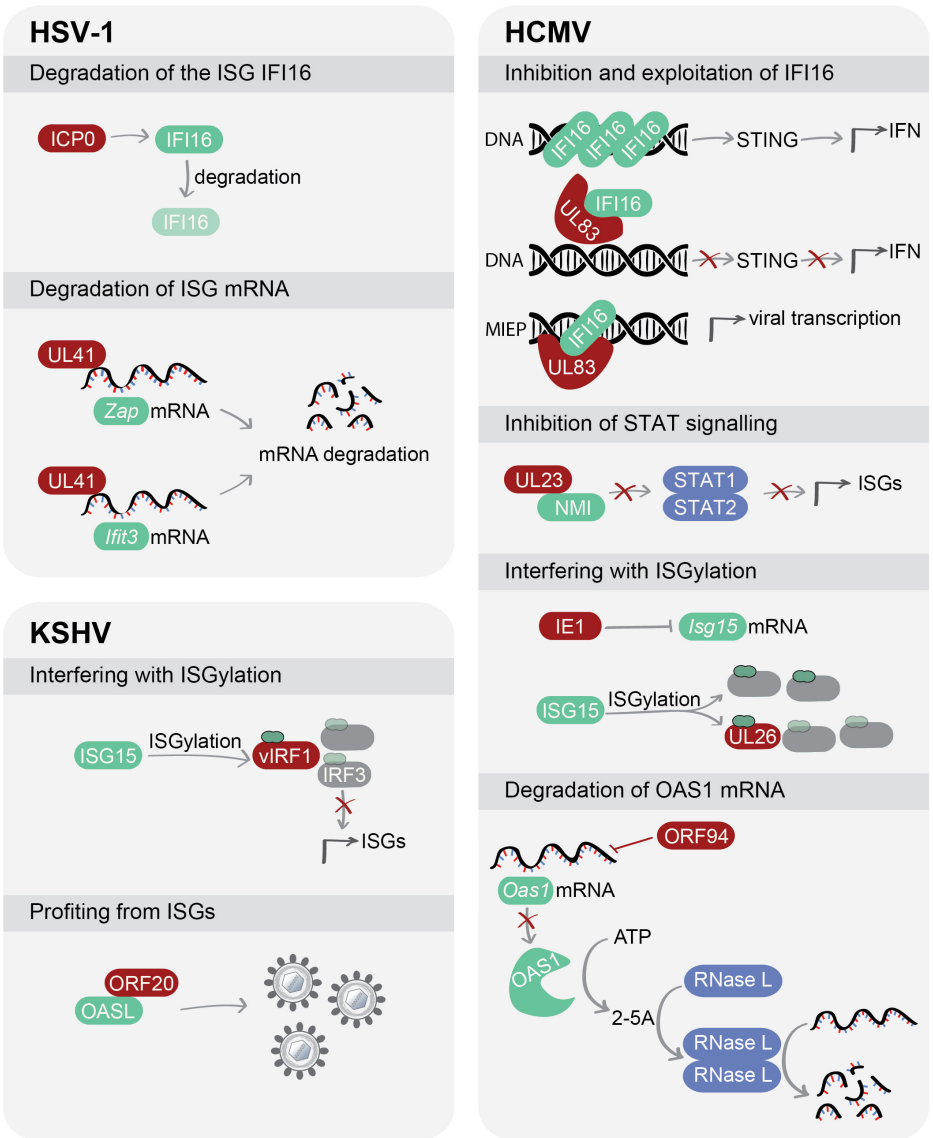

FIGURE 1 | Herpesviruses use a variety of strategies to manipulate ISGs. Viral proteins can interfere with protein expression and stability of ISGs, inhibit signalling pathways exerted by ISGs or, in some cases, exploit ISGs for their own benefit. Viral proteins are depicted in red, while ISGs are shown in green. Abbreviations: HSV-1, herpes simplex virus type 1; HCMV, human cytomegalovirus; KSHV, Kaposi's sarcoma-associated herpesvirus; IFI16, gamma-interferon-inducible protein 16; STING, stimulator of interferon genes; IFN, interferon; ZAP, zinc finger CCCH-type antiviral protein; IFIT3, interferon-induced protein with tetratricopeptide repeats 3; NMI, N-myc-interactor; STAT1/2, signal transducer and activator of transcription 1/2; ISG15, interferon-stimulated gene 15; IRF, interferon regulatory factor; OASL, 2′-5′-Oligoadenylate synthetase like; OAS1, 2′-5′-Oligoadenylate synthetase 1; RNase L, ribonuclease L.

that contain ARE in their 3′-UTR which have been shown to be incapacitated by UL41 (**Figure 1**).

The zinc finger CCCH-type antiviral protein is a non-canonical ISG (Schoggins et al., 2014), meaning that its expression can also be induced in the absence of type I IFN production. ZAP exerts antiviral activity against a diverse range of viruses such as retroviruses, alphaviruses, filoviruses, hepatitis B virus and Japanese encephalitis virus by binding to RNA and indirectly mediating its degradation (Bick et al., 2003; Muller et al., 2007; Zhu et al., 2011; Mao et al., 2013; Takata et al., 2017; Chiu et al., 2018). However, ZAP fails to control other viruses, e.g., influenza A virus (Liu et al., 2015; Tang et al., 2017) or enterovirus A71 (Xie et al., 2018).

In the case of HSV-1, ectopic expression of both rat and human forms of ZAP does not affect HSV-1 infection (Bick et al., 2003), which suggested that a viral antagonist may counteract the antiviral activity of ZAP. Accordingly, a luciferase-based assay in 293T cells identified the HSV-1 UL41 protein as a ZAP antagonist (Su et al., 2015). In accordance with previous observations regarding the nuclease activity of UL41, this viral protein was shown to degrade ZAP mRNA during HSV-1 infection. Correspondingly, growth of a mutant virus

TABLE 1 | Viral antagonists of interferon-stimulated genes (ISGs).

Virus	Strain	Viral antagonist	Target ISG	Cell type	Viral evasion strategy	References
HSV-1	KOS	ICP0	IFI16	HFF	Degrades IFI16	Orzalli et al., 2012
	17+	ICP0	IFI16	U-2 OS	Does not degrade IFI16	Cuchet-Lourenco et al., 2013
	KOS, 17, F	UL41 (vhs)	IFI16	HFF NOK, HeLa, U-2 OS	ICP0 degrades IFI16 in a cell-type specific manner; UL41 also reduces protein levels of IFI16 (presumably by RNA degradation)	Orzalli et al., 2016
	F	UL41	ZAP	293Trex-hZAPL/S	Degrades ZAP mRNA through its endoribonuclease activity, preferentially binds ARE motifs	Su et al., 2015
	F	UL41	IFIT3	293T	Decreases IFIT3 expression levels by degrading IFIT3 mRNA, does not target IFIT1 or IFIT2	Jiang et al., 2016
HCMV	AD169	UL83 (pp65)	IFI16	HFF	Interacts with IFI16 to block its oligomerisation and prevents signalling; promotes transcription of immediate early genes by exploiting the binding capacity of IFI16 to DNA	Li et al., 2013
	TB40/E					Biolatti et al., 2016
	AD169					Cristea et al., 2010
	Towne (BAC-derived)	UL23	NMI	U251	Inhibits ISG transcription by binding to NMI and disrupting its association with STAT1	Feng et al., 2018
	Towne	IE1 (UL123)	ISG15	HF	Inhibits HCMV-induced ISG15 expression and thereby prevents ISGylation	Kim et al., 2016
	AD169	UL26	ISG15	HF	Reduces the accumulation of ISGylated proteins by acting as a decoy target for ISG15	Kim et al., 2016
	Towne	ORF94 (UL126a)	OAS1	HF	Inhibits mRNA and protein expression of OAS1, leading to reduced viral RNA degradation	Tan et al., 2011
KSHV	iSLK.219 harbouring rKSHV.219*	vIRF1	ISG15	293, 293-TLR, BCBL PEL, iSLK.219	Reduces ISGylation of cellular target proteins, leading to IRF3 instability and decreased ISG transcription; acts as a decoy target for ISG15	Jacobs et al., 2015
	HuARLT2 harbouring rKSHV.219*	ORF20	OASL	293T, HeLa, HFF, HuARLT2-rKSHV.219	ORF20 and OASL interact; ORF20 increases RIG-I dependent OASL expression; OASL and ORF20 concomitantly enhance KSHV infection	Bussey et al., 2018

**These studies used iSLK or HuARLT2 cells that were latently infected with recombinant rKSHV.219 (Vieira and O'Hearn, 2004; Myoung and Ganem, 2011; Lipps et al., 2017). HFF, human foreskin fibroblasts; HF, human fibroblasts.*

lacking UL41 expression was impaired in the presence of ZAP (Su et al., 2015).

Similarly, IFIT3 was reported to have no effect on HSV-1 infection (Jiang et al., 2016). As for ZAP, human IFIT proteins with the family members IFIT1, IFIT2, and IFIT3 belong to the subgroup of non-canonical ISGs (Schoggins et al., 2014). IFIT3 mediates the association of TBK1 with mitochondrial antiviral-signalling protein (MAVS) at the mitochondria (Liu et al., 2011), enhancing the MAVS-TBK1 signalling axis. Notably, IFIT3 inhibits the replication of HSV-1 lacking UL41 expression, underlining the importance of UL41 in evading the antiviral effect of IFIT3. The authors showed that UL41 degrades IFIT3 mRNA, but not that of IFIT1 or IFIT2 (Jiang et al., 2016), indicating that HSV-1 may specifically target IFIT3 to prevent the MAVS-TBK1 association, thus suppressing downstream signalling.

Human Cytomegalovirus

Human cytomegalovirus (HCMV), also called human herpesvirus 5 (HHV-5), is a member of the *Betaherpesvirinae* subfamily. HCMV usually causes only mild disease in immunocompetent individuals. However, in immunosuppressed individuals such as AIDS or transplant patients, HCMV infection can cause severe complications (reviewed in Arvin and National Center for Biotechnology, 2007). HCMV infection during pregnancy can cause long-term sequelae in newborns, such as hearing loss, vision abnormalities, microcephaly or global development delays.

Targeting DNA Sensing: UL83 Hijacks the ISG IFI16

Human cytomegalovirus, as for HSV-1, interferes with DNA sensing by targeting IFI16 via the UL83 encoded tegument protein pp65. Upon HCMV infection, IFI16 is activated in the nucleus and undergoes oligomerisation, which is a prerequisite for it to promote the immune response (Cristea et al., 2010; Li et al., 2013). Accordingly, siRNA-mediated silencing of IFI16 dampens cytokine transcription in response to HCMV infection (Li et al., 2013). However, UL83 prevents IFI16 oligomerisation, thus disarming the antiviral effect of IFI16 during HCMV infection (Li et al., 2013). UL83 even goes a step further in its manipulation of host responses: it exploits the binding capacity of IFI16 to DNA in order to form a complex with the major immediate early promoter (MIEP) of HCMV, thereby triggering viral transcription in the early stages of infection (Cristea et al., 2010; Biolatti et al., 2016). In this manner, UL83 not only prevents the antiviral activity of IFI16 but also hijacks it to promote HCMV gene expression (**Figure 1**). This viral protein serves as a stellar example of the resourcefulness of herpesviruses in encoding a protein that can simultaneously inhibit a host antiviral strategy while

exploiting this same host response factor to promote its own replication.

Fooling the Type I IFN Response: UL23 Inhibits ISG Transcription by Targeting the ISG NMI

The ISG N-myc interactor (NMI) interacts with all STATs, except STAT2, and enhances the recruitment of co-activators, such as the transcription factors CREB-binding protein (CBP)/p300, to the STAT complex. NMI specifically modulates IFN-induced signalling to foster efficient STAT-dependent transcription (Zhu et al., 1999). Recently, the HCMV tegument protein UL23 was reported to inhibit the transcription of ISGs by targeting NMI. Through a yeast two-hybrid screen, NMI was identified as an interacting partner of UL23, which was confirmed by co-immunoprecipitation in HCMV-infected U251 cells (Feng et al., 2018). Using a combination of immunofluorescence, cell fractionation and immunoblotting, the authors showed that the binding of UL23 to NMI disrupts its association with STAT1, thereby preventing the translocation of both proteins to the nucleus (**Figure 1**). Infection of U251 cells with an HCMV UL23-null mutant resulted in enhanced transcription of antiviral genes and controlled viral replication.

HCMV Finds Ways to Avoid ISGylation: Both IE1 and UL26 Target ISG15

Interferon-stimulated gene 15 (ISG15) encodes an ubiquitin-like protein that, in a similar way to ubiquitin, covalently conjugates to lysine residues, thereby regulating protein function (reviewed in Jeon et al., 2010). ISG15 modification is known as ISGylation, which marks proteins for either degradation or stabilisation. ISG15 is one of the most abundantly produced transcripts upon induction of the type I IFN response (Der et al., 1998; Potter et al., 1999) and exerts antiviral effects against DNA and RNA viruses (Lenschow, 2010; Morales and Lenschow, 2013).

Human cytomegalovirus infection induces ISG15 expression, which, through ISGylation, inhibits viral replication (Kim et al., 2016). HCMV employs two proteins with two separate strategies to evade this process (**Figure 1**). First, the viral immediate-early protein IE1 suppresses ISG15 transcription (Kim et al., 2016). However, this effect is only partial, and therefore some ISG15 protein is still expressed to carry out ISGylation, which is protected from the antagonistic activity of IE1. To counteract this remnant ISG15, HCMV expresses the tegument protein UL26, which reduces the accumulation of other viral ISGylated proteins by acting as a decoy for ISGylation itself (Kim et al., 2016). UL26 is known as an antagonist of the NF-κB pathway (Mathers et al., 2014), but ISGylated UL26 can no longer antagonise NF-κB signalling (Kim et al., 2016), suggesting that the virus sacrifices one of its own proteins to avoid ISGylation of other viral proteins. Why UL26 is more prone to ISGylation compared to other viral proteins, and the impact of the loss of its effect on NF-κB signalling during HCMV infection remains unclear at this stage. This in turn raises the question of whether the dominant role of UL26 is to inhibit NF-κB signalling or to act as an ISGylation decoy, since these seem to be opposing functions.

Targeting an Essential Player of the Innate Immune Response: ORF94 Against the ISG OAS1

Human cytomegalovirus expresses several genes during latency to avoid immune recognition of infected cells (Jenkins et al., 2004; Cheung et al., 2009), the so-called CMV latency-associated transcripts (CLTs). These products are also expressed during lytic HCMV infection. HCMV ORF94 (also known as UL126a) is one such transcript, and its localization in the nucleus suggests a potential role in cellular gene regulation (White et al., 2000). ORF94 was shown to inhibit both the transcription and translation of the ISG 2′-5′-oligoadenylate synthetase 1 (OAS1) (Tan et al., 2011). OAS1, together with OAS2, OAS3, OAS-like (OASL), and cGAS, forms the OAS family (Justesen et al., 2000). Upon detection of double stranded RNA (dsRNA), OAS1-3 proteins are activated and oligomerise ATP into 2′,5′-linked oligoadenylate products (2-5A). This leads to the activation of endoribonuclease L (RNase L), which in turn degrades viral and cellular RNA. Thus, expression of HCMV ORF94 reduces OAS mRNA and protein levels and consequently the formation of 2-5A during productive infection in human fibroblasts (**Figure 1**; Tan et al., 2011). However, as ORF94 is expressed in both the productive and latent phases of HCMV infection, it could potentially contribute to latency by modulating the immune response, which would be an intriguing avenue of further research.

Kaposi's Sarcoma-Associated Herpesvirus

Kaposi's sarcoma-associated herpesvirus (KSHV), also called human herpesvirus 8 (HHV-8), belongs to the *Gammaherpesvirinae* subfamily. KSHV is one of the seven known human oncoviruses. It can cause multiple malignancies, namely Kaposi's sarcoma, primary effusion lymphoma, multicentric Castleman's disease, or KSHV inflammatory cytokine syndrome (Chang et al., 1994; Ablashi et al., 2002; Ganem, 2006).

Targeting ISGylation: vIRF1 and ISG15

Kaposi's sarcoma-associated herpesvirus encodes four viral homologs of cellular interferon regulatory factors (vIRFs) (Jacobs and Damania, 2011). In 2013, Jacobs et al. (2013) showed that vIRF1 inhibits the type I IFN response. By performing affinity purification coupled to mass spectrometry with cells expressing vIRF1 and in which TLR3 signalling was activated, the authors identified the cellular ISG15 E3 ligase, HERC5, as an interaction partner of vIRF1 (Jacobs et al., 2015). HERC5 interacts with the C-terminus of vIRF1. Moreover, vIRF1 reduces total ISG15 conjugation levels on cellular target proteins, which in turn inhibits IRF3 function as it relies on ISGylation for stabilisation (**Figure 1**; Shi et al., 2010). Additionally, siRNA-mediated knockdown of ISG15 or HERC5 increases KSHV replication upon reactivation. Therefore, it is possible that vIRF1 negatively regulates ISGylation by interacting with HERC5, leading to a decrease in IRF3 stability and reduced transcription of ISGs. Interestingly, the authors observed by immunoprecipitation that vIRF1 is conjugated to ISG15 at multiple sites, suggesting a role as a viral ISGylation target similar to the HCMV protein UL26 (Kim et al., 2016),

which may reflect a conservation of this function between herpesvirus subfamilies.

While KSHV vIRFs inhibit IFN signalling, type I IFN is not always detrimental for herpesviruses as it plays an important role for the maintenance of latency (Zhang et al., 2004; De Regge et al., 2010; Dag et al., 2014; Holzki et al., 2015). In line with these findings, vIRF2 has been recently described to manipulate the innate immune response. vIRF2 regulates the expression of 51 genes known to be involved in innate or intrinsic defences, boosting the formation of the antiviral cellular state to restrict KSHV early lytic protein expression and promote latency (Koch et al., 2019). This is an intriguing illustration of the fine-tuned balance between herpesviruses and their host, which dictates the outcome of the infection course.

Profiting From ISGs: ORF20 Fancies the ISG OASL

The OAS family member OASL shares a highly conserved N-terminal OAS-like domain with the OAS enzymes, but it lacks enzymatic activity and has a unique C-terminus composed of two ubiquitin-like domains (Hartmann et al., 1998). In addition, OASL binds dsRNA (Ibsen et al., 2015). OASL was identified as an ISG with targeted, but not broad antiviral specificity against a variety of RNA viruses (Schoggins et al., 2011, 2014). Its role for HSV-1 is more controversial - while one study observed no role for OASL on HSV-1 replication (Marques et al., 2008), another reported that OASL inhibited HSV-1 (Zhu et al., 2014).

We showed that the KSHV protein ORF20 interacts with OASL, presumably in the nucleoli given their subcellular localization (Bussey et al., 2018). Interestingly, stable expression of OASL enhances KSHV replication in an ORF20-dependent manner (**Figure 1**). Since both proteins interact with ribosomal proteins and co-sediment with ribosomal subunits, which are involved in the formation of active ribosomal complexes, ORF20 may manipulate OASL so that KSHV can seize control of the host translational machinery. However, further studies are needed to understand the mechanism by which KSHV ORF20 usurps OASL. It is worth noting that the expression of ORF20 in 293T cells specifically enhances OASL mRNA and protein levels. This may be congruent with the observation of a recent study that OASL negatively affects the DNA-binding ability of the DNA sensor cGAS (Ghosh et al., 2019), which is a crucial sensor of KSHV infection (Wu et al., 2015). Thus, enhanced levels of OASL during lytic KSHV replication may inhibit cGAS-mediated activation of the innate immune response and therefore provide a more conducive environment for infection.

FINAL REMARKS

The complex interaction between herpesviruses and their host is essential for the outcome of infection. In the case of ISGs, understanding the mechanisms by which herpesviruses manipulate these effectors gives an insight into both how viruses establish lifelong infections and the role that ISGs play in immune defence. The importance of ISGs for antiviral defence is indisputable, given that IFNAR knockout mice readily succumb to infection with herpesviruses (Strobl et al., 2005; Lenschow et al., 2007; Rasmussen et al., 2007). Interestingly, several studies reported only minor defects in mice lacking individual ISGs (Lenschow et al., 2007), supporting the notion that at least some ISGs may act in concert to exert their full effect, or the existence of ISGs with redundant functions. Moreover, a recent study revealed the complex network that ISGs create during viral infection, not just by binding to other ISGs, but also to many other cellular proteins (Hubel et al., 2019), adding an even greater level of complexity to the host immune response against infection.

We have only just crossed the starting line to understanding whether certain ISGs are proviral or antiviral in the context of herpesviral infections. This investigation into the role and mechanism of action of ISGs is challenging. Overexpression studies may give some valuable insights into the function of these ISGs. However, since viral infections induce the expression of multiple ISGs that may cooperate, studies on a single ISG may not reflect reality or at least may not reveal the full potential of the individual ISG tested. Ideally, tagged, endogenously expressed ISGs would be used for co-immunoprecipitation studies in infected cells to identify viral and/or cellular binding partners of them. To expand on these studies, analysis of single and combined ISG knockouts will help to determine whether ISGs have a proviral role, an antiviral role, or neither. Another point to consider is that some ISGs may have diverse functions in different cell types while other ISGs may be species-specific.

Herpesviruses are a very valuable tool in the endeavour to uncover the role that ISGs play in antiviral defence as they are highly adapted and have likely developed multiple antagonists (**Table 1**). However, viral antagonists can be friend or foe: while the function of ISGs may only be revealed in the absence of viral antagonists, these opponents may be key to our greater understanding of how cellular defence is regulated. Through our bid to decipher the intricacies of this complex interplay between herpesviruses and the tailored ISG response to individual infections, we may uncover novel targeted therapies against these masters of immune escape and manipulation.

AUTHOR CONTRIBUTIONS

AG-P conducted the literature research, critically analyzed the published data, planned the concept of the review with MB, prepared the table and figure, and wrote the manuscript. BC and MB wrote parts of the Introduction and Conclusion. MS, BC, and MB critically discussed and revised the manuscript together with AG-P.

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DNA Sensors' Signaling in NK Cells During HHV-6A, HHV-6B and HHV-7 Infection

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Objectives: The host DNA sensor proteins TLR9, STING, IFI16 are central signaling molecules that control the innate immune response to cytosolic nucleic acids. Here we propose to investigate how Natural killer (NK) cell infection by human herpesvirus (HHV)-6A, HHV-6B or HHV-7 is able to modify DNA sensor signaling in NK cells.

Methods: We infected the NK92 cell line and primary NK cells with cell-free inocula of HHV-6A, HHV-6B or HHV-7 and evaluated TLR9, STING, and IFI16 pathway expression by Real-Time PCR, Western Blot, immunofluorescence and flow cytometry for 1, 2, 3, and 6 days post-infection. We evaluated NK cell cytokine-producing by Real-Time PCR and enzyme immunosorbent assay.

Results: NK92 and primary NK cells were promptly infected by three viruses, as demonstrated by virus presence (DNA) and transcription (RNA) analysis. Our data show STING/STAT6 up-modulation in HHV-6A infected NK cells. NK cells infected with HHV-6B and HHV-7 up-regulated CCL3, IFN-alpha, TNF-alpha, IL-8 and IFN-gamma and slightly induced IL-4, and CCL4. HHV-6A infected NK cells up-regulated IL-4 and IL-13 and slightly induced IL-10, TNF-alpha, IFN-alpha, and IFN-gamma.

Conclusion: For the first time, we demonstrate that HHV-6A, HHV-6B, and HHV-7 infections have a differential impact on intracellular DNA sensors. HHV-6B and HHV-7 mainly lead to the active control of *in vivo* viral spreading by pro-inflammatory cytokine secretion via TLR9. HHV-6A infected NK cells conversely induced STING/STAT6 pathway, as a mechanism of anti-viral activation, but they were characterized by a Th2 type response and a non-cytotoxic profile, suggesting a potential novel mechanism of HHV-6A-mediated immunosuppression.

Keywords: DNA sensors, human herpesvirus, natural killer cells, HHV-6A, HHV-6B, HHV-7

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INTRODUCTION

Three herpesviruses gaining medical interest are the human herpesvirus-6 (HHV-6) A and B and human herpesvirus-7 (HHV-7). They are members of the *Herpevirales* order, *Herpesviridae* family, *Betaherpesvirinae* subfamily, and *Roseolavirus* genus.

HHV-6, as HHV-6A and HHV-6B are commonly called when they are not separated into two species, has a wide cell tropism inducing a lifelong latent infection in humans (Ablashi et al., 2014;

Eliassen et al., 2017). HHV-6A/B replicate preferentially in CD4+ T lymphocytes and utilize distinct cell surface receptors: HHV-6A uses CD46, a regulator of complement activation expressed on all nucleated cells, while HHV-6B uses CD134 (also called OX40), a member of the tumor necrosis factor (TNF) receptor superfamily. HHV-6 infects also CD8+ T lymphocytes, NK cells, astrocytes, microglial cells oligodendrocytes, liver cells, human fibroblasts, epithelial cells, endothelial *in vitro* cells (De Bolle et al., 2005).

Human herpesvirus-7 has a narrow tropism for CD4+ T-cells, where it uses the glycoprotein CD4 for cell entry (Lusso et al., 1994).

Human herpesvirus-6 and HHV-7 are immune-modulating and modify the secretion of chemokines and cytokines, with a significant effect on host immune response (Lusso, 2006; Yoshikawa et al., 2009).

Currently, few studies are available on HHV-6 and HHV-7 infection of Natural killer (NK) cells, probably due to the absence of reliable animal models.

Natural killer cells are able to kill tumor cells and virus-infected cells independently of MHC restriction. Patients lacking NK cells are subject to multiple infections by HHV, evidencing their importance in viral immuno-surveillance *in vivo* (van Erp et al., 2019). Several studies demonstrate NK-cell-dependent protective effects during viral infections (Vidal et al., 2011), with a direct killing of infected target cells and production of cytokines (e.g., interferon (IFN)-γ) (Blanc et al., 2011).

HHV-6A/B can infect NK cells (Rizzo et al., 2017). We have reported that NK cells are permissive to both HHV-6A and HHV-6B viruses establishing a lytic replication. Both viruses affect the expression of miRNAs implicated in NK cell development, maturation and functions (miR-146, miR-155, miR-181, miR-223). Moreover, HHV-6A/6B infections modify the expression of transcription factors, with both species increasing ATF3, JUN, and FOXA2, whereas HHV-6A inducing POU2AF1 decrease, and HHV-6B FOXO1 increase, and ESR1 decrease. HHV-6B evades the elimination of infected cells by suppressing surface expression of ligands for NK cell receptors NKG2D and NKp30 (Schmiedel et al., 2016). Meanwhile, the up-regulation of IL-15 production induced by HHV-6A/B and HHV-7 infection results in NK cell antiviral activity (Atedzoe et al., 1997).

Human herpesvirus-7 U21 protein reduces NK activation and cytotoxicity interacting with the NK cell activating ligand ULBP1 that is rerouted to the lysosomal compartment, and down-regulating the surface expression of the NK activating ligands MICA and MICB (Schneider and Hudson, 2011).

The germline-encoded pattern recognition receptors (PRR) and DNA sensors facilitate the NK cells recognition of pathogens during the initial stages of infection, activating downstream signaling cascades and the secretion of type I IFN and pro-inflammatory cytokines.

Endosomal DNA-sensor Toll-like receptor (TLR)-9 has been shown to recognize microbial DNA and induces the host defense against infections (Kawai and Akira, 2010), such as Human cytomegalovirus (HCMV), Herpes simplex virus (HSV)-1 (Hochrein et al., 2004) and HSV-2 (Lund et al., 2003). The hexamers containing unmethylated CpG (cytosine-phosphate-guanine dideoxynucleotide) motifs are the preferential ligands of TLR9 (Hemmi et al., 2000).

Upon HHV infection, viral DNA or aberrantly localized cellular DNA are recognized by the DNA sensor cyclic GMPAMP (cGAMP) synthase (cGAS) that forms the second messenger 2′3′-cGAMP (Diner et al., 2013). cGAMP interacts with the endoplasmic reticulum (ER)-resident adaptor protein stimulator of interferon genes (STING) that dimerizes and translocates from the ER to the Golgi apparatus (Dobbs et al., 2015). Here, Tank-binding kinase 1 (TBK1) is recruited for the interferon regulatory factor 3 (IRF3) phosphorylation. IRF3 dimerizes (Tanaka and Chen, 2012) and translocates into the nucleus, inducing the expression of type I IFN. STING can also recruit Signal transducer and activator of transcription (STAT)6 to the endoplasmic reticulum, where it dimerizes and translocates to the nucleus, inducing target genes involved in immune cell homing, such as chemokines (Chen et al., 2012). Gamma-interferon-inducible protein 16 (IFI16) is a cytosolic DNA sensor (Diner et al., 2013) of the Pyrin and HIN domain (PYHIN) protein family. In the presence of HHV infection, IFI16 translocates to the cytoplasm where it induces STING-mediated signaling (Almine et al., 2017) or synergizes with cGAS as a DNA co-sensor (Almine et al., 2017; Dunphy et al., 2018).

The role of DNA sensors in NK cell anti-HHV-6 and HHV-7 response is unclear and additional studies are needed to understand the biological consequences on pathway signaling. Here, we examine the role of DNA sensors in human NK cells infected by HHV-6 and HHV-7.

MATERIALS AND METHODS

NK Cells

Natural killer 92 (ATCC CRL-2407) cell line was grown in MEM-Alpha medium (Minimal Essential Medium, Gibco BRL, Invitrogen Corporation, Carlsbad, CA, United States) supplemented with 20% of FCS (fetal calf serum, Euroclone, Pero, MI, Italy), 0.1 mM 2-Mercaptoethanol (Gibco BRL, Invitrogen Corporation, Carlsbad, CA, United States), 100 U/mL penicillin, 100 μg/mL streptomycin and 150 U/mL of IL-2. Cell cultures were maintained at 37°C in humidified atmosphere of 5% CO_2 in air. For stimulation of DNA sensors we used 2′,3′-cGAMP (Sigma-Aldrich, St. Louis, MO, United States) 1 μM for 30 min in digitonin permeabilization buffer (50 mM HEPES, 100 mM KCl, 3 mM MgCl2, 0.1 mM DTT, 85 mM sucrose, 0.2% BSA, 1 mM ATP, 0.1 mM GTP, pH 7.0) (Srikanth et al., 2019).

Human primary NK cells were obtained from the peripheral blood of healthy blood donors. This study was approved by the "Ferrara Ethics Committee" and we collected written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Primary NK cells were separated from peripheral blood samples using the negative magnetic cell separation (MACS) system (Miltenyi Biotec, Gladbach, Germany) (Marci et al., 2016). The analysis of purified cell fraction by flow cytometry with CD3-PerCp-Cy5.5, CD56-FITC moAbs (e-Bioscience,

Frankfurt, DE), demonstrated that the NK cell content was >90% (data not shown). NK cells were treated with different mRNA sensors or DNA sensors antagonists/inhibitors. We used: ODN 2087 (Miltenyi Biotec), TLR7 and TLR8 antagonist (0.5 μM); TLR3.CI (Calbiochem, Merck, Darmstadt, Germany), TLR3/dsRNA Complex Inhibitor (30 nM); ODN 2088 (Miltenyi Biotec), TLR7, TLR8, TLR9 antagonist (0.5 μM); H-151 (Invivogen; San Diego, CA, United States) STING antagonist (0.5 μM) (Haag et al., 2018); A15117499 (Sigma-Aldrich, Saint Louis, MS, United States) STAT6 inhibitor (100 nM) (Chiba et al., 2009). To confirm the efficacy of mRNA sensors antagonists/inhibitors, we used synthetic agonists. R-848 (Invivogen), a TLR-7/8 agonist (Gorden et al., 2005) (28), was dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, United States) at a concentration of 10 mM and stored at 4°C. It was used at the concentration of 3 μM (Gorski et al., 2006). Poly I:C (Sigma-Aldrich), an agonist of TLR3, was reconstituted at 2.5 mg/ml at 50°C and re-annealed before storage at −20°C. It was used at the concentration of 25 μg/ml.

HHV-6A, HHV-6B and HHV-7 Infection

Cell-free virus inocula were obtained as previously described: HHV-6 variant A (strain U1102) was grown in the J-Jhan cell line (Rotola et al., 1998); HHV-6 variant B (strain Z29) and HHV-7 (strain CZ) (Portolani et al., 1995) were grown in the Sup T1 cell line.

RNA cell extraction was performed with the RNeasy kit (Qiagen, Hilden, Germany). The absence of contaminant DNA in the extracted RNA was assured by DNase treatment and control β-actin PCR without retrotranscription reverse transcription (Caselli et al., 2017; Rizzo et al., 2017). The analysis of virus transcripts was preformed by RNA reverse transcription with the RT2 First strand kit (Qiagen, Hilden, Germany) using cDNA aliquots obtained from 200 ng RNA (Menegazzi et al., 1999; Caselli et al., 2012). We used specific primers for HHV-6 or HHV-7 U42 gene, respectively (Caselli et al., 2012). The sequences are reported in **Table 1**. Each sample was run in duplicate.

We also evaluated the transcription of latent (EBNA1, EBNA-2, LMP1) and lytic (BAL2) genes of Epstein-Barr virus (EBV), that latently infect NK92 cell line. We used specific primers, as previously reported (Isobe et al., 2004). The lymphoblastoid cell line LCL-B95.8 (kind gift of Professor R. Dolcetti) was used as control of EBV gene expression, after viral cycle activation using TPA (12-*O*-tetradecanoylphorbol-13-acetate) (Sigma-Aldrich), used at 20 ng/ml.

Immunofluorescence Assay

Human herpesvirus-6 gp116 and HHV-7 KR4 late antigens' expression was analyzed by immunofluorescence with anti-gp116/64/54 FITC antibody (Ab) (Clone 6A5) (Santa Cruz, United States) or KR4 (a kind gift of HHV-6 foundation), as previously described (Caselli et al., 2012). STING/STAT expression was evaluated with anti-STING PE Ab (Clone T3-680) (BD Biosciences, Italy) and anti-STAT6 FITC (Clone D-1) (Santa Cruz, United States).

TABLE 1 | U42 primers.

Gene	Primers
HHV-6 U42 (Mirandola et al., 1998)	Forward 3′ACGATGGACATGGCTTGTTG5′ Reverse 3′ACCTTACAACGGAGACGCC5′
HHV-7 U42 (Menegazzi et al., 1999)	Forward 3′AAGCTGCAAGACGGAGTTGT5′ Reverse 3′AGTATTCCGGTGAAGCACGA5′

RNA and DNA Sensor mRNA Analysis

Toll-like receptor 3, TLR7, and TLR8 mRNA were analyzed using the set of primers: TLR3 (F:5′-GAGGCGGGTGTTT TTGAACTAGAA-3′, R:5′-AAGTCAATTGTCAAAAATAGG CCT-3′) (Menager et al., 2009); *TLR7* (F:5′-AGTGTCTAAA GAACCTGG-3′, R:5′-CTTGGCCTTACAGAAATG-3′); and *TLR8* (F:5′-CAGAATAGCAGGCGTAACACATCA-3′,R:5′ATG TCACAGGTG CATTCAAAGG -3′) (Hart et al., 2005).

Toll-like receptor 9 and STING mRNA were analyzed using the set of primers: TLR9 5′-CCGTGACAATTACCTGGC CTTC-3′ (forward) and 5′-CAGGGCCTTCAGCTGGTTTC-3′ (reverse) (Bao et al., 2016); STING: Fw: 5′-GCTGCTG TCCATCTATTTCTACT-3′ (forward) and 5′-GCCGCAGATAT CCGATGTAATA-3′ (reverse) (Gram et al., 2017). Actin was used as house-keeping gene and was analyzed with the set of primers: 5′-GATGGAGTTGAAGGTAGTTT-3′ (forward) and 5′- TGC-TATCCAGGCTGTGCTAT-3′ (reverse) (Rizzo et al., 2017).

Cytokine mRNA Analysis

Uninfected or HHV infected NK92 cells were stimulated with CpG 25 μg/ml CpG (ODN 2006, TIB MOLBIOL) ml 3d.p.i., and the cells were collected 24 h after stimulation. Cytokine mRNAs were analyzed with Real-Time PCR assays for human genes: IFN-alpha: Hs03044218-g1; IL-6: Hs00174131; IL-8: Bt03211906; IL-22: Hs01574154; TNF-alpha: Hs02621508 (Applied Byosystems; ThermoFisher Scientific; United States).

Cytokine/Chemokine Enzyme Immunosorbent Assay

Levels of CCL3, CCL4, CCL5, IL-4, IL-8, IL-10, IL-13, IFN-alpha, TNF-alpha, IL-8, IFN-gamma were assessed in duplicates in cell culture supernatants using commercial human specific enzyme-linked immunosorbent assays (ELISAs) (myBiosource, United States) following manufacturer's protocols.

Western Blot Analysis

Whole cell lysates were prepared by using the RIPA buffer containing proteinase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, United States). Proteins were quantified by means of the Bradford assay (Bio-Rad; Segrate, MI, Italy) using bovine albumin (Sigma-Aldrich) as standard. Twenty μg of total proteins were loaded in each well and evaluated in denaturating conditions in 10% TGX-Pre-cast gel (Bio-Rad), with subsequent electroblotting transfer onto a PVDF membrane (Millipore, MA, United States). The membrane was incubated with a specific antibody for the protein to be analyzed, then with a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (1:5000; Amersham Biosciences, NJ, United States)

and developed with the ECL kit (Amersham Biosciences, NJ, United States). The images were acquired by Geliance 600 (Perkin Elmer, MA, United States). The specific antibodies used were: anti-Myd88 (Clone 4D6), anti-STING (Clone TMEM173), anti-cGAS (CL3605), anti-IFI16 (Clone 2E3), anti-TBK1 (Clone 108A429), anti-IRF3 (Clone SD2062), anti-STAT6 (Clone 177C322.1) (Novus Biologics; Italy), anti-IRF3 Ser396 (Clone 4D4G), and anti STAT6 Tyr641 (Clone D859Y) (Cell signaling Technology; United States). The complete Western Blots are reported in **Supplementary Figures S2**, **S3**.

Intracellular TLR9 Expression by Flow Cytometry

Intracellular expression of TLR9 was quantified fixing NK92 cells with 2% formaldehyde and permeabilizing them for intracellular staining with anti-TLR9-PE (Clone anti-GJ15A7) (BD Biosciences). Cells were analyzed by flow cytometry (FACS Canto II, BD) and FlowJo software (Tree Star Inc). Viable cells were assessed by propidium iodide. Approximately 10^5 cells were collected for each individual sample.

Statistical Analysis

Since the biological variables presented a normal distribution (Kruskal–Wallis test, $p > 0.05$), they were evaluated by Student t test by Graph pad software. A p-value < 0.05 was defined statistically significant.

RESULTS

HHV-6A, HHV-6B and HHV-7 Infect NK Cells

As previously reported, HHV-6A and HHV-6B viruses can infect NK cells (Rizzo et al., 2019). Here we showed that NK92 cells are permissive to HHV-6A, HHV-6B, and HHV-7, with a high viral amount 3 days post infection (d.p.i.) using 100 multiplicity of infection (m.o.i.) (**Figure 1A**). In **Figures 1B,C**, the expression of DNA and mRNA of U42, an immediate early HHV-6 viral gene, increased during the 6 d.p.i. that were evaluated. Similarly, using HHV-7 U42 specific primers, the expression of both DNA and mRNA increased significantly. We selected the time point 3d.p.i. to perform the subsequent experiments, since it coincides with a high viral amount for both HHV-6 and HHV-7. When we looked at viral late antigens at 3 d.p.i., in particular gp116 for HHV-6 and KR4 for HHV-7, we observed their expression (**Figure 1D**).

Since NK92 cells arbor a latent EBV infection, we wanted to be sure that it did not affect the results observed. The analysis of latent (EBNA1, EBNA-2, LMP1) and lytic (BZLF1) EBV genes showed no mRNA transcription (**Supplementary Figure S1**), supporting the absence of any confounding effect of EBV latent infection.

HHV-6A, HHV-6B and HHV-7 Infection Affects TLR9 Expression

To further dissect and understand the modification induced by HHV-6 and HHV-7 infection of NK cells, we performed the analysis on NK92 cell line to have reproducible data not affected by individual differences in NK cell subpopulations. First, we considered the DNA sensor protein TLR9 (Roda et al., 2005). We infected NK cells with HHV-6A, HHV-6B or HHV-7, and evaluated TLR9 mRNA expression at 3 d.p.i. comparing TLR9 mRNA levels in HHV infected cells with those in uninfected NK92 cells.

At mRNA level, we observed that HHV-6A inhibited the expression of TLR9 mRNA and protein ($p < 0.001$; Student t test) (**Figures 2A,B**). HHV-6B and HHV-7 did not affect the TLR9 mRNA expression but we observed a decrease in protein expression after HHV-6B infection ($p = 0.023$; Student t test) (**Figures 2A,B**). When we looked at the TLR9 pathway, we considered the key component Myd88. No modifications were observed in the expression levels of this protein in the NK92 cells infected with any of the three viruses (**Figure 2C**). These data suggest that the decrease in TLR9 protein expression observed in HHV-6A infected NK92 cells is restricted to the TLR9 gene. Since the expression of TLR9 is fundamental for the pathway activation and transcription of IFN-alpha and pro-inflammatory cytokines (e.g., IFN-alpha, TNF-alpha, IL-6, IL-8, and IL-22), we evaluated the cytokines' mRNA expression by NK92 cells 3 d.pi. HHVs infection and 24 h of stimulation with unmethylated CpG DNA motif, the ligand for TLR9 activation. We observed that NK92 cells express TLR3, TLR7, and TLR8 mRNA sensing molecules (**Supplementary Figure S2B**). To be sure that their activation does not affect the results obtained, we inhibited them with specific antagonists (ODN2087: TLR7, TLR8 antagonist; TLR3.CI: TLR3 antagonist). The efficacy of RNA sensors' antagonists was demonstrated by activating the cells with the corresponding agonists (**Supplementary Figure S2C**). We used synthetic agonists, R-848 (Invivogen), a TLR-7/8 agonist and Poly I:C (Sigma-Aldrich), an agonist of TLR3. We tested the relative mRNA expression of type I interferon (IFN-alpha) (Tabeta et al., 2004; Uematsu and Akira, 2007), as marker of TLR3, TLR7, TLR-8 activation in NK92 cells treated with TLR7/8, TLR3 agonists with or without antagonists treatment. We observed an increase in IFN-alpha levels in TLR7/8, TLR3 agonists treated cells, while the antagonist treatment inhibits the induced expression of IFN-alpha (**Supplementary Figure S2C**). We showed an increase in IFNA-alpha, TNF-alpha, and IL-8 expression in HHV-6B and HHV-7 infected NK92 cells (**Figure 2D**), with the highest levels reached by IL-8 with HHV-7 infection ($p < 0.001$; Student t test). On the contrary, TNF-alpha and IL-8 expression was slightly modified in HHV-6A infected NK92 cells (**Figure 2D**). IL-6 and IL-22 were not induced by the viruses (data not shown).

HHV-6A, HHV-6B and HHV-7 Infection Affects STING Expression and Activation Pathway

We then considered the DNA sensor protein STING. The stimulation of the cytoplasmic DNA sensing pathways was performed with 2′,3′-cGAMP. At mRNA level, we observed increased levels of STING expression during HHV-6A infection

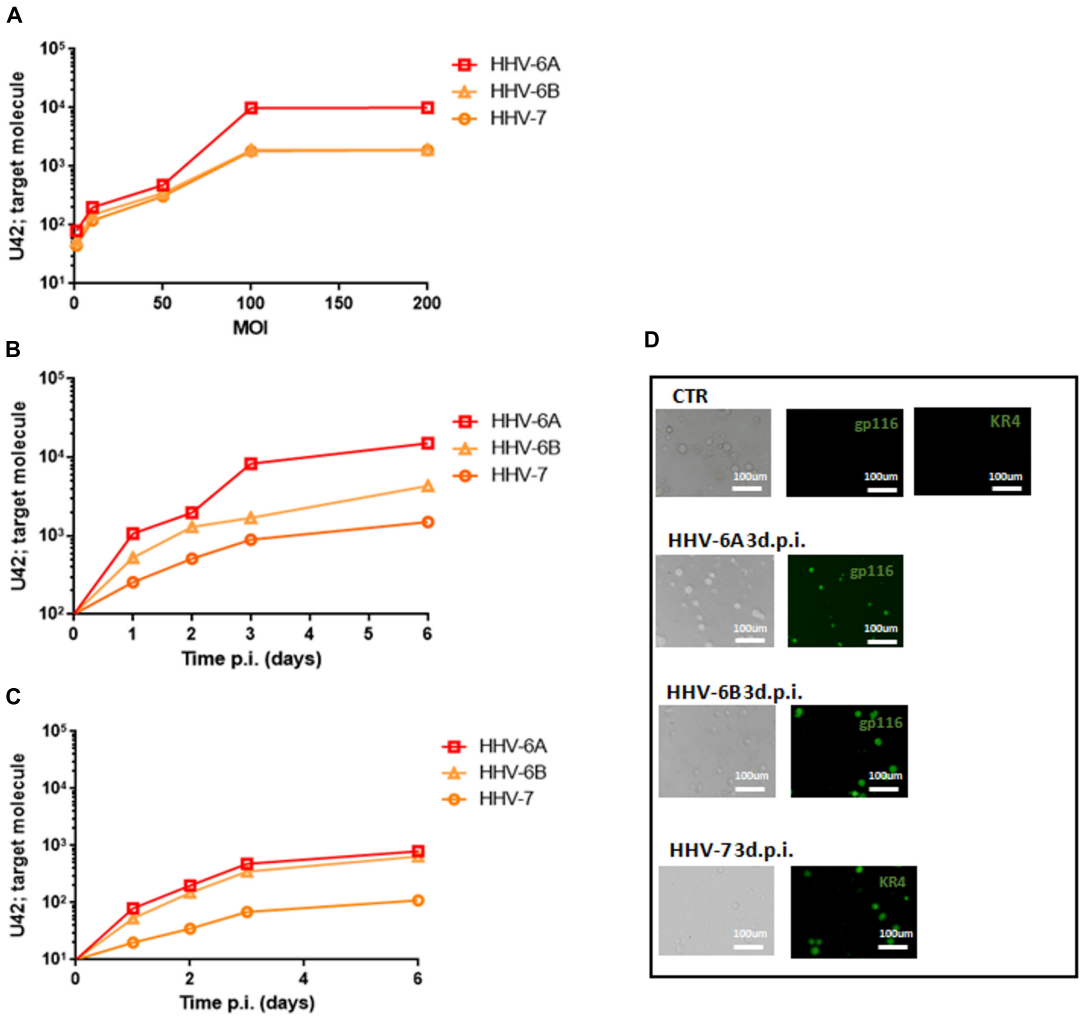

FIGURE 1 | NK92 infection by HHV-6A, HHV-6B and HHV-7. **(A)** NK92 cells were infected with HHV-6A, HHV-6B or HHV-7 at different multiplicity of infection (1.0, 10.0, 50.0, 100.0, 200.0 m.o.i.). The virus presence (U42 DNA) was evaluated 3 days post infection (d.p.i.). Virus **(B)** presence (DNA) and **(C)** transcription (RNA) were evaluated, respectively, by qPCR and RT-qPCR performed on U42 virus gene, at 1, 2, 3, and 6 d.p.i., as already detailed. The infection was performed with 100 m.o.i. **(D)** HHV-6A and HHV-6B infected NK92 cells were characterized by immunofluorescence for gp116 (late viral protein) expression 3d.p.i. HHV-7 infected NK92 cells were characterized by immunofluorescence for KR4 (late viral protein) expression 3d.p.i. Uninfected NK92 cells (CTR) were used as control. Images were taken in bright field (*left panels*) or fluorescence (*right panels*) (Nikon Eclipse TE2000S) equipped with a digital camera. Original magnification 20×.

and cGAMP treatment ($p < 0.001$; Student t test), while HHV-6B and HHV-7 did not affect STING mRNA expression (**Figure 3A**). When we considered protein expression, we did not observe any induction of STING expression in all the four conditions (**Figure 3B**).

When we looked at STING pathway, we considered the key components cGAS and IFI16 as up-stream regulators of STING activation, TBK1 and IRF3 for the NFkB activation pathway and STAT6 for the STAT6-dependent pathway.

cGAs protein expression was not modified by HHV-6A and HHV-6B and only slightly decreased by HHV-7 ($p = 0.038$; Student t test). IFI16 protein expression was not modified by HHV-7, while it was down-modulated by HHV-6A and HHV-6B infection ($p = 0.033$; Student t test) (**Figure 3C**). NK92 cell infection induced TBK1 expression (**Figure 3C**), mainly after HHV-6A infection ($p = 0.011$; Student t test). Notably, the Western Blot of TBK1 presented the control lane with a lighter background in comparison with the HHV lanes. We hypothesize that TBK1 moAb might cross-reacts with some HHV proteins and creates a darker background. IRF3 protein expression and Ser396 phosphorylation were not up-regulated by the three viruses. On the contrary, STAT6 Tyr641 phosphorylation was induced by HHV-6A (**Figure 3C**) ($p = 0.022$; Student t test). HHV-6B and HHV-7 did not induce STAT6 expression (**Figure 3C**). The peculiar activation of STING/STAT6 pathway in HHV-6A infected NK92 cells seems to be confirmed by the different cellular localization of STING and STAT6 (**Figure 3D**). We observed that already after 3d.p.i. STING and STAT6 co-localized in the peri-nuclear/cytoplasmic region of the HHV-6A infected NK92 cells, that express the late

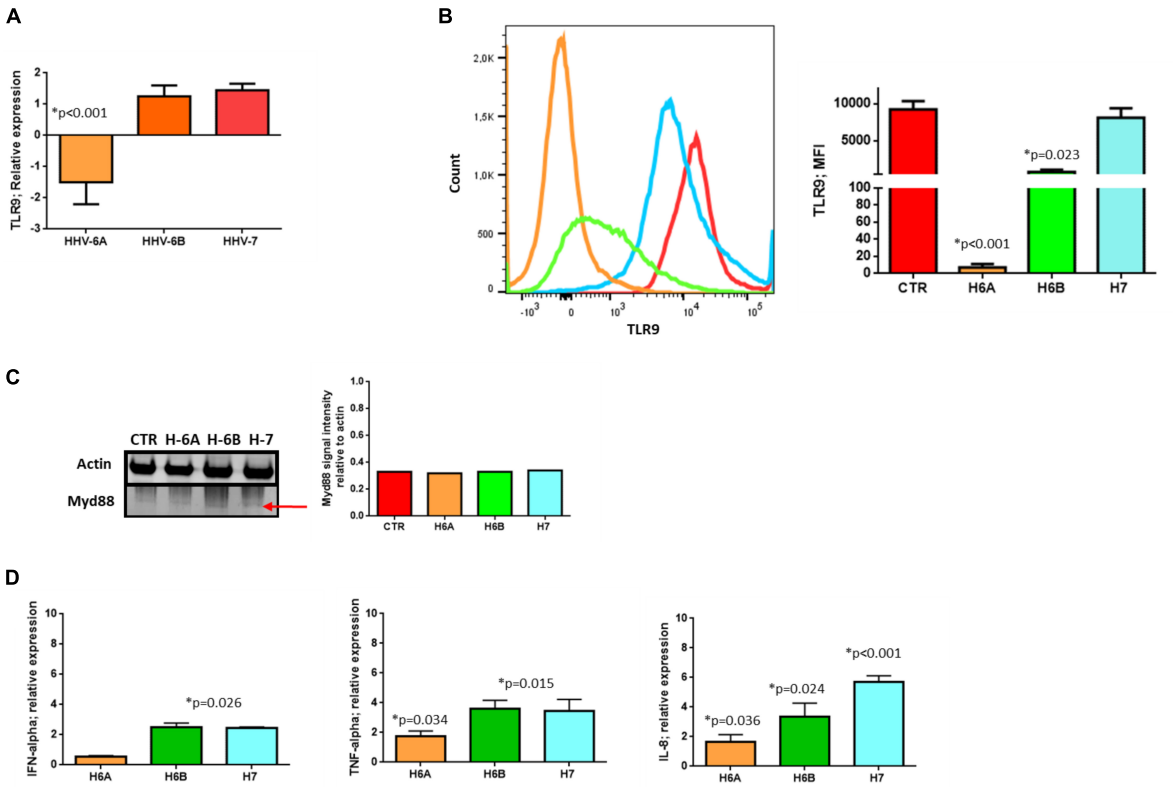

FIGURE 2 | TLR9 analysis. TLR9 **(A)** mRNA relative expression and **(B)** endosomal protein expression in uninfected, HHV-6A, HHV-6B or HHV-7 infected NK92 cells 3 d.p.i. NK92 cells were intracytoplasmic stained for anti-TLR9-PE (Clone anti-GJ15A7). Representative histograms are reported. The histograms showed the MFI (mean fluorescence intensity) values of three independent experiments. *p values Student t test. **(C)** Western Blot analysis for house-keeping actin (upper blot) and Myd88 (lower blot, a red arrow indicates the localization of the bands of interest) expression in uninfected (CTR), HHV-6A, HHV-6B or HHV-7 infected NK92 cells 3 d.p.i. The molecular weights were determined by protein ladder (14.4-97.4kDa) (BioRad). Actin was evidenced at 44kDa, Myd88 at 33kDa. The images were acquired by Geliance 600 (Perkin Elmer, MA, United States). The complete Western Blots are reported in **Supplementary Figure S2**. **(D)** Relative mRNA expression of IFN-alpha, TNF-alpha, IL-8 in HHV-6A, HHV-6B or HHV-7 infected NK92 cells in comparison with uninfected NK92 cells. The cytokines' mRNA expression was evaluated in NK92 cells 3 d.pi. HHVs infection, 24 h of stimulation with unmethylated CpG DNA motif and with RNA sensor antagonists (ODN 2087 (Miltenyi Biotec), TLR7 and TLR8 antagonist (0.5 μM); TLR3.CI (Calbiochem, Merck, Darmstadt, Germany), TLR3/dsRNA Complex Inhibitor (30 nM).

gp116 viral antigen (**Figure 3D**). On the contrary, the localization of STING and STAT6 in control NK92 cells was prevalently cytosolic (**Figure 3D**, lower panel).

HHV-6A, HHV-6B and HHV-7 Infection Affects NK Cell Cytokines/Chemokines Secretion

The activation of DNA sensor proteins is responsible for the expression of cytokines and chemokines (Diner and Cristea, 2015). We evaluated the effect of the activation of the different pathways (TLR9, STING, STAT6) due to viral infections on the cytokine and chemokine expression by primary NK cells. We purified NK cells from peripheral blood samples of five control subjects and infected them with 100 m.o.i. for 3 days (**Supplementary Figure S4**), in the presence of mRNA sensor molecules antagonists/inhibitors (ODN2087: TLR7, TLR8 antagonist; TLR3.CI: TLR3 inhibitor) differently combined with DNA sensor molecules antagonists/inhibitors (ODN2088: TLR9 antagonist; H151: STING antagonist; AS1517499: STAT6 inhibitor).

We observed a different cytokine/chemokine expression during viral infections in the presence of different inhibitors. In the presence of mRNA sensor molecules antagonists/inhibitors, HHV-6A infected NK cells up-regulated IL-4 and IL-13 and slightly induced IL-10, TNF-alpha, IFN-alpha, and IFN-gamma (**Figure 4A**). NK cells infected with HHV-6B and HHV-7 up-regulated CCL3, IFN-alpha, TNF-alpha, IL-8, and IFN-gamma and slightly induced IL-4 and CCL4.

When we added the TL9 antagonist ODN2088, we observed no more induction in TNF-alpha, IFN-alpha, IL-8, and IFN-gamma in HHV-6A infected NK cells (**Figure 4B**). The TLR9 antagonists drastically reduced the secretion of CCL3, IFN-alpha, TNF-alpha, IL-8, and IFN-gamma in HHV-6B and HHV-7 infected NK cells (**Figure 4B**). The addition of STING antagonist H151 reduced cytokine/chemokine secretion by HHV-6A infected NK cells, maintaining only IFN-alpha levels unaltered (**Figure 4C**). IN HHV-6B and HHV-7 infected NK cells there was only a slight reduction in IFN-alpha, TNF-alpha, and IL-8 secretion (**Figure 4C**). The addition of STAT6 inhibitor AS1517499 in HHV-6A infected NK cell cultures resulted in a similar reduction of cytokine/chemokine secretion observed with

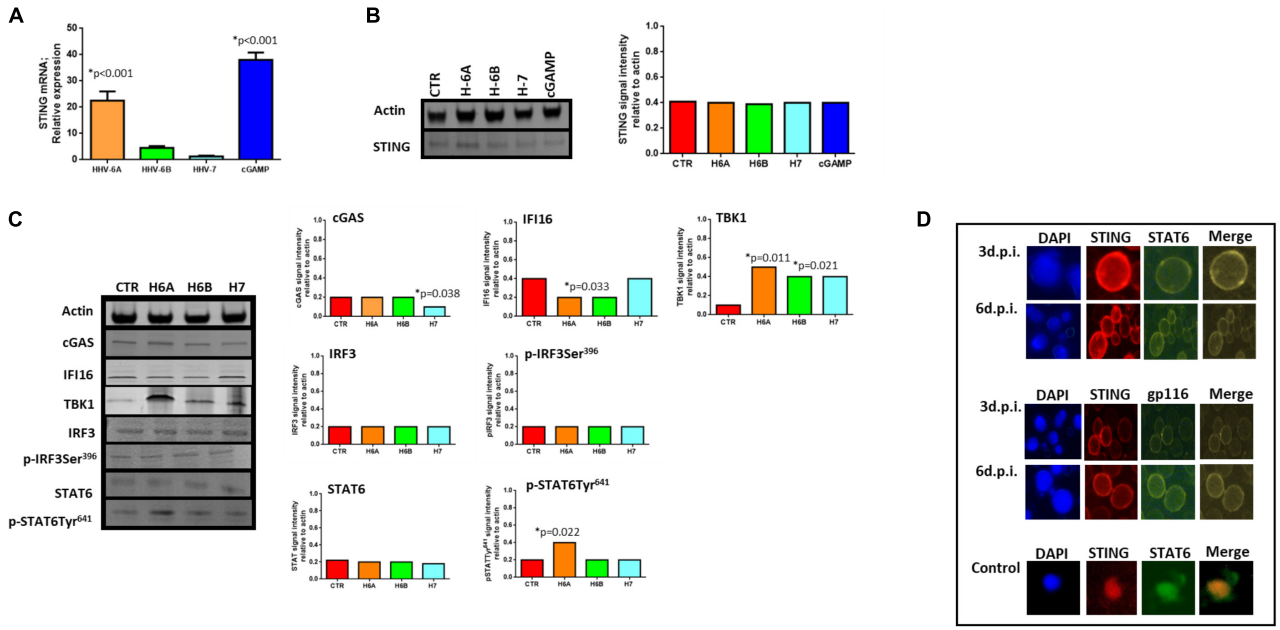

FIGURE 3 | STING pathway analysis. **(A)** STING mRNA relative expression in uninfected (CTR), HHV-6A, HHV-6B or HHV-7 infected NK92 cells 3d.p.i. *p values Student t test; **(B)** Western Blot analysis for house-keeping actin (upper blot) and STING (lower blot) expression in uninfected (CTR), HHV-6A, HHV-6B or HHV-7 infected NK92 cells 3d.p.i. For stimulation of the cytoplasmic DNA sensing pathways, we used 2′,3′-cGAMP. The molecular weights were determined by protein ladder (14.4-97.4kDa) (BioRad). Actin was evidenced at 44kDa, STING 35kDa. The images were acquired by Geliance 600 (Perkin Elmer, MA, United States). The complete Western Blots are reported in **Supplementary Figure S3**. *p value Student t test. The histogram represents the STING band intensity in relation with the corresponding actin band. **(C)** Western Blot analysis for house-keeping actin (upper blot) and cGas, IFI16, TBK1, IRF3, pIRF3Ser396, STAT6, pSTAT6Tyr641 (lower blots) expression in uninfected (CTR), HHV-6A, HHV-6B or HHV-7 infected NK92 cells 3 d.p.i. The molecular weights were determined by protein ladder (25-250kDa; 14.4-97.4 kDa). Actin was evidenced at 44kDa, cGas at 68kDa, IFI16 at 100kDa, TBK1 at 80kDa, IRF3 at 55kDa, STAT6 at 120kDa. The histogram represents the STING band intensity in relation with the corresponding actin band. *p value Student t test. The images were acquired by Geliance 600 (Perkin Elmer, MA, United States). The complete Western Blots are reported in **Supplementary Figure S3**. **(D)** HHV-6A infected NK92 cells were characterized by immunofluorescence for STING [anti-STING PE Ab (Clone T3-680)], STAT6 [anti-STAT6 FITC (Clone D-1)] and gp116 (Clone 6A5) expression. (Nikon Eclipse TE2000S) equipped with a digital camera. Original magnification 100×. Uninfected NK92 cells were used as control.

the STING antagonist H151 (**Figure 4D**). AS1517499 slightly reduced the secretion of CCL3, CCL4, TNF-alpha, and IL-8 in HHV-6B and HHV-7 infected NK cells (**Figure 4D**).

DISCUSSION

Here, we evaluated the effect of HHV-6A, HHV-6B, and HHV-7 NK cell infection on DNA sensor molecules. We used, for the first set of experiments, NK92 cell line to avoid individual differences in NK cell subpopulations. The confirmatory experiments were performed on primary NK cells obtained from control subjects. We found that the three viruses affect the DNA sensors in NK cells differently.

TLR 9 mRNA and protein levels were inhibited by HHV-6A infection but no effect was observed in the protein expression of Myd88, a downstream mediator of the TLR9 pathway. These data suggest a particular impairment in the TLR9 pathway during HHV-6A infection, that is slightly evident only during HHV-6B but not HHV-7 infection. As a proof of concept, the analysis of the TLR9 down-stream genes' activation (Huang and Yang, 2010), in the presence of mRNA sensor molecules (TLR3, TLR7, TLR8) antagonists/inhibitors, showed that HHV-6B and HHV-7 infections induced an increase in TNF-alpha and IL-8 expression, with the highest levels reached by IL-8 during HHV-7 infection. On the contrary, HHV-6A infection slightly modified TNF-alpha and IL-8 expression. TLR9 signaling is essential for the early cytotoxicity of NK cells during infections (Liese et al., 2007). The reduction of TLR9 expression in HHV-6A infected NK cells leads to an impaired cytokine expression that might prevent NK cells activation toward target cells and slow down the inflammatory response needed to fight the infection. On the contrary HHV-6B and HHV-7 induced TNF-alpha and IL-8 secretion by NK cells, as previously reported for astrocyte cultures (Chi et al., 2012), where HHV-6 infection promoted transforming growth factor β (TGF-β), IL-6, IL-8, and TNF-alpha production. During HHV-6B and HHV-7 infection, we observed a high expression of IL-8, as previously reported in HHV-6 infected Hep G2 liver cells (Inagi et al., 1996), where a significant induction of IL-8 gene expression was observed. These data suggest that HHV-6B and HHV-7 may induce a cytokine-mediated inflammatory response infecting NK cells. On the contrary, HHV-6A reduced pro-inflammatory cytokine release, which could result in NK cell dysfunction *in vivo*.

STING mRNA expression was increased during HHV-6A infection, but not during HHV-6B and HHV-7 infection.

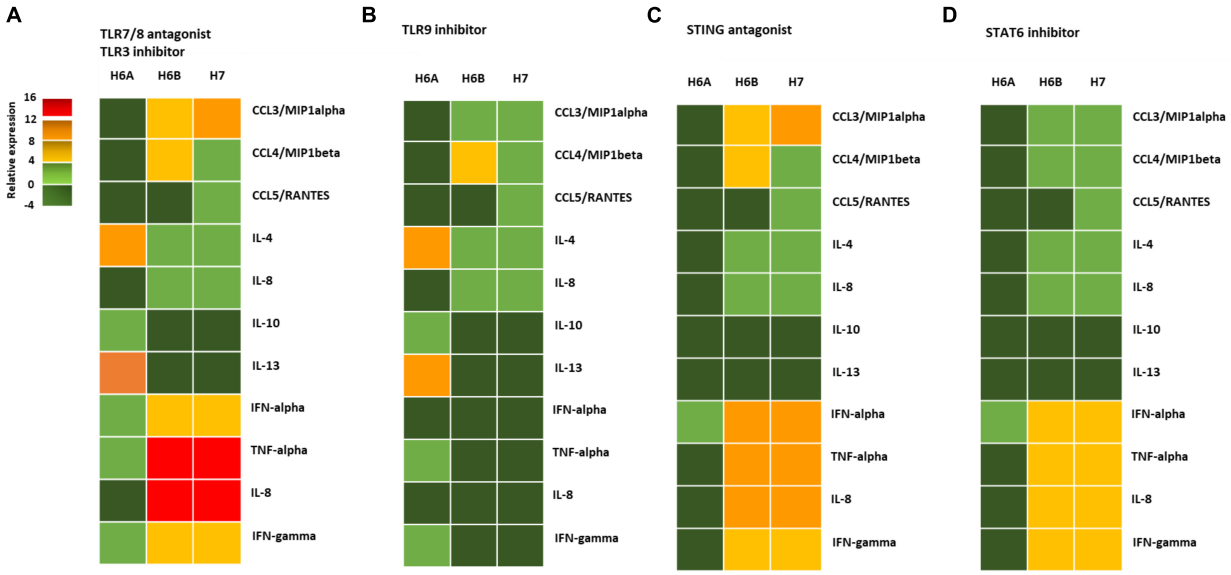

FIGURE 4 | NK cell cytokine/chemokine protein amount. The concentrations of cytokines in the culture supernatants of HHV-6A, HHV-6B or HHV-7 infected primary NK cells 3d.p.i. were quantified by immunoassays (CCL3, CCL4, CCL5, IL-4, IL-8, IL-10, IL-13, IFN-alpha, TNF-alpha, IL-8, IFN-gamma) (myBiosource, United States. The data are presented as relative to the expression of uninfected primary NK cells. Five donors have been analyzed and the data are the mean of the results obtained. The cell were treated with RNA/DNA sensor antagonists/inhibitors: **(A)** ODN 2087 TLR7 and TLR8 antagonist (0.5 μM) + TLR3.CI TLR3/dsRNA Complex Inhibitor (30 nM); **(B)** TLR3.CI TLR3/dsRNA Complex Inhibitor (30 nM) + ODN 2088 (Miltenyi Biotec), TLR7, TLR8, TLR9 antagonist (0.5 μM); **(C)** ODN 2087 TLR7 and TLR8 antagonist (0.5 μM) + TLR3.CI TLR3/dsRNA Complex Inhibitor (30 nM) + H151 STING antagonist (0.5 μM); **(D)** ODN 2087 TLR7 and TLR8 antagonist (0.5 μM) + TLR3.CI TLR3/dsRNA Complex Inhibitor (30 nM) + AS1517499 STAT6 inhibitor (100 nM). The green squares are representative of slight differences that do not reach the a significant statistical value.

Strangely, STING protein levels were not modified by all the three viruses, suggesting that the over-production of STING mRNA during HHV-6A infection is degraded, probably to convey the transduction machinery to the viral mRNAs. Similarly, 2′,3′-cGAMP affected only mRNA but not protein expression. Since STING is already efficiently expressed, we can hypothesize that 2′,3′-cGAMP affects the transcription of mRNA that is then degraded.

When we looked at STING pathway, we observed that TBK1 protein expression is induced by all three viruses, but without an enhanced IRF3 Ser^{396} phosphorylation. On the contrary, STAT6 Tyr^{641} phosphorylation was induced only by HHV-6A with a perinuclear co-localization of STING and STAT6 at 3 d.p.i. in HHV-6A infected cells, as suggested by the co-localization with the gp116 late antigen. It has been already shown that viruses or cytoplasmic nucleic acids trigger STING to recruit STAT6 to the ER, where it is phosphorylated on Ser^{407} by TBK1 and on Tyr^{641} by IL-4/IL-13 pathway (Chen et al., 2003). Dimerized STAT6 then translocates to the nucleus where it induces target genes responsible for immune cell homing. We have evaluated only STAT6 Tyr^{641} phosphorylation since there are no commercial antibodies available toward Ser^{407}. However, since TBK1 is induced in HHV-6A infected NK92 cells, we can hypothesize that both Ser^{407} and Tyr^{641} might be phosphorylated in HHV-6A infected NK92 cells and lead to down-stream genes' transcription. As a proof of concept, Atf3 transcription factor, that we have previously found up-modulated by HHV-6A infection of NK cells, is induced by IL-4 through STAT6 (Chen et al., 2003), supporting the activation of STING/STAT6 pathway.

cGAS protein levels were down-modulated by HHV-7 infection, while HHV-6A and HHV-6B remained at a basal level. IFI16 protein expression was down-modulated by HHV-6A and HHV-6B infection, while it remained at a basal level during HHV-7 infection. These results might confirm previous papers on HSV-1 that induced the degradation of IFI16 by a proteasome and apparently ICP0-dependent mechanism (Orzalli et al., 2012). Similarly, HHV-6A and HHV-6B seem to maintain the cGAS up-stream activation of STING pathway. On the contrary, HHV-7 data support the role of IFI16 as the primary HHV DNA sensor and restriction factor (Gariano et al., 2012), where cGAS has an indirect role in the presence of nuclear HHV DNA by interacting and stabilizing IFI16 (Orzalli et al., 2015).

The analysis of cytokines/chemokines secretion in HHV infected primary NK cells, showed a different behavior in the presence of the different viruses. The NK cell response seem to be similar in the presence of both HHV-6B and HHV-7 viral infection. NK cells express mainly CCL3, IFN-alpha, TNF-alpha, IL-8, and IFN-gamma. The use of DNA sensors antagonists assigns to TLR9 the main effect on cytokines/chemokines expression. On the contrary, HHV-6A infection of NK cells induced IL-4, IL-10, and IL-13. The addition of antagonists/inhibitors against STING and STAT6 reduced drastically the secretion of these cytokines by NK cells, supporting the activation of STING/STAT6 pathway as predominantly implicated in the response of NK cells to HHV-6A infection. These results suggest a chemoattractant role for cytokine/chemokine secreted by HHV-6B and HHV-7 infected

NK cells, while HHV-6A infected NK cells showed a viral-driven Th2 response (Kaiko et al., 2010).

Collectively, these results show an implication of TLR9 DNA sensor in the cytokine/chemokine expression by NK cells infected with HHV-6B and HHV-7. These might lead to the active control of *in vivo* viral spreading. HHV-6A infected NK cells conversely induced STING/STAT6 pathway, as a mechanism of anti-viral activation, but were characterized by a Th2 type response, providing a potential new mechanism used by HHV-6A to induce immunosuppression and immune evasion (Horan et al., 2013; Christensen and Paludan, 2017). To confirm these results, further studies are needed, to dissect the viral mechanism that leads to differential response of NK cells in the presence of different HHV infections.

In conclusion, we have shown that HHV-6A, HHV-6B, and HHV-7 infection of NK cells interact differently with cellular DNA sensors. Strikingly, HHV-6B behaves similarly to HHV-7 compared to HHV-6A, confirming the difference of HHV-6A and -6B in their molecular, epidemiological and biological properties (Ablashi et al., 2014).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

DB and RR analyzed the results and wrote the manuscript. DB performed the DNA sensor and cytokine/chemokine analysis. EC, IS, and MD'A performed viral infection. VG and AR performed viral titration and *in vitro* experiments. IB and RR performed cytofluorimetry. MS performed cell cultures. DD revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00226/full#supplementary-material

FIGURE S1 | EBV mRNA expression of latent (EBNA1, EBNA-2, LMP1) and lytic (BALF2) EBV genes in NK92 cell line. The lymphoblastoid cell line LCL-B95.8 (kind gift of Professor R. Dolcetti) was used as control of EBV gene expression, after viral cycle activation using TPA (12-*O*-tetradecanoylphorbol-13-acetate) (Sigma-Aldrich), used at 20 ng/ml.

FIGURE S2 | (A) Complete Western Blots for **Figure 2C**. **(B)** RT-PCR for TLR3, TLR7, and TLR8 expression in NK92 cell line. **(C)** Relative mRNA expression of IFN-alpha in the culture supernatants of NK92 cells untreated or treated with synthetic agonists (R-848, TLR7/8 agonist; Poly I:C, TLR3 agonist) with or without ODN 2087 TLR7 and TLR8 antagonist (0.5 μM) + TLR3.CI TLR3/dsRNA Complex Inhibitor (30 nM).

FIGURE S3 | Complete Western Blots for **Figures 3B,C**.

FIGURE S4 | Virus **(A)** presence (DNA) and **(B)** transcription (RNA) were evaluated, respectively, by qPCR and RT-qPCR performed on U42 virus gene, at 1, 2, 3, and 6 d.p.i., as already detailed. The infection was performed with 100 m.o.i. in primary NK cells.

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HSV-2 Infection of Human Genital Epithelial Cells Upregulates TLR9 Expression Through the SP1/JNK Signaling Pathway

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It is known that herpes simplex virus type 2 (HSV-2) triggers the activation of Toll-like receptor (TLR) 9 signaling pathway and the consequent production of antiviral cytokines in dendritic cells. However, the impact of HSV-2 infection on TLR9 expression and signaling in genital epithelial cells, the primary HSV-2 targets, has yet to be determined. In the current study, by using both human genital epithelial cell lines and primary genital epithelial cells as models, we found that HSV-2 infection enhances TLR9 expression at both mRNA and protein levels. Such enhancement is virus replication-dependent and CpG-independent, while the HSV-2-mediated upregulation of TLR9 does not activate TLR9 signaling pathway. Mechanistically, a SP1 binding site on TLR9 promoter appears to be essential for HSV-2-induced TLR9 transactivation. Upon HSV-2 infection, SP1 translocates from the cytoplasm to the nucleus, and consequently binds to TLR9 promoter. By using specific inhibitors, the JNK signaling pathway is shown to be involved in the HSV-2-induced TLR9 transactivation, while HSV-2 infection increases the phosphorylation but not the total level of JNK. In agreement, antagonism of JNK signaling pathway inhibits the HSV-2-induced SP1 nuclear translocation. Taken together, our study demonstrates that HSV-2 infection of human genital epithelial cells promotes TLR9 expression through SP1/JNK signaling pathway. Findings in this study provide insights into HSV-2-host interactions and potential targets for immune intervention.

Keywords: herpes simplex virus type 2, toll-like receptor 9, genital epithelial cells, specificity protein 1, JNK pathway

INTRODUCTION

Herpes simplex virus type 2 (HSV-2) is a large double-stranded DNA (dsDNA) virus that primarily infects genital epithelial cells during the lytic cycle and can also eslishes a lifelong latency in the sacral ganglia (1). HSV-2 infection causes clinical manifestations such as genital ulcers, blindness and encephalitis (2, 3). HSV-2 is epidemiologically proven to enhance HIV-1 acquisition, transmission and disease progression (4–6). An implication of HSV-2 to the pathogenesis of

Kaposi's sarcoma has also been proposed (7). To date, there are still no preventative vaccines or curative measures available against HSV-2.

During its infection and replication, HSV-2 can trigger innate immunity through various recognition signaling pathways including Toll-like receptor (TLR)-dependent and independent pathways (8–10). TLRs are type I transmembrane proteins that recognize pathogen-associated molecular patterns (PAMPs) and signal via MyD88-dependent or TRIF-dependent pathways. Different TLRs can recognize different HSV-2 components. For instance, TLR2 recognizes glycoproteins gH/gL and gB (11), TLR3 recognizes viral double-stranded RNA (dsRNA) (12) and TLR9 recognizes unmethylated CpG motifs in viral dsDNA (13). Activation of TLR-mediated signaling pathways leads to the production of inflammatory cytokines.

Recognition of HSV-2 by TLRs, in particular TLR9, has been reported by several studies. For instance, in plasmacytoid dendritic cells (pDCs), both HSV-1 and HSV-2 can stimulate IFN-α expression via TLR9/MyD88 signaling pathway, and this stimulation is not viral replication-dependent (13). Certain strains of HSV-1/2 are sequentially recognized by TLR2 and TLR9 in conventional DCs (cDCs), but not pDCs, for the induction of IL-6 and IL-12 (14). However, most of these studies adopted immunocompetent cells like DCs as models, and discrepancies have been observed (15). Given that HSV-2 predominantly infects epithelial cells at the portal of viral entry, the findings obtained from DCs may not well represent the events during its primary infection. In addition, previous studies mainly focused on whether and how HSV-2 infection triggers the activation of TLR9 signaling pathway, with little attention being paid to the regulation of TLR9 expression.

In this study, by using human genital epithelial cell lines and primary genital epithelial cells as models, we investigated the impact of HSV-2 infection on TLR9 expression and signaling. We demonstrated that HSV-2 upregulates TLR9 expression but does not activate TLR9 signaling pathway. We further revealed that HSV-2 enhances TLR9 expression in a viral replication-dependent manner by promoting TLR9 promoter activation via SP1/JNK signaling pathway.

MATERIALS AND METHODS

Cells, Virus, and Inhibitors

Vero cell line and human cervical epithelial cell lines ME-180 and HeLa were purchased from American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% FBS and antibiotics. HSV-2 (G strain) was obtained from LGC standards, propagated in ME-180, and titrated in Vero cells. Titrated virus stocks were aliquoted and stored at −80°C until use. Signaling inhibitors specifically targeting TBK1/IKKε (BX795), IκB-α (BAY11-7082), JNK (SP600125), and p38 (SB203580) were purchased from InvivoGen and used according to the manufacturer's instructions.

Plasmids

Human TLR9 promoter sequence (−2,577/+77) was amplified from genomic DNA and cloned into pGL3-Basic luciferase reporter vector (Promega) and designated as pGL3-TLR9. Truncations on TLR9 promoter were made based on pGL3-TLR9, and designated as (−1,577/+77)TLR9, (−1,077/+77)TLR9, (−577/+77)TLR9, (−377/+77)TLR9, (−177/+77)TLR9, and (−77/+77)TLR9, respectively. Mutations of transcription factor binding sequences on TLR9 promoter were made based on full length pGL3-TLR9 using QuickChange II Site-Directed Mutagenesis Kit (Agilent), and designated as 5′PU MUT, 3′PU MUT, 3′AP MUT, 5′AP+3′AP MUT, SP1 MUT and C/EBP MUT, respectively. Full open reading frames of transcription factor specificity protein 1 (SP1) and TLR9 were amplified from human cDNA library and cloned into pcDNA3.1(+) (Thermo Fisher Scientific) and designated as pcDNA3.1-SP1 and pcDNA3.1-TLR9, respectively. The pRL-TK Renilla luciferase control reporter vector was purchased from Promega. All primers used for plasmid construction were listed in **Table S1**.

Isolation and Infection of Primary Foreskin Epithelial Cells

All protocols involving human subjects were reviewed and approved by the Research Ethics Committee of Wuhan Institute of Virology, Chinese Academy of Sciences. Informed written consents from the human subjects were obtained in this study, and informed written parental consents were obtained for all participants under the age of 18.

Foreskin samples were obtained from teenagers who underwent circumcision in Wuhan Medical and Healthcare Center for Women and Children, and foreskin epithelial cells were isolated as previously described (16). For infection assay, cells were infected with HSV-2 at an MOI of 0.5 for 24 h. For signaling pathway inhibition, inhibitors were introduced into the culture 1–2 h after infection and maintained until 24 h. After 24 h of infection, cells were lysed and TLR9 expression was determined by Western blot.

HSV-2 Infection

Human cervical epithelial cell line ME-180 or primary foreskin epithelial cells were preseeded in 24-well-plates 1 day before infection. In most cases, cells were infected with HSV-2 at an MOI of 0.5 for 24 h. For infection dose assay, cells were infected with ascendant HSV-2 doses ranging from 0 to 2 MOI. For infection time course, cells were infected with HSV-2 for ascendant time periods ranging from 0 to 30 h. UV-inactivated HSV-2 (UV-HSV-2) was obtained by exposing viruses to UV irradiation for 15 min as previously described (17). To separate HSV-2 virus particles from cytokine-containing medium, virus stocks were filtrated through a 100 kD ultracentrifugal filter tube (Thermo Fisher Scientific Pierce) by centrifuging at 1,000 g for 20 min at 4°C. Filter-through fraction (HSV-2 free cytokine fraction) was collected directly while the membrane-retained fraction (cytokine-free HSV-2 fraction) was diluted with fresh medium and collected for infection.

Transfection and Luciferase Reporter Gene Assay

All plasmid transfections in this study were conducted using Lipofectamine 2000 (Thermo Fisher Scientific Invitrogen) according to the manufacturer's instructions. For luciferase reporter gene-based promoter activation, constructs carrying promoter of interest in full-length, truncations or with mutations were co-transfected with the Renilla-expressing control plasmid pRL-TK into ME-180 cells. Four to six hours post transfection, cells were infected with HSV-2 or UV-HSV-2 for another 24 h. Afterwards, cells were lysed and firefly luciferase and Renilla luciferase activities were measured using the Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions. For siRNA knockdown, siRNAs were transfected 24 h before plasmid transfection using X-tremeGENE^{TM} siRNA Transfection Reagent (Roche) according to the manufacturer's instructions. For signaling pathway inhibition, signaling pathway inhibitors were added 1–2 h after virus infection and maintained until luciferase measurement. For CpG treatment, 4–6 h after plasmid transfection, cells were treated with CpG (ODN 2395, Miltenyi Biotec) or GpC (ODN 5328, Miltenyi Biotec) according to the manufacturer's instructions.

RNA Extraction and Semi-Quantitative RT-PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen) and then reverse-transcribed into cDNA using High-capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), both according to the manufacturers' instructions. TLR9 mRNA level was semi-quantified by SYBR Green RT-PCR, as previously described with modifications (18). In brief, reaction was prepared using SsoAdvanced^{TM} Universal SYBR Green Supermix (Bio-Rad) and PCR was run on a Bio-Rad CFX Connect platform. GAPDH was used as an internal control and $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression of TLR9. The following primer pairs were used. TLR9, forward: 5′-CGTCTTGAA GGCCTGGTGTTGA-3′, reverse: 5′-CTGGAAGGCCTTGGT TTTAGTGA-3′; GAPDH, forward: 5′-GCCAAGGTCATCCAT GACAACTTTGG-3′, reverse: 5′-GCCTGCTTCACCACCTTC TTGATGTC-3′.

ELISA

IL-6 expression by ME-180 and peripheral blood mono-nuclear cells (PBMCs) in response to HSV-2 infection or GpC stimulation was quantified by ELISA. In brief, PBMCs were isolated from single buffy coats of healthy donors obtained from NHS Blood and Transplant using Histopaque (Sigma-Aldrich). ME-180 cells with or without TLR9 overexpression and PBMCs were either infected with HSV-2 or stimulated with CpG or control GpC for 24 h. Cell culture supernatants were collected and IL-6 was quantified by ELISA using human IL-6 ELISA kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Western Blot

For detection of protein expression at whole cell level, cell lysates were prepared using Pierce IP Lysis buffer (Thermo Fisher Scientific) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Santa Cruz). For detection of protein expression in cytoplasm and nucleus, cytoplasmic and nuclear fractions were prepared using Pierce NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. For Western blot analysis, protein samples were first separated by 4–15% SDS-PAGE gel (Bio-Rad), and then transferred onto a 0.45 μm PVDF membrane. After blocking with 5% non-fat milk, membrane was incubated sequentially with primary and HRP-conjugated secondary antibodies. Following the final incubation, membrane was extensively washed, and immuno-bands were visualized using ECL substrate (Millipore) under a CCD camera (LAS 4000, Fujifilm). The following primary antibodies were used: mouse anti-human TLR8 (Santa Cruz), rabbit anti-human TLR9 (Cell Signaling Technology), mouse anti-human β-actin (Santa Cruz), rabbit anti-human MyD88 (Cell Signaling Technology), mouse anti-human SP1 (Santa Cruz), mouse anti-human HDAC1 (Santa Cruz), mouse anti-human JNK (Santa Cruz), rabbit anti-human p-JNK (Cell Signaling Technology). The following HRP-conjugated secondary antibodies were used: goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP (both from Abcam).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP was used to test the binding of SP1 to TLR9 promoter using Piece Magnetic ChIP Kit (Thermo Fisher Scientific), as previously described with modifications (19). In brief, cells with or without HSV-2 infection were crosslinked with 1% formaldehyde and harvested. Pelleted cells were then lysed and digested with MNase in the presence of protease/phosphatase inhibitors. After digestion, nuclear fraction was harvested, and fragmentized chromatin was released from nuclei by sonication. Immunoprecipitation was performed with a ChIP grade rabbit anti-SP1 antibody (Merck Millipore) or a normal rabbit IgG (negative control, Merck Millipore) overnight at 4°C and pulled down with protein A/G magnetic beads. Recovered DNA samples were used for PCR using TLR9 promoter specific primers. Forward: 5′-AAGAGGAAGGGGTGAAGGAG-3′, reverse: 5′-TTCCCACAGGGGCAGCAGCG-3′.

Statistical Analysis

All data in this study were expressed as mean ± standard deviation (SD). Statistical analyses were performed with GraphPad Prism 7.02. Mann–Whitney test was used for comparison between two groups while Kruskal–Wallis test was used when three or more groups were compared. For all comparisons, a $p < 0.05$ was considered statistically significant.

RESULTS

HSV-2 Infection Increases TLR9 Transcription and Translation

It is known that HSV-2 activates several TLRs in pDCs (13). Here we investigated the impact of HSV-2 infection on TLR7, 8, and 9 activation in human genital epithelial cells, the main HSV-2 targets during primary infection. We constructed luciferase-carrying plasmids under the control of TLR7, 8 or 9 promoter (named as pGL3-TLR7, pGL3-TLR8, and pGL3-TLR9, respectively) and examined the responses to HSV-2 infection in

cervical epithelial cells ME-180. As shown in **Figure 1A**, HSV-2 infection significantly induced TLR9 promoter activation. After HSV-2 infection, TLR7 promoter was also moderately activated but no apparent activation was observed for TLR8 promoter. Since TLR9 promoter showed the highest level of activation upon HSV-2 infection, we focused on HSV-2 infection-induced TLR9 upregulation. Western blot results showed that HSV-2-induced activation of TLR9 promoter also led to the increase of TLR9 expression at protein level in both ME-180 (**Figure 1B**) and primary foreskin epithelial cells (**Figure 1C**).

To exclude possible involvement of cytokines in the virus stock, HSV-2 virus stock was filtered through a 100 kD Amicon ultracentrifugal unit. Cytokine-free viruses and virus-free supernatants were harvested separately and used to treat cells transfected with pGL3-TLR9. Results showed that only virus-containing fraction (cytokine-free HSV-2), but not HSV-2-free cytokines induced TLR9 promoter activation, indicating that the TLR9 induction was mediated by HSV-2 but not cytokines in the samples (**Figure 1D**).

Further infection dose assay showed that TLR9 promoter activation was enhanced when HSV-2 dose increased (**Figure 1E**). Time-course assay revealed that HSV-2 induced TLR9 promoter activation in an infection time-dependent manner, which peaked around 24 h after infection (**Figure 1F**). Consistent results were also observed at both mRNA and protein levels (**Figures 1G–J**). In addition, ME-180 cells infected with 0.5 MOI of HSV-2 showed a much higher percentage of infection compared to those infected with 0.1 MOI of HSV-2, which was consistent with the levels of TLR9 mRNA and protein, suggesting that TLR9 expression was upregulated in HSV-2-infected cells (**Figures 1G,I** and **Figure S1**).

Taken together, our data indicate that HSV-2 induces the activation of TLR9 promoter and consequently leads to the upregulation of TLR9 at both the mRNA and protein levels.

HSV-2-Induced TLR9 Upregulation Is Viral Replication-Dependent

As a pattern recognition receptor, TLR9 is activated by unmethylated CpG-rich DNA sequence and leads to the secretion of pro-inflammatory cytokines. HSV-2, as a DNA virus, has abundant CpG motifs in its genome. To assess if HSV-2-induced TLR9 upregulation was a result of CpG-triggered TLR9 activation, TLR9 induction by UV inactivated HSV-2 and synthesized CpG ODNs was analyzed. As shown in **Figures 2A,D**, only replicative HSV-2, but not UV-treated HSV-2 or CpG ODN triggered the activation of TLR9 promoter. Consistent results were observed at both mRNA and protein levels (**Figures 2B,C,E,F**). These data indicate that HSV-2-induced TLR9 upregulation is HSV-2 replication-dependent and CpG-independent.

HSV-2 Induces TLR9 Expression Without Activating TLR9 Signaling Pathway

Since HSV-2 infection induced TLR9 expression, we next investigated whether this induction could activate TLR9 signaling pathway and cause the production of pro-inflammatory cytokines like IL-6. We constructed a firefly luciferase reporter plasmid under the control of IL-6 promoter and tested its response to HSV-2 infection. Our data showed that HSV-2 infection induced the promoter activation of TLR9 but not that of IL-6 (**Figure 3A**). To further confirm these results, we treated ME-180 cells with HSV-2 or CpG ODNs and measured the IL-6 concentration in the supernatants. As shown in **Figure 3B**, neither HSV-2 infection nor CpG treatment induced IL-6 expression. As a positive control, CpG treatment significantly increased IL-6 in the PBMCs (**Figure 3B**). Upon activation, TLR9 binds to its adaptor protein MyD88, which subsequently activates downstream signaling pathway. To assess if HSV-2 infection-upregulated TLR9 was activated and able to bind to MyD88, co-immunoprecipitation with anti-MyD88 antibody was performed. As shown in **Figure 3C**, no interaction between HSV-2-upregulated TLR9 and MyD88 was detected. However, TLR9 overexpression by transfection with pTLR9 plasmid showed binding of the two proteins. In consistent, pTLR9 transfection alone also triggered high level of IL-6 expression (**Figure S2**). Taken together, these data indicate that HSV-2 infection induces TLR9 expression without activating the TLR9 signaling pathway.

SP1-Binding Site in TLR9 Promoter Is Involved in HSV-2-Induced TLR9 Expression

Since TLR9 promoter could be transactivated by HSV-2 infection, we next investigated whether one or more *cis*-elements in the TLR9 promoter was involved in this transactivation. 5' serial truncations of TLR9 promoter showed that HSV-2 induced luciferase activity was lost when−177 to−77 bp of the promoter were deleted (**Figure 4A**). Additional bioinformatics analysis predicted a few transcription factor binding sites in this region, including PU box, AP1, SP1, and C/EBP (**Figure 4B**). Removal of these sites by point-direct mutations showed that only the mutation of the SP1 binding site demolished the responsiveness of TLR9 promoter to HSV-2 infection, indicating that the SP1 binding site within−177 to−77 bp is essential for the HSV-2-induced TLR9 promoter activation (**Figure 4C**).

SP1 Binds to TLR9 Promoter After HSV-2 Infection

Given the importance of SP1 binding site in TLR9 transactivation upon HSV-2 infection, we proposed that HSV-2 infection likely activates TLR9 promoter by promoting SP1 binding to SP1 binding site in the promoter. To test this hypothesis, ChIP assay was performed and TLR9 promoter fragment containing the SP1 binding site was amplified by PCR. As shown in **Figure 5A**, a positive amplification of the fragment was only seen in the pulldown by anti-SP1 antibody in HSV-2 treated cells, but not by control IgG or in cells without HSV-2 treatment. This indicates that HSV-2 infection enhances TLR9 expression through promoting SP1 binding to TLR9 promoter.

To further confirm the role of SP1 on TLR9 promoter transactivation, the activation level of TLR9 promoter was measured under the condition of SP1 overexpression. As

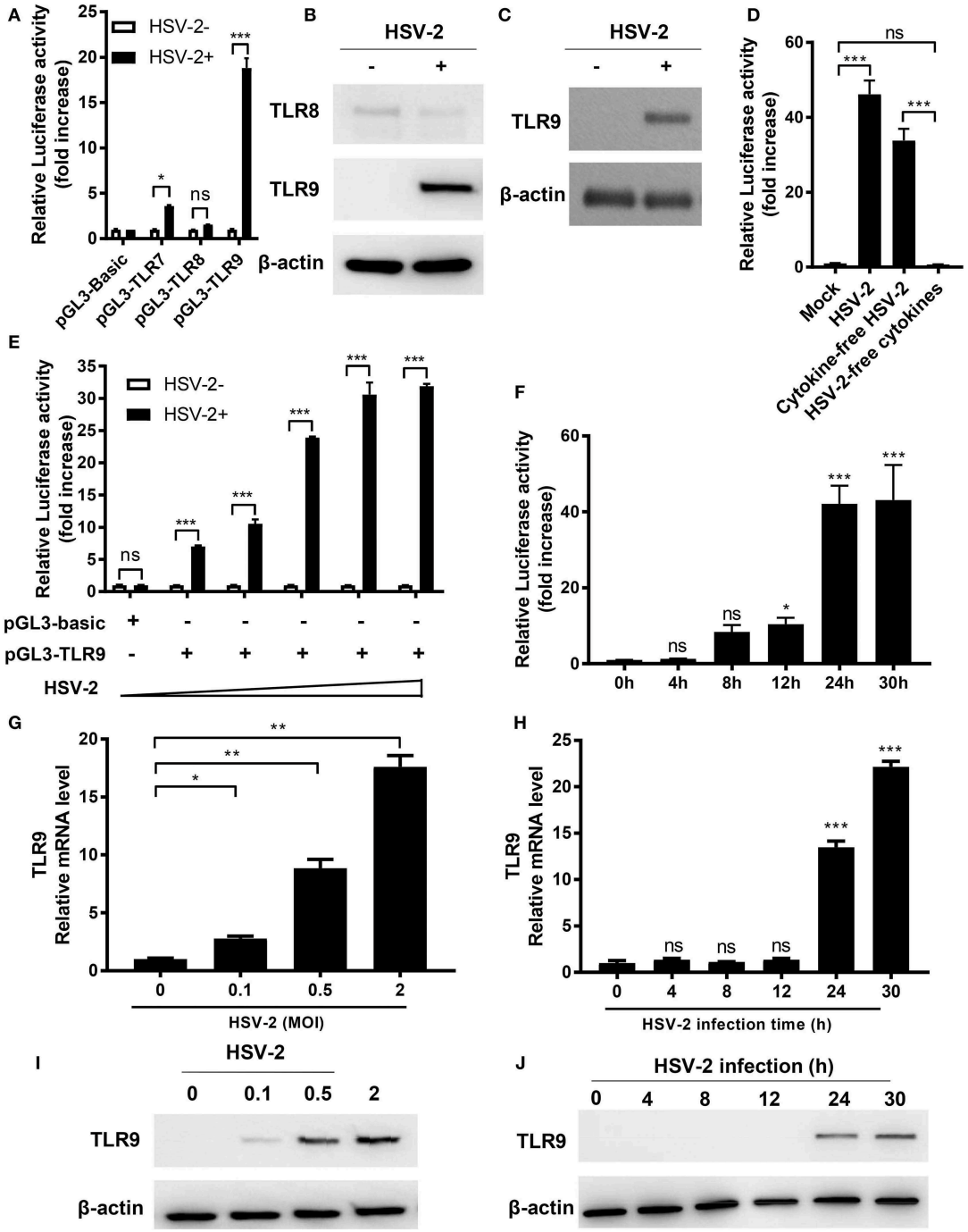

FIGURE 1 | HSV-2 infection induces TLR9 expression in genital epithelial cells. **(A)** ME-180 cells were transfected with reporter plasmid pGL3-TLR7, pGL3-TLR8 or pGL3-TLR9 and infected with or without HSV-2. Twenty-four hours later, relative luciferase activity was measured. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. **(B,C)** ME-180 **(B)** and primary foreskin epithelial cells **(C)** were infected with HSV-2 for 24 h and the expression of TLR8 and TLR9 was determined by Western blot. One representative experiment out of three is shown. **(D)** HSV-2 stock was fractionized into cytokine-free viruses and virus-free cytokines by ultrcentrifugation and both fractions were used to infect ME-180 cells transfected with pGL3-TLR9. Twenty-four hours after infection, relative luciferase activity was measured. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. **(E,F)** ME-180 cells were transfected with or without pGL3-TLR9 were infected with ascending doses of HSV-2 for 24 h **(E)** or with 0.5 MOI HSV-2 for ascending infection time periods **(F)**. After incubation, relative luciferase activity was measured. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. **(G–J)** ME-180 cells were infected with or without ascending doses of HSV-2 for 24 h **(G,I)** or infected with 0.5 MOI HSV-2 for ascending infection time periods **(H,J)**. After incubation, TLR9 mRNA level **(G,H)** and protein level **(I,J)** were determined by RT-PCR **(G,H)** and Western blot **(I,J)**, respectively. For RT-PCR results, data shown are mean ± SD of three independent experiments with each condition performed in duplicate. For Western blot results, one representative experiment out of three is shown. ns, statistically not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

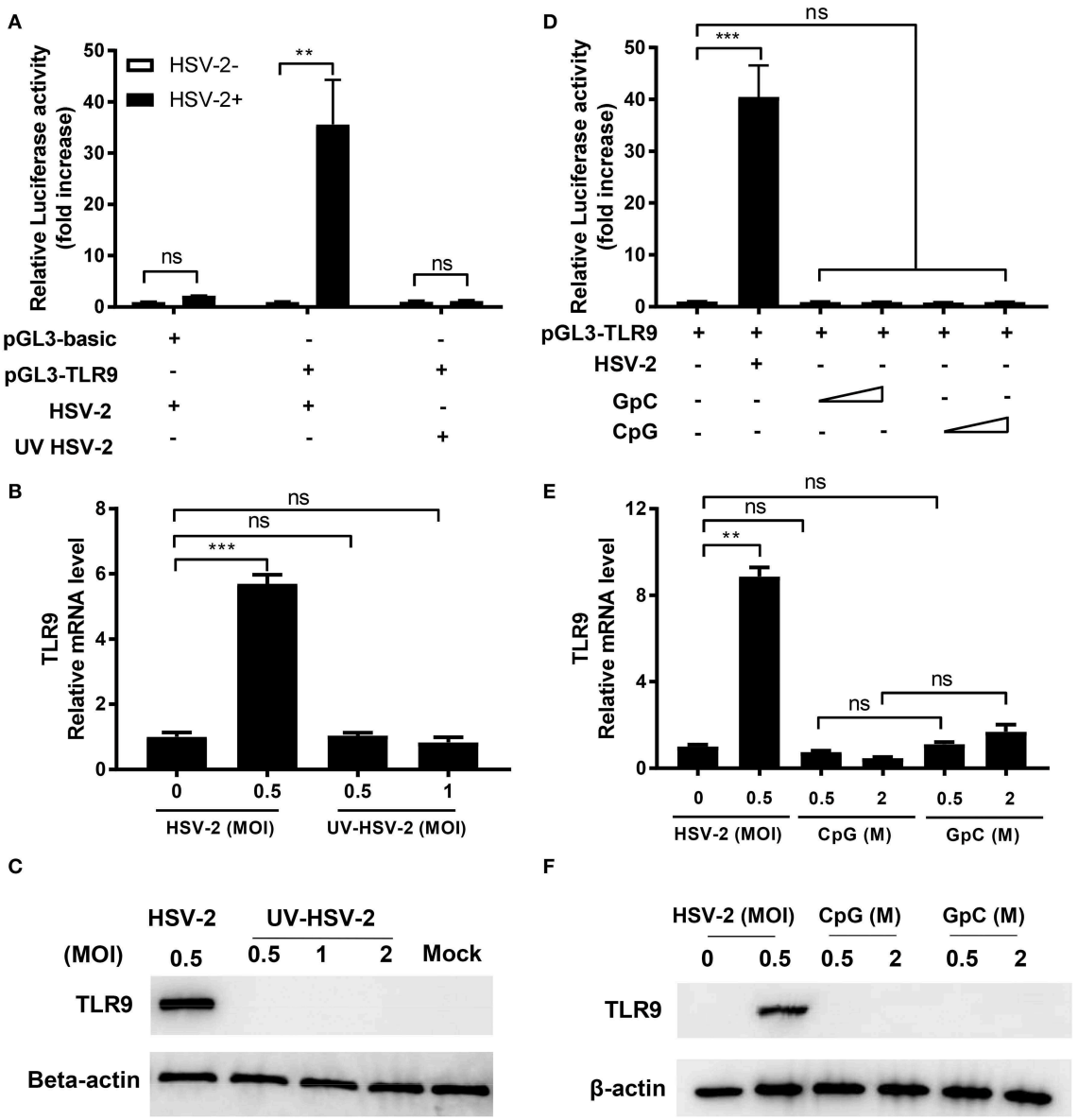

FIGURE 2 | HSV-2-induced TLR9 expression is viral replication-dependent. ME-180 cells were transfected with or without pGL3-TLR9 and infected with HSV-2 **(A–C)** or treated with CpG or GpC ODNs **(D–F)**. Twenty-four hours after infection or treatment, relative luciferase activity **(A,D)**, relative TLR9 mRNA level **(B,E),** and TLR9 protein level **(C–F)** were determined by dual luciferase activity assay **(A,D)**, RT-PCR **(B,E)**, and Western blot **(C,F)**, respectively. For luciferase assay and RT-PCR, data shown are mean ± SD of three independent experiments with each condition performed in duplicate. For Western blot results, one representative experiment out of three is shown. ns, statistically not significant; $^{**}p < 0.01$; $^{***}p < 0.001$.

shown in **Figure 5B**, SP1 overexpression significantly enhanced TLR9 promoter activity. The essential role of SP1 in HSV-2-induced TLR9 transactivation was also confirmed by siRNA interference of SP1. Luciferase reporter gene assay showed that HSV-2-induced transactivation of TLR9 promoter was largely impaired when SP1 was knocked down by siRNAs (**Figure 5C**). Western blot analysis showed consistent results, revealing a SP1-dependent TLR9 expression under the condition of HSV-2 infection (**Figure 5D**). In order to regulate promoter activity, transcription factors need to translocate from the cytoplasm to the nucleus. We next investigated whether HSV-2 infection also enhanced SP1 nuclear translocation. As shown in **Figure 5E**, a near full nuclear translocation of SP1 was observed when cells were infected with HSV-2. In addition, upon HSV-2 infection, an increased SP1 phosphorylation level (top band) (20) was seen in the nuclear fraction, indicating that HSV-2 infection also enhanced SP1 phosphorylation (**Figure 5E**). Taken together, these data here indicate that HSV-2 infection induces TLR9 expression via promoting SP1 binding to TLR9 promoter.

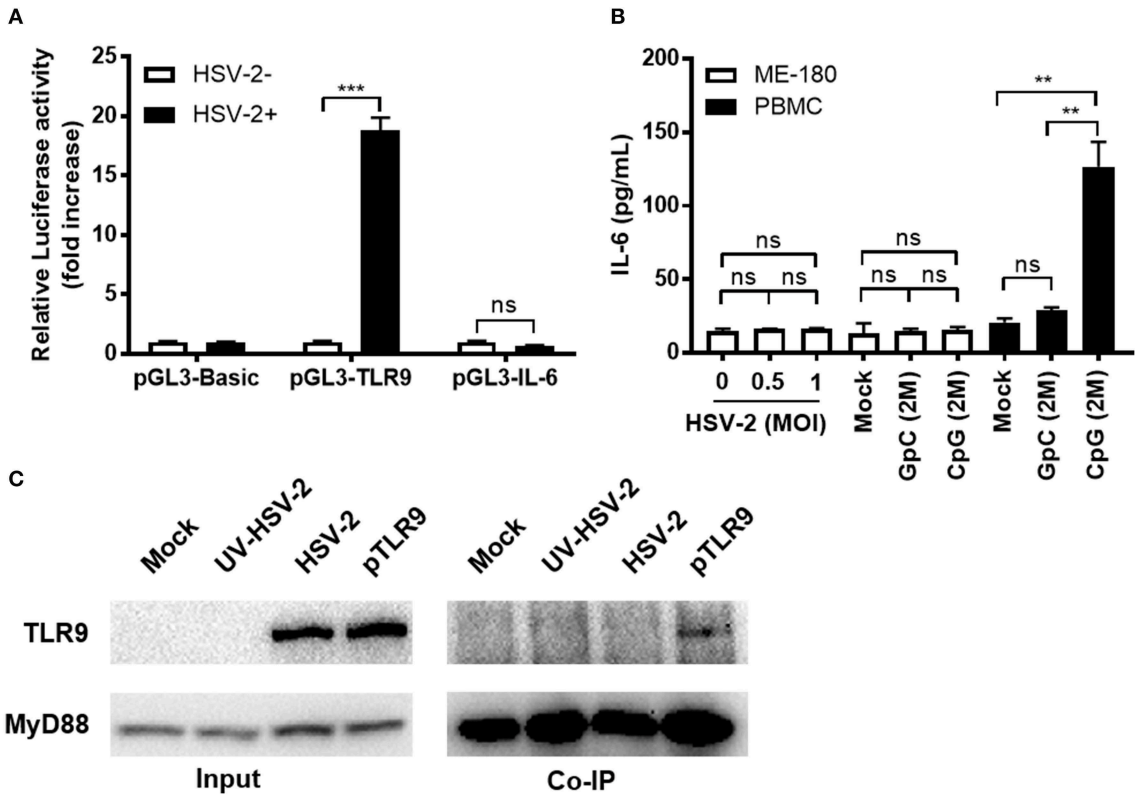

FIGURE 3 | HSV-2-induced TLR9 expression does not activate TLR9 signaling pathway. **(A)** ME-180 cells were transfected with pGL3-TLR9 or pGL3-IL-6 and infected with 0.5 MOI HSV-2 for 24 h. After incubation, relative luciferase activity was measured. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. **(B)** ME-180 cells or PBMCs were infected with or without HSV-2 or treated with or without CpG or GpC ODNs for 24 h. After incubation, IL-6 concentration in the cell culture supernatants were measured by ELISA. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. ns, statistically not significant; $^{**}p < 0.01$; $^{***}p < 0.001$. **(C)** ME-180 cells were either mock-infected, or infected with UV-HSV-2, HSV-2 or transfected with pTLR9. Twenty-four hours later, cells were lysed and immunoprecipitation was performed with anti-MyD88 antibody and the presence of TLR9 and MyD88 in the pulldown was detected by Western blot. One representative experiment out of three is shown.

HSV-2 Induces TLR9 Expression by Activating JNK Signaling Pathway

To determine which signaling pathway(s) was involved in HSV-2-induced TLR9 upregulation, inhibitors targeting TBK1/IKKε (BX795), IκBα (BAY11-7082), JNK (SP600125), and p38/MAPK (SB203580) were used. As shown in **Figure 6A**, only the addition of SP600125 significantly reduced the luciferase activity, indicating a JNK-dependent signaling pathway. To further confirm these findings, signaling inhibition assay was repeated in both ME-180 and primary foreskin epithelial cells where TLR9 expression was determined by Western blot. In consistent with the luciferase reporter gene assay, SP600125, but not SB203580 could significantly decrease TLR9 expression in both ME-180 (**Figure 6B**) and foreskin epithelial cells (**Figure 6C**).

We next determined the impact of HSV-2 infection on the activation of JNK signaling pathway. As shown in **Figure 6D**, HSV-2 infection did not show apparent impact on JNK expression, but significantly increased its phosphorylation level. The impact of inhibiting JNK signaling pathway on SP1 phosphorylation and nuclear translocation was also investigated. As shown in **Figure 6E**, SP600125, but not SB203580, significantly decreased SP1 nuclear translocation. Furthermore, upon SP600125 treatment, SP1 phosphorylation level was also considerably decreased in the nucleus.

Collectively, our study has revealed that replicative, but not UV-inactivated HSV-2 induces TLR9 expression in human genital epithelial cells, and this induction is through promoting SP1 binding to TLR9 promoter via JNK signaling pathway.

DISCUSSION

It is known that HSV-2 is recognized by TLR2/3/9 signaling pathway in immunocompetent cells like DCs, NK cells and macrophages, leading to the production of antiviral inflammatory cytokines, such as type I IFNs, IL-6, and IL-12. However, little attention has been paid to the interaction between HSV-2 and TLRs in genital epithelial cells. In the current study, we reveal that HSV-2 infection triggers TLR9 expression in both human genital epithelial cell lines and primary cells. We demonstrate that HSV-2-induced TLR9 expression is mediated by promoting SP1 binding to TLR9 promoter via JNK signaling pathway. Since TLR9 expression is mainly manifested at 24 h post infection, it is possible that HSV-2 drives a response which subsequently activates TLR9 promoter. Although it remains to

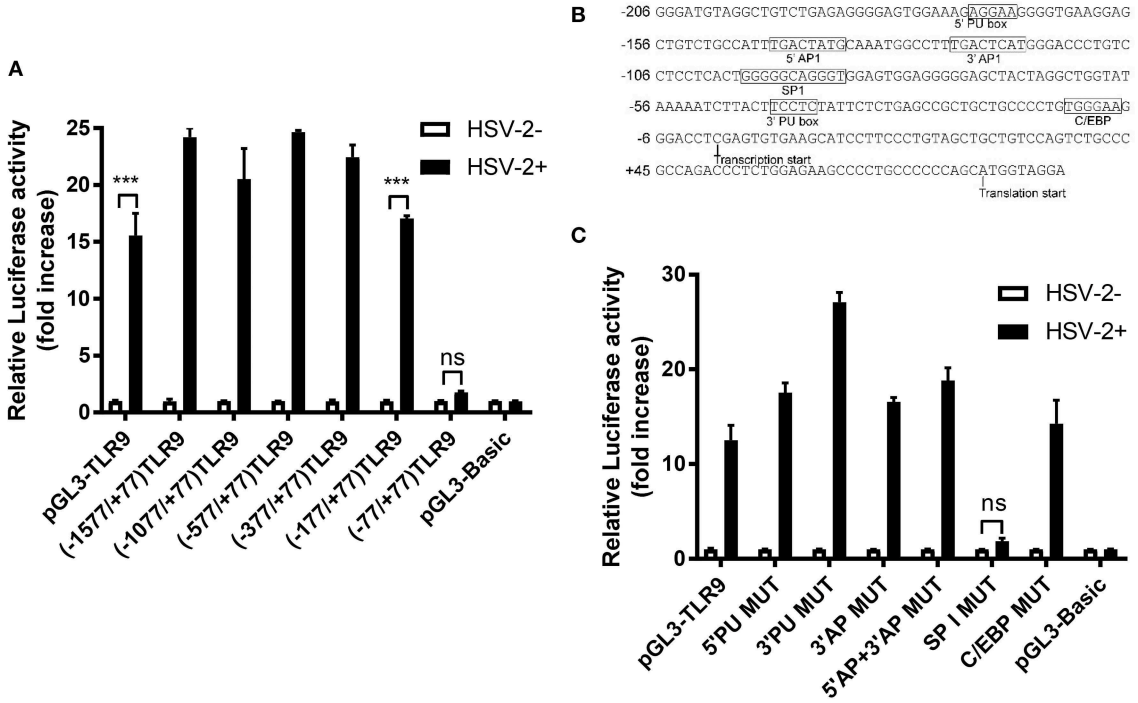

FIGURE 4 | SP1 binding site on TLR9 promoter is involved in HSV-2-induced TLR9 transactivation. **(A)** ME-180 cells were transfected with reporter plasmids with 5′ serial deletions within the TLR9 promoter region and infected with or without 0.5 MOI HSV-2 for 24 h. After incubation, relative luciferase activity was measured. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. **(B)** TLR9 promoter sequence was analyzed by Gene2promoter software and the predicted transcription binding sites were shown. **(C)** ME-180 cells were transfected with reporter plasmids with or without mutations within the TLR9 promoter region and infected with or without 0.5 MOI HSV-2 for 24 h. After incubation, relative luciferase activity was measured. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. ns, statistically not significant; ***$p < 0.001$.

be determined whether a secondary response is involved in the HSV-2-induced TLR9 expression, we can largely rule out the involvement of HSV-2-induced cytokines in the process, as we found that cytokines in the viral stock did not activate TLR9 promoter. Given that HSV-2 in our current study was propagated in ME-180, the cell line also used for the subsequent infection experiments, the cytokine composition induced in the infection experiments would be similar to that in the viral stock and unlikely to impact TLR9 expression.

TLRs recognize pathogens and activate downstream signaling members to initiate innate immune responses. In general, increased TLR expression can increase pathogen recognition, which leads to enhanced immune response (21). For instance, upregulation of TLR4 expression by IL-27 enhances proinflammatory cytokine production in human monocytes (22). GM-CSF-promoted expression of TLR3 and TLR7 increases the release of IL-13 and IL-6 in mast cells (23). Of interest, in our current study, although a significant increase of TLR9 expression was observed when epithelial cells were infected with HSV-2, we did not observe an apparently increased activation of TLR9 signaling pathway. In contrast, TLR9 overexpression in the absence of HSV-2 did trigger the activation of the signaling pathway in genital epithelial cells, showing that TLR9 overexpression in ME-180 cells activated the TLR9 signaling, which resulted in enhanced IL-6 secretion in the cell culture (**Figure S2**). Moreover, we found that genital epithelial cells with ectopic overexpression of TLR9 were resistant to HSV-2 infection (**Figure S3**), indicating a TLR9 mediated anti-HSV-2 capability in the genital epithelial cells. Nevertheless, HSV-2-induced TLR9 expression appeared not to trigger the activation of the downstream signaling pathway, suggesting that HSV-2 may have evolved a mechanism to antagonize the TLR9 signaling pathway.

We found that HSV-2-induced TLR9 expression is HSV-2 replication-dependent and CpG-independent, indicating that TLR9 expression is induced by HSV-2 infection rather than the CpG motifs within the viral genome. It has been shown that TLR9 can antagonize affinity maturation by preventing B cells from antigen capture and presentation (24). TLR9 was also reported to negatively modulate antifungal functions in macrophages (25). Although further investigation is required, it is probable that HSV-2 increases TLR9 expression as a mechanism to interrupt host adaptive immune response. It is known that HSV-2 progeny virus packaging happens in the Golgi apparatus which uses intracellular membrane system for virus release (26). During this process, the virus usually adopts cellular functions and pathways to facilitate its release by specifically interacting with host cell lipids and proteins (27). Since TLR9 is mainly present in the intracellular vesicles and circulates within the membrane system (28, 29), future study is warranted to determine whether HSV-2 can hijack TLR9 to enhance virus transportation.

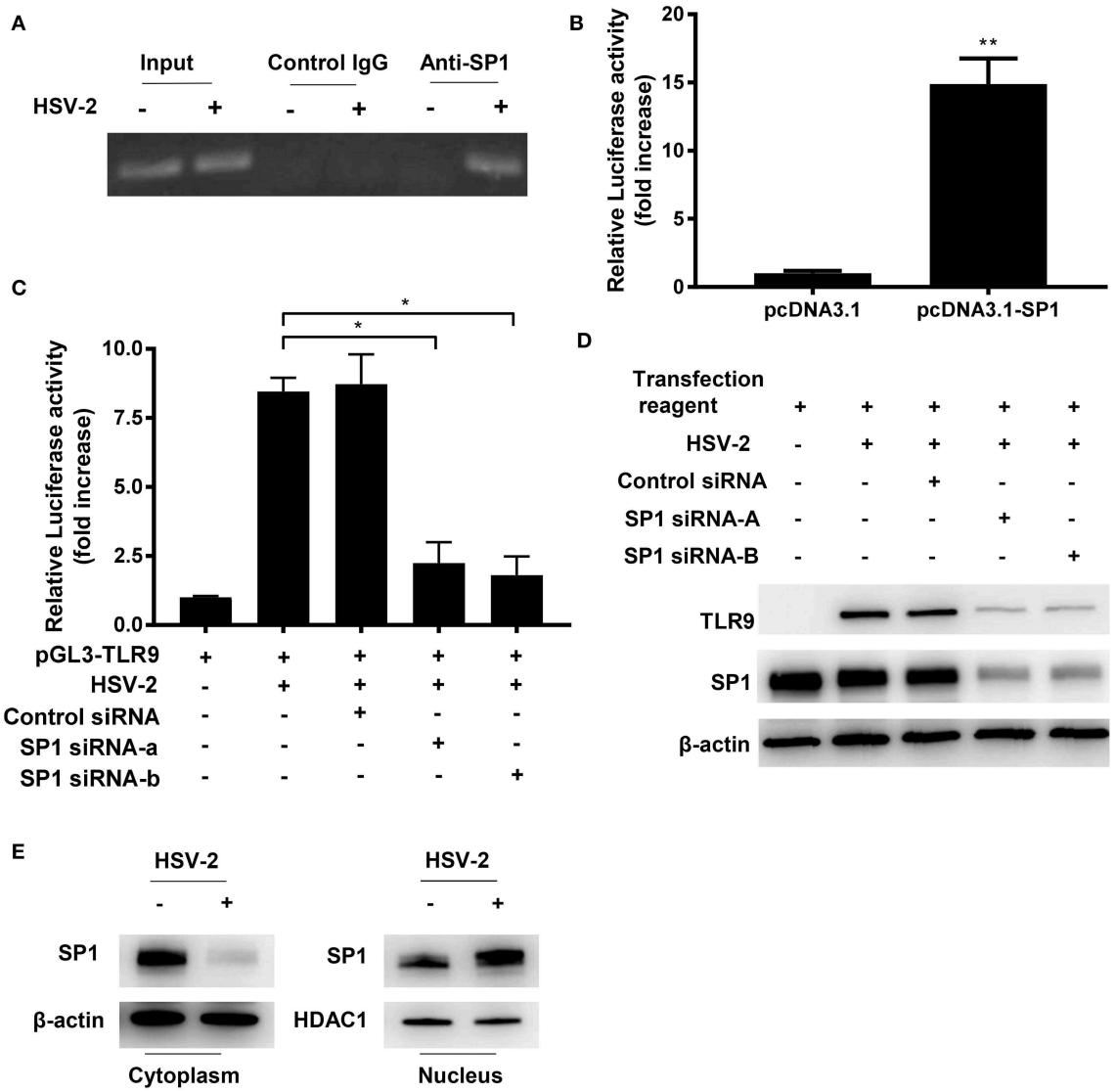

FIGURE 5 | HSV-2 infection promotes SP1 phosphorylation and nuclear translocation. **(A)** ME-180 cells were infected with or without 0.5 MOI HSV-2 for 24 h and chromatin immunoprecipitation assay was performed using anti-SP1 antibody and control IgG. The amplification of input samples was also shown. One representative experiment out of three is shown. **(B)** ME-180 cells co-transfected with pGL3-TLR9 and pcDNA3.1-SP1 or pcDNA3.1 for 24 h, and then relative luciferase activity was measured. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. **(C,D)** ME-180 cells were sequentially transfected with SP1 siRNAs or control siRNA **(C,D)** and pGL3-TLR9 **(C)**, and then infected with HSV-2. Twenty-four hours after infection, relative luciferase activity **(C)** and TLR9 and SP1 expression were determined by dual luciferase assay **(C)** and Western blot **(D)**, respectively. For luciferase assay and RT-PCR, data shown are mean ± SD of three independent experiments with each condition performed in duplicate. For Western blot results, one representative experiment out of three is shown. **(E)** ME-180 cells were infected with or without HSV-2 for 24 h. Cell cytoplasmic and nuclear fractions were isolated, and SP1 expression was determined by Western blot. One representative experiment out of three is shown. ns, statistically not significant; $^{*}p < 0.05$; $^{**}p < 0.01$.

The impact of HSV-2 infection on TLR9 activation in pDCs has been previously described, the findings of which are quite different from those observed in our current study using genital epithelial cells as models. In pDCs, both live and UV-inactivated HSV-2 induced IFN-α production by activating TLR9/MyD88 signaling pathway (13), whereas in genital epithelial cells, only live HSV-2 upregulated TLR9 expression and neither live nor UV-inactivated HSV-2 activated TLR9 signaling pathway. Such discrepancy may be attributed to differences in cell targets. DCs as immunocompetent antigen-presenting cells have high level of TLR9 expression (30), and upon HSV-2 infection, TLR9 can be activated by viral dsDNA before viral antagonism takes place. In contrast to that in immune cells, TLR9 expression in epithelial cells is less ubiquitous (30). We observed that, prior to HSV-2 infection, TLR9 expression was hardly detectable in genital epithelial cells at both mRNA and protein levels. The lack of TLR9 expression may explain why UV-inactivated HSV-2 or CpG

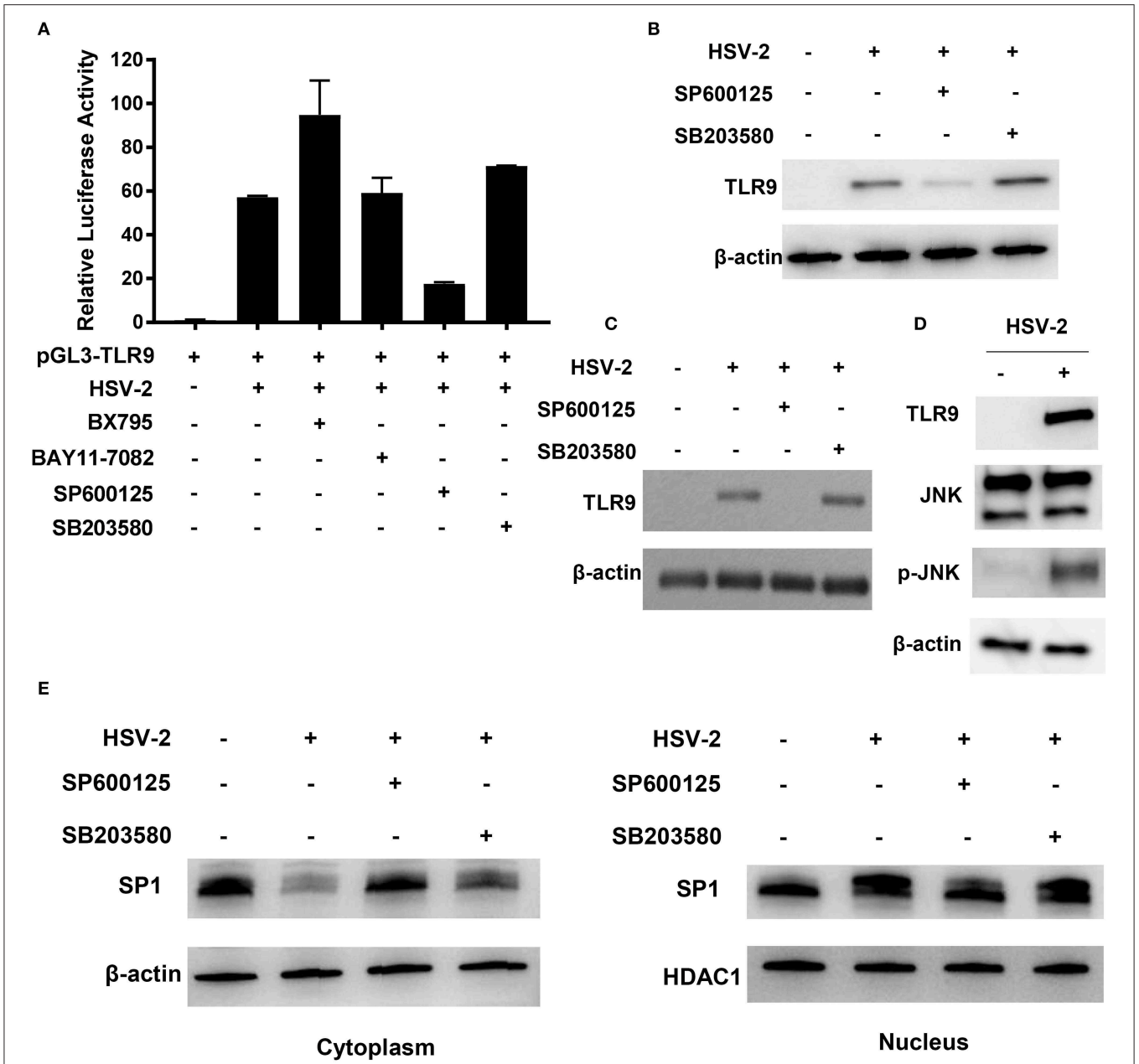

FIGURE 6 | JNK signaling pathway is involved in HSV-2-induced TLR9 expression. **(A)** ME-180 cells were transfected with pGL3-TLR9, infected with HSV-2 and treated with various signaling pathway inhibitors. Twenty-four hours after treatment, relative luciferase activity was measured. Data shown are mean ± SD of three independent experiments with each condition performed in duplicate. **(B–E)** ME-180 **(B,D,E)** and primary foreskin epithelial cells **(C)** were infected with HSV-2 and treated with various signaling pathway inhibitors. Twenty-four hours later, TLR9 **(B,C)** and JNK and p-JNK **(D)** expression were determined by Western blot. **(E)** Cytoplasmic and nuclear fractions were isolated and SP1 expression was determined by Western blot. One representative experiment out of three is shown.

ODN was unable to activate TLR9 signaling pathway in genital epithelial cells.

It is not yet clear whether HSV-2 infection upregulated TLR9 expression is important to viral replication or simply a byproduct during its replication. However, the upregulated TLR9 may shed light on the understanding of HSV-2 enhanced HIV-1 infection. It has been reported that TLR9 can transactivate HIV-1 LTR and initiate viral replication (31, 32). In addition, TLR9 polymorphism is related to HIV-1 progression (33, 34). In a separate study, we have also observed that replicative HSV-2 but not UV-treated HSV-2 activates HIV-1 LTR and this activation is sensitive to TLR9 siRNA treatment (unpublished data). Although

further investigations are needed, these findings imply that HSV-2 may promote HIV-1 replication in a TLR9-dependent manner.

In conclusion, our study has revealed that replicative, but not UV-inactivated HSV-2 induces TLR9 expression in genital epithelial cells, and that such induction is through promoting SP1 binding to TLR9 promoter via JNK signaling pathway.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee of Wuhan Institute of Virology, Chinese Academy of Sciences. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KH and QH conceived the study and wrote the manuscript. KH, MF, JW, SL, MB, RS, TC, ML, MZ, XG, and JX conducted experiments and analyzed the data. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00356/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
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Tuning the Orchestra: HCMV vs. Innate Immunity

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Understanding how the innate immune system keeps human cytomegalovirus (HCMV) in check has recently become a critical issue in light of the global clinical burden of HCMV infection in newborns and immunodeficient patients. Innate immunity constitutes the first line of host defense against HCMV as it involves a complex array of cooperating effectors – e.g., inflammatory cytokines, type I interferon (IFN-I), natural killer (NK) cells, professional antigen-presenting cells (APCs) and phagocytes – all capable of disrupting HCMV replication. These factors are known to trigger a highly efficient adaptive immune response, where cellular restriction factors (RFs) play a major gatekeeping role. Unlike other innate immunity components, RFs are constitutively expressed in many cell types, ready to act before pathogen exposure. Nonetheless, the existence of a positive regulatory feedback loop between RFs and IFNs is clear evidence of an intimate cooperation between intrinsic and innate immunity. In the course of virus-host coevolution, HCMV has, however, learned how to manipulate the functions of multiple cellular players of the host innate immune response to achieve latency and persistence. Thus, HCMV acts like an orchestra conductor able to piece together and rearrange parts of a musical score (i.e., innate immunity) to obtain the best live performance (i.e., viral fitness). It is therefore unquestionable that innovative therapeutic solutions able to prevent HCMV immune evasion in congenitally infected infants and immunocompromised individuals are urgently needed. Here, we provide an up-to-date review of the mechanisms regulating the interplay between HCMV and innate immunity, focusing on the various strategies of immune escape evolved by this virus to gain a fitness advantage.

Keywords: human cytomegalovirus, innate immunity, interferon system, apoptosis, restriction factors, NK cells, antigen presenting cell (APC)

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INTRODUCTION

The innate immune response is a fundamental defense mechanism, shielding the host from constant attacks of invading pathogens of different origin, whether they are bacterial, fungal, transposon or viral (Akira et al., 2006; Yan and Chen, 2012). Thus, for a virus, successful invasion and efficient subversion of the host immediate immune response are critical steps to achieve productive infection.

Some viruses, such as herpesviruses, have succeeded in establishing lifelong persistence in humans by evading immune surveillance (Stempel et al., 2019). For example, human cytomegalovirus (HCMV), a notorious opportunistic pantropic betaherpesvirus with a worldwide seroprevalence of 50 to > 90% in adults (Cannon et al., 2010), has the remarkable ability to manipulate and evade immune detection, literally transforming the host cellular environment into an ideal niche in which to thrive (Griffiths et al., 2015). This is achieved through sophisticated manipulations of cellular gene expression or elegant evasion strategies evolved by the virus during its long lasting co-evolution with the host (Wang et al., 2007; Loewendorf and Benedict, 2010; Rossini et al., 2012).

Even though HCMV infection is asymptomatic in immunocompetent individuals, it may lead to several life-threatening conditions in immunosuppressed subjects, such as organ and stem cell transplant recipients or AIDS patients. Furthermore, it can cause severe morbidity in congenitally infected children and elderly people (Cannon et al., 2010; Manicklal et al., 2013; Tu and Rao, 2016; Britt, 2018). Additionally, spontaneous reactivation of latent endogenous virus and/or superinfection with multiple viral strains can contribute to the overall burden and individual disease severity, as neither a vaccine nor an effective cure is currently available (Schleiss et al., 2017).

Although several viral polymerase inhibitors acting upon lytic replication (e.g., ganciclovir, cidofovir, and foscarnet) are widely used to treat HCMV infections, they are characterized by high hematopoietic toxicity and poor bioavailability, which prevents their use in pregnant women and congenitally infected newborns (Britt and Prichard, 2018). In addition, targeting latent HCMV remains an unsolved issue in patient clinical management. To make matters worse, the number of drug-resistant HCMV mutants has increased dramatically over the last decade (Piret and Boivin, 2019).

The outcome and severity of HCMV infection depends predominantly on initial virus-host interactions, occurring early upon infection, when intrinsic innate immunity comes into play to fight off the virus. As a frontline defense and earliest reaction measure, innate immunity avail itself of a complex array of effector cells and soluble factors, including pro-inflammatory cytokines and type I interferon (IFN-I), natural killer (NK) cells, professional antigen-presenting cells (APCs) and phagocytes, all operating in a fine-tuned and balanced manner (Luecke and Paludan, 2015; Patel et al., 2018).

Intrinsic cellular restriction factors (RFs) are constitutively expressed and play physiological roles in uninfected cells by cooperating with innate immune effectors, as some of them appear to be IFN-inducible, thus contributing to early host defense (Bieniasz, 2004; Duggal and Emerman, 2012).

Finally, triggered cell suicide processes (i.e., apoptosis and pyroptosis), resulting in death and removal of HCMV-infected cells, can also have a major impact on viral infection progression (Brune and Andoniou, 2017).

Ultimately, the orchestra formed by these innate immune components fine-tunes a highly efficient adaptive immune response that keeps HCMV infection at bay. However, HCMV often becomes the conductor of this orchestra, and as such it can manipulate to its liking all the various components of the immune response to make the cellular environment more permissible to viral replication and survival, thereby achieving persistence, latency and ultimately seroprevalence.

HCMV has an extremely large genome, and its enhanced encoding capacity allows for generating multiple viral proteins, involved in modulation and subversion of multiple signaling pathways (Stern-Ginossar et al., 2012; Brune and Andoniou, 2017). The exact mechanisms of action and role of this large number of viral proteins have not been yet completely elucidated, although many of them are probably involved in immune evasion.

In this regard, the fact that HCMV has developed a number of ingenious strategies directed against NK cells and APCs underscores the overall importance of these cells in innate immunity. For example, NK cells can release cytotoxic granules triggered by natural or antibody-dependent cytotoxicity (ADCC) or produce cytokines upon engagement of activating and inhibitory NK cell receptors. Even though NK cells are the major cytotoxic arm of innate immunity, their contribution in shaping T cell-mediated immune responses and generating memory cells is now well established (Netea et al., 2016; Nikzad et al., 2019). Since NK cells are efficient eliminators of HCMV-infected cells, it is not surprising that HCMV has devised multiple strategies to evade recognition by these cells (Babić et al., 2011; Goodier et al., 2018; Zingoni et al., 2018). Likewise, APCs from the myeloid and epithelial compartments [i.e., monocytes, macrophages, and dendritic cells (DCs)], are well-known targets of HCMV, serving as vehicles upon infection to facilitate viral dissemination (Jackson and Sparer, 2018). In particular, HCMV is able to interfere with MHC class I (MHC-I) and II (MHC-II) antigen presentation, thereby subverting the immunological functions of APCs.

This review provides an in-depth description of the complex interplay between the host innate immune responses and HCMV, highlighting multiple viral feedback mechanisms that modulate and counteract the various arms of innate immunity.

THE IFN SYSTEM AND HCMV: A STORMY RELATIONSHIP

Upon HCMV sensing, intracellular pattern recognition receptors (PRRs) trigger downstream signaling events leading to the production of type I IFN and release of inflammatory cytokines. Type I IFNs (IFN-I) are a group of cytokines comprising IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, IFN-ζ, and IFN-τ (Mesev et al., 2019).

IFN-I signaling pathways have long been considered key limiting factors of HCMV infection and replication. Despite their complexity, these defense mechanisms occur early after pathogen entry into the host and, in most cases, they can eradicate the pathogen before it can overwhelm the host immune defenses (Goodwin et al., 2018).

Cellular sensors capable of detecting HCMV include toll-like receptor 2 (TLR2) and CD14 receptors, both able to interact

with HCMV envelope glycoproteins (Compton et al., 2003), most of DNA sensors and the newly described group of PRRs, able to stimulate transcription of IFN-I *via* the key adaptor protein stimulator of interferon genes (STING). In particular, the DNA sensor cyclic guanosine monophosphate (GMP)–adenosine monophosphate (AMP) synthase (cGAS)/STING axis is crucial for activating the IFN-I signaling (Diner et al., 2016; Paijo et al., 2016; Jønsson et al., 2017; Biolatti et al., 2018b). On the other hand, HCMV has evolved a wide range of proteins with which to manipulate and counteract the host IFN response (Biolatti et al., 2018c; Goodwin et al., 2018; Marques et al., 2018; Stempel et al., 2019). This complex and intertwined relationship between HCMV and IFN has been addressed by a number of studies discussed below and schematically represented in **Figure 1**.

The HCMV tegument protein pp65 –also identified as pUL83, encoded by *UL83* – best exemplifies the multifaceted interplay between viral and host proteins (Biolatti et al., 2018a). Specifically, pp65 has been shown to modulate nuclear factor-κB (NF-κB) and interferon regulatory factors 3 (IRF3) activities, which cooperate to induce transcription of several cytokines such as IFN-β, which then counteracts HCMV infection (Iwanaszko and Kimmel, 2015).

The recent finding that IκB kinases, the main regulators of NF-κB pathway, exerts antiviral activity (Goodwin and Munger, 2019) adds a level of complexity to this scenario. In this regard, pp65 is able to inhibit NF-κB but not IRF3 nuclear translocation (Browne and Shenk, 2003). This is in disagreement with findings by Abate et al. (2004) showing that pp65 reduces IRF3 phosphorylation preventing its nuclear translocation.

Recent results obtained by our group have demonstrated that the pyrin association domain (PAD) of pp65 binds cGAS, thereby inhibiting its enzymatic activity upon HCMV infection. This phenomenon leads to impairment of the cGAS/STING axis and downregulation of IFN-β production (Biolatti et al., 2018b). In good agreement with these findings, the HCMV tegument protein pUL31 (encoded by *UL31*), similar to pp65, can interact with nuclear and cytoplasmic cGAS in HCMV-infected HFFs and HEK293T cells. Results from Huang et al. (2018) have shown how pUL31 can interact directly with cGAS in HEK293T cells, which is followed by disassociation of DNA from cGAS leading to decreased cGAMP production and consequent downregulation of IFN-I gene expression.

The HCMV tegument protein pp71 (i.e., pUL82, encoded by *UL82*) also contributes to evade the IFN response. According to Fu et al. (2017), pp71 interacts with the inactive rhomboid protein 2 (iRhom2) and STING to disrupt STING trafficking. Particularly, pp71 prevents STING translocation from the ER to the perinuclear microsomes, an essential step of STING-mediated signaling.

The HCMV glycoprotein US9, encoded by *US9*, inhibits IFN-I by targeting mitochondrial antiviral-signaling protein (MAVS) and STING pathways (Choi et al., 2018). In this regard, Choi et al. (2018) have proposed that US9 inhibits IRF3 nuclear accumulation by preventing STING dimerization. Moreover, the overexpression of US9 disrupts the mitochondrial membrane integrity and its membrane potential.

Moreover, the HCMV immediate early (IE) 86 kDa protein (IE86), negatively affects IFN-β mRNA transcription by preventing NF-κB binding to the IFN-β promoter (Taylor and Bresnahan, 2006). Intriguingly, a recent study by Kim et al. (2017) has shown that IE86 downregulates STING protein, suggesting that IE86 may also target STING for proteasomal degradation. Interestingly, STING levels were restored upon treatment with the peptide aldehyde MG132, which prevents the proteolytic activity of the proteasome complex. However, no interaction between STING and IE86 during HCMV infection could be detected.

Finally, HCMV tegument proteins have also been proposed to affect the modulation of type II IFN (also known as IFN-γ) signaling, which is an aspect not well studied. In this regard, Feng et al. (2018) have reported that the human N-myc interactor (Nmi) protein, which is important for the activation of IFN-γ, specifically interacts with the viral tegument protein UL23, encoded by *UL23*, leading to a decrease in IFN-γ expression, thus facilitating viral immune evasion.

To summarize, HCMV has evolved sophisticated mechanisms to modulate the host IFN response, especially that through IFN-I. This new evidence contributes to our understanding of the molecular mechanisms employed by HCMV to evade the innate immune response (**Table 1**).

RESTRICTION FACTORS VS. HCMV: A NEVER ENDING FIGHT

During the last few years, RFs have emerged as main players of the host antiviral response against HCMV (Paludan et al., 2011). RFs are intrinsic antiviral factors, which are sometimes regarded as integral part of the innate immune response or some other times an autonomous third branch of the immune system (Yan and Chen, 2012). Unlike other classical components of innate immunity, they are constitutively expressed within the host cells and are generally IFN inducible, thus allowing an immediate response against viral infection through specific targeting of viral/cellular components (Bieniasz, 2003; Hotter and Kirchhoff, 2018). Interestingly, during HCMV infection a subset of classical IFN-stimulated genes (ISGs) may be also induced or upregulated independently of IFN (Ashley et al., 2019).

Similar to what observed for the IFN system, HCMV has devised clever strategies to sidestep the antiviral activity of RFs, among which IFN-γ-inducible protein 16 (IFI16), nuclear domain 10 (ND10) and virus inhibitory protein ER-associated IFN-inducible (viperin) are among the best characterized (Biolatti et al., 2018c). This list has been in the last few years expanded to include apolipoprotein B editing catalytic subunit-like 3 (APOBEC3), survival time-associated PHD protein in ovarian cancer 1 (SPOC1), Galectin-9 (Gal-9) and human myxovirus resistance 2 (MX2) gene product MxB (**Figure 2**).

Unexpectedly, BST2/tetherin, considered to be the pioneer among RFs due to its long established antiviral activity against human immunodeficiency virus (HIV), does not display restriction activity against HCMV, but it rather enhances the susceptibility of hematopoietic cells to HCMV infection, thereby

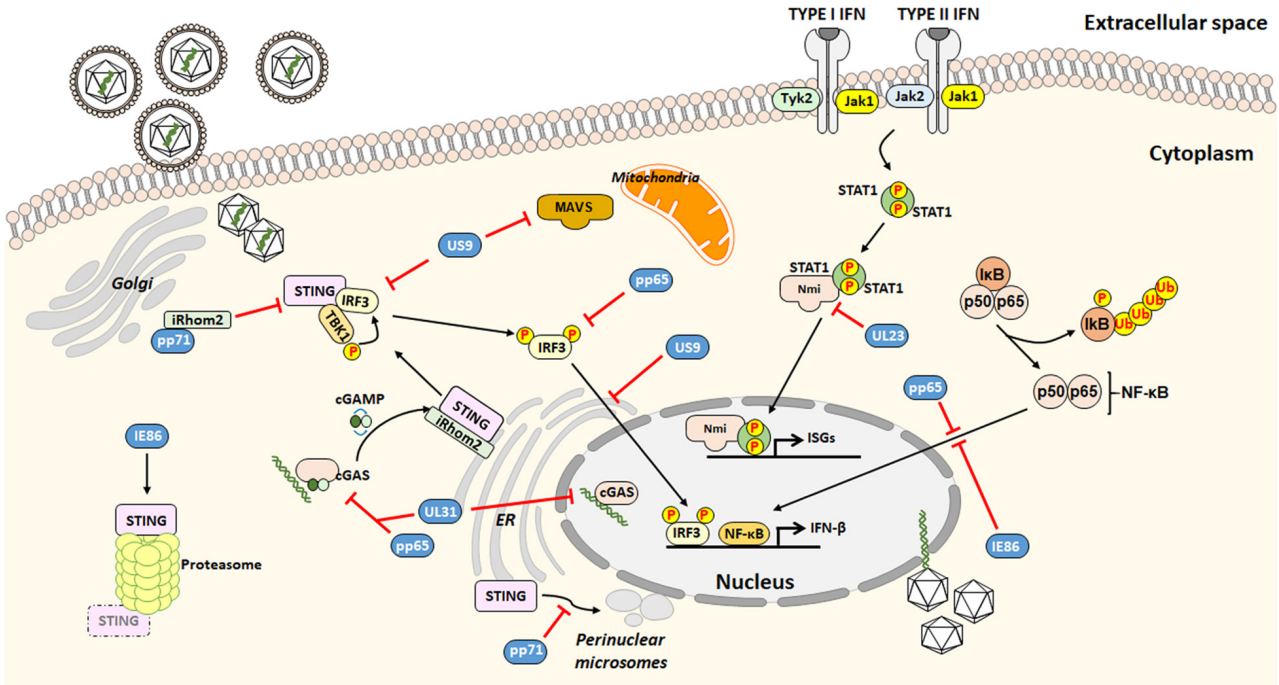

FIGURE 1 | Outline of the HCMV strategies to evade from the interferon (IFN)-associated antiviral activity.

TABLE 1 | Summary of studies describing HCMV evasion strategies from IFN antiviral activity.

Viral protein (viral gene)	Host target	Suggested mechanism	Type of IFN	References
pp65 (UL83)	NF-κB	Reduced nuclear relocalization	IFN-β	Browne and Shenk, 2003
	IRF3	Reduced phosphorylation and relocalization	IFN-β	Abate et al., 2004
	cGAS	Reduced enzymatic activity	IFN-β	Biolatti et al., 2018a
pUL31 (UL31)	cGAS	Dissociation of cGAS from DNA	IFN-β	Huang et al., 2018
pp71 (UL82)	iRhom	Distruption of translocation complex	IFN-β	Fu et al., 2017
	STING	Distruption of translocation complex	IFN-β	
US9 (US9)	MAVS	Attenuation of MAVS signaling	IFN-β	Choi et al., 2018
	STING/TBK1	Prevention of STING oligomerization	IFN-β	
	IRF3	Dysfunctional nuclear relocalization	IFN-β	
IE86 (UL122)	NF-κB	Preventing interaction with IFN-β promoter	IFN-β	Kim et al., 2017
	STING	Proteasome degradation	IFN-β	Taylor and Bresnahan, 2006
UL23 (UL23)	Nmi	Disruption of Nmi/STAT1 interaction	IFN-γ	Feng et al., 2018

favoring viral hematogenous spread (Viswanathan et al., 2011). Similarly, IFN-inducible transmembrane proteins (IFITMs) 1, 2, and 3, capable of blocking the entry of a broad variety of RNA viruses, fail to inhibit the entry of DNA viruses, such as HCMV, HPV16 and human adenovirus type 5, pointing to an evolutionarily preserved mechanism shared by some DNA viruses to circumvent the antiviral function of IFITMs (Warren et al., 2014). This is however a controversial point, as a more recent study has shown that HCMV, instead of taking part in the entry process, exploits IFITMs at later time points of its viral cycle to facilitate the formation of the virion assembly compartment (vAC), which enhances virion assembly (Xie et al., 2015). Finally, a very recent work elegantly described the ability of HCMV to actively stimulate the cellular RNA-binding protein Roquin in inhibiting the innate immune response through the suppression of IRF1 antiviral activity (Song et al., 2019).

IFI16

In the past decade, our group and others have extensively investigated the antiviral activity of IFI16 against HCMV. In particular, we have shown that IFI16 inhibits HCMV replication at early-late phases through blockade of Sp1 binding to the HCMV DNA polymerase promoter (UL54) (Gariano et al., 2012). At late stages of infection, we also found that HCMV is able to promote IFI16 nuclear delocalization through UL97-mediated IFI16 phosphorylation. Phospho-IFI16 is then redirected from the nucleus to the vAC where it is incorporated into newly formed viral particles (Dell'Oste et al., 2014).

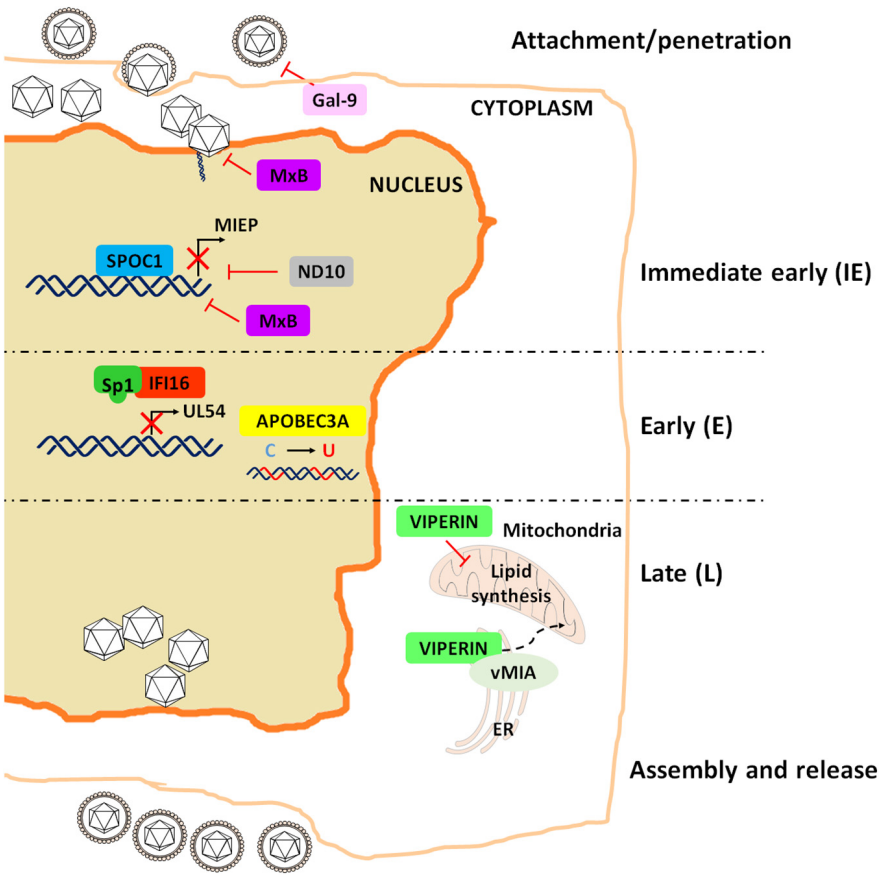

FIGURE 2 | Schematic representation of the restriction activities played by the major RFs to impair HCMV replication.

This unexpected behavior raised the important question of why HCMV chooses to incorporate an RF (i.e., IFI16) into its virions. A partial answer to this riddle came from experiments on pp65 showing that at early stages of HCMV infection this tegument protein can interact with IFI16 at the major immediate-early promoter/enhancer (MIEP), promoting viral gene transcription. Thus, entrapping cytoplasmic IFI16 into virions might after all confer a fitness advantage to the virus (Cristea et al., 2010). However, more recent findings have shown that pp65 can also protect IFI16 from degradation, thereby favoring the inhibitory effect of this latter on the promoter region of *UL54* (Biolatti et al., 2016). Interestingly, it has been recently demonstrated that IFI16 is rapidly targeted during the establishment of viral latency in a US28-dependent manner, but only in undifferentiated myeloid cells, a natural site of latent carriage (Elder et al., 2019). These authors have indeed proposed that the consequent downregulation of IFI16 is beneficial to the establishment of latency, since IFI16 overexpression drives MIEP activity and IE gene expression via NF-κB.

In addition to its antiviral activity, IFI16 is also able to induce IFN-β expression through cGAS interaction (Diner et al., 2016). cGAS activity plays a major role in the STING/tank-binding kinase (TBK-1)/IRF3 pathway, activated by herpes simplex virus type 1 (HSV-1) and HCMV infection (Diner et al., 2016; Biolatti et al., 2018c). Therefore, it does not come as a surprise that also in this case HCMV has been able to develop a strategy to counteract cGAS activity. Indeed, HCMV UL31 has been recently identified as a cGAS inhibitor, acting through direct protein-protein interaction followed by DNA dissociation from cGAS and reduced cGAMP production (Huang et al., 2018).

ND10 Complex

One of the best characterized HCMV RFs is certainly the ND10 complex, formed by the proteins PML, hDaxx, and Sp100 (Zhang and van Drunen Littel-van den Hurk, 2017). In addition to these components, other molecules, such as the nuclear matrix protein microrchidia family CW-type zinc-finger 3 (MORC3/NXP-2), have been shown to associate with the ND10 complex and exert antiviral activity through an unknown mechanism (Sloan et al., 2016).

During HCMV infection, the viral genome is accumulated at the periphery or within the central core of ND10 bodies, and all the ND10 components are recruited at the site of viral replication to exert their antiviral activity (Tavalai et al., 2008; Adler et al., 2011; Cosme et al., 2011; Glass and Everett, 2013). This is achieved by forming a transcriptionally inactive chromatin complex binding the MIEP, which then silences IE gene expression (Preston and Nicholl, 2006; Woodhall et al., 2006;

Lukashchuk et al., 2008; Shin et al., 2012). Moreover, PML is an E3 ligase mediating IE1 SUMOylation, thereby blocking the antagonistic effect of IE1 on STAT-mediated IFN response (Reuter et al., 2017).

Although PML, hDaxx, and Sp100 act as RFs during HCMV lytic replication, they do not seem to affect HCMV latency, as demonstrated by silencing experiments in non-differentiated THP-1 monocytes (Wagenknecht et al., 2015). Meanwhile, other have shown that hDaxx can act as an RF in several latency cellular models, such as NT2 and THP-1 cells, myeloblastic cell lines and primary human $CD34^+$ cells (Saffert and Kalejta, 2006).

Also in this instance, HCMV has developed fine-tuned strategies to subvert the gatekeeping functions of ND10. Perhaps the most surprising solution adopted by HCMV relies on IE1, probably because this viral protein is also the main target of the ND10 complex. Specifically, IE1 can block ND10 SUMOylation (Xu et al., 2001; Lee et al., 2004; Schilling et al., 2017), thereby preventing ND10 oligomerization and activation (Korioth et al., 1996; Ahn and Hayward, 1997; Wilkinson et al., 1998). Moreover, the viral latency-associated gene product (LUNA), encoding a deSUMOylase activity, promotes the disruption of cellular ND10 bodies during latency (Poole et al., 2018).

Other strings to the bow of HCMV are its tegument proteins. Indeed, HCMV pp71 prevents hDaxx-mediated repression of MIEP by binding this protein and stimulating its proteasome degradation, leading to disruption of the ND10-MIEP complex (Hofmann et al., 2002; Cantrell and Bresnahan, 2005). In addition, two other tegument proteins, UL35 and UL35a, have been found to cooperate in regulating pp71activity. In particular, UL35 interacts with pp71, and this interaction has two different effects: at early steps of viral replication, this complex activates IE gene transcription (Schierling et al., 2004), whereas at later stages UL35 independently remodels ND10 and co-localizes with the remodeled structures, thus facilitating pp71-mediated hDaxx disruption. Intriguingly, this activity appears to be negatively regulated by UL35a, which prevents UL35 from shaping ND10 and delivers pp71 to the cytoplasm (Salsman et al., 2011).

Viperin

Another early identified HCMV RF is the IFN-inducible iron-sulfur (4Fe-4S) cluster-binding protein viperin, whose main antiviral activity is exerted during late phases of HCMV life cycle (Chin and Cresswell, 2001). A curious aspect of this interplay is that HCMV is not just able to inhibit viperin RF activity but it has learned how to take advantage of it in different ways. Firstly, HCMV promotes viperin translocation from the ER to the mitochondria by encoding the viral mitochondria-localized inhibitor of apoptosis (vMIA) protein. Once in the mitochondria, viperin can inhibit viral replication by modulating the host metabolism through three distinct mechanisms: (1) inhibition of fatty acid β-oxidation; (2) downregulation of ATP levels; and (3) rearrangement of the actin cytoskeleton (Seo et al., 2011). To this end, viperin transcriptionally activates several mediators of fatty acid metabolism, such as AMP-activated protein kinase (AMPK) and GLUT4 (Seo and Cresswell, 2013). This processes leads to enhanced lipid production in HCMV-infected cells, which in turn favors viral envelope formation and virion release.

APOBEC3

Together with tetherin, cytidine deaminases belonging to the APOBEC3 family are considered fundamental antiviral proteins, known for their antiviral activity against HIV-1 (Blanco-Melo et al., 2012). Over the years, their antiviral activity has also been shown to affect DNA viruses, including HCMV (Harris and Dudley, 2015). Specifically, the APOBEC3 family member APOBEC3A (A3A) is upregulated in the maternal decidua upon HCMV infection or IFN-β administration and displays a strong inhibitory effect against HCMV replication (Weisblum et al., 2017). Furthermore, A3A cytidine deamination activity is responsible for hypermutations in the viral genome of HCMV-infected epithelial cells, thereby impairing HCMV replication through a poorly defined mechanism, presumably involving IFN-β (Weisblum et al., 2017).

The observation that A3A is not the only APOBEC3 isoform induced by HCMV comes from one of our recent studies showing that A3G is also strongly upregulated in HCMV-infected HFFs, an induction apparently mediated by IFN-β (Pautasso et al., 2018). However, the fact that the HCMV genome almost totally lacks A3G motifs (i.e., CCC) rules out the possibility that this protein is a *bona fide* HCMV RF, raising the hypothesis that host-virus coevolution might have shaped the nucleotide composition of HCMV DNA to generate viruses able to dodge A3G-mediated immune surveillance.

SPOC1

SPOC1, also known as PHF13 (PHD finger 13), was characterized for the first time in patients with epithelial ovarian cancer (Mohrmann et al., 2005). Many cellular functions of this protein can be attributed to its ability to bind and modulate chromatin by cooperating with several heterochromatin proteins. By doing so, SPOC1 differentially regulates subsets of genes mainly involved in DNA binding and chromatin organization, cell cycle and differentiation (Kinkley et al., 2009; Bördlein et al., 2011; Chung et al., 2016). SPOC1 is also a DNA repair factor as it accumulates at DNA double-strand breaks and regulates the DNA damage response (Mund et al., 2012). A restriction activity of SPOC1 has been observed against human adenovirus type 5 (HAdV5) (Schreiner et al., 2013) and HIV-1 (Hofmann et al., 2017). In these specific contexts, SPOC1 inhibits viral replication, but it is also degraded by viral proteins as a negative feedback mechanism. Furthermore, SPOC1 inhibits early steps of HCMV replication by specifically binding MIEP and driving the recruitment of heterochromatin-building factors, in line with its chromatin remodeling activity. Intriguingly, HCMV but not HIV-1 and AdV5 infection promotes and early transient upregulation of SPOC1 through an IE1-mediated mechanisms independent of protein stabilization. At later steps of infection, SPOC1 levels start to decline upon phosphorylation by the serine-threonine kinase glycogen synthase kinase 3β (GSK-3β) (Hofmann et al., 2017). However, contrary to HIV-1 infection, where Vpr has already been identified as the viral protein involved in SPOC1 degradation (Reichel et al., 2018), the mechanism of HCMV-mediated downregulation of SPOC1 still remains obscure.

Gal-9

Among the most recently identified HCMV-RFs, Gal-9 is of particular interest. It belongs to the widely expressed protein family of galectins, playing an important role in both innate and adaptive immunity (Rabinovich et al., 2007; Rabinovich and Toscano, 2009). The immunomodulatory role of Gal-9 is due to the presence of glycan structures on the surface of both host cells and microorganisms, thus enabling galectins to orchestrate antiviral immunity as well as host-virus interactions. For example, Gal-1 and Gal-9 have shown antiviral activity against Epstein-Barr virus (EBV), murine CMV infection (MCMV), Nipah virus (NIV), enterovirus, HIV-1, influenza virus, and dengue virus in a number of *in vivo* and *in vitro* models of infection (reviewed in Merani et al., 2015).

Even though galectins can either enhance or inhibit viral infection, a restriction activity of Gal-9 during HCMV infection has been recently observed (Machala et al., 2019). In experiments where Gal-9 was added at different time points after HCMV infection it functioned as an antiviral lectin binding the virions and blocking entry of HCMV into the host cell without influencing post-entry events (Machala et al., 2019). On the other hand, the same authors observed increased concentrations of soluble Gal-9 in the plasma of hematopoietic stem cell transplantation (HSCT) recipients during HCMV reactivation, raising the possibility that Gal-9 may also exert a restriction activity *in vivo* (Machala et al., 2019).

MxB

The Mx GTPases MxA and MxB are best known as RFs of several RNA viruses, including influenza A virus, vesicular stomatitis virus (VSV), measles virus (MeV) (Haller and Kochs, 2011), and HIV-1 (reviewed in Staeheli and Haller, 2018). The antiviral activity of Mx against herpesviruses is somewhat more controversial. Indeed, while it has recently been demonstrated a pan-herpesvirus restriction activity for MxB against IE viral gene expression, the precise mechanisms it relies on has not yet been fully clarified (Schilling et al., 2018). The most consistent hypothesis is that of a direct action of MxB during the uncoating process aimed at targeting viral capsids or components of the nuclear pore complexes, similarly to what happens during HSV-1 infection (Crameri et al., 2018).

ANTIGEN PRESENTING CELLS: A TWO-EDGED SWORD

APCs are often defined as sentinels of the body, essential for initiating the immune response against pathogens. They, however, play an enigmatic role during HCMV infection. On the one hand, many APCs, including monocytes, macrophages and DCs, are critical to trigger specific T-cell responses. On the other hand, they are permissive to HCMV infection, serving as vehicles for viral spread during the first steps of infection, and then becoming cozy and protective niches for virus replication and persistence at later stages. Conversely, components of the lymphoid lineage, such as NK cells and plasmacytoid DCs (pDCs) are not just resistant to HCMV infection but they are also activated early upon infection by viral components, triggering an antiviral response. Despite the presence of these defense mechanisms, HCMV has put in place multiple strategies to evade APC-mediated immune control so as to establish latency and persistence within the host (Sinclair and Reeves, 2014).

Dendritic Cells (DCs)

DCs are specialized APCs mediating immune response induction and maintenance. The major subsets in humans include classical DCs (cDCs), which comprise Langerhans cells (LCs) and pDCs, the main producers of IFN-I, and monocyte-related DCs (mDCs) (Collin et al., 2013). The role of DCs during HCMV infection remains somewhat controversial because, despite being critical components for the establishment of an antiviral NK and T-cell response, they are also targeted by HCMV for immune escape.

HCMV interacts with DCs in a pleiotropic manner. It is in fact well established that HCMV strains with an intact UL128-131A locus can infect DCs *in vitro* (Jahn et al., 1999; Riegler et al., 2000). In addition, circulating mDCs isolated from healthy seropositive donors can also support HCMV infection (Reeves and Sinclair, 2013), a process probably favored by the expression of the viral chemokine receptor-like protein US28, which drives DC recirculation (Farrell et al., 2018). In contrast, by using co-culture approaches, it has been shown that mDCs or monocyte-derived macrophages can restrict HCMV with interferon-unrelated mechanisms (Kasmapour et al., 2017; Becker et al., 2018).

For pDCs, the scenario is even more complex. Different subpopulations of pDCs obtained either from tonsils (tpDCs) or blood (bpDCs) react to HCMV-infection in opposite ways (Schneider et al., 2008). For instance, tpDCs are fully permissive for HCMV replication despite the fact that their IFN-α production and expression of costimulatory and adhesion molecules are ultimately affected by HCMV. In contrast, bpDCs appear to be resistant to HCMV infection (Schneider et al., 2008).

HCMV can latently infect DC precursors and then undergo reactivation by taking advantage of chromatin remodeling during differentiation of DC progenitors into mature DCs (Reeves et al., 2005). Conversely, in undifferentiated myeloid precursors, viral lytic genes are inhibited as a consequence of histone modifications of the MIEP, leading to a repressive chromatin structure eventually preventing IE transcriptional activity (Sinclair, 2010). Furthermore, proinflammatory factors, such as IL-6 and the ERK/MAPK pathway have been linked to the reactivation of latent HCMV in DCs and other permissive cells (Reeves and Compton, 2011).

The interplay between HCMV and DCs interaction can have different outcomes in terms of immune response. For instance, HCMV infection of mDCs *in vitro* triggers IFN and IL-12 release in a cGAS-dependent manner (Renneson et al., 2009; Paijo et al., 2016). Subsequently, other immune mediators are recruited to the infection site to amplify the immune reaction. HCMV infection in mDCs can also modulate TLR3 signaling, but this effect is more evident at later times post-infection (Mezger et al., 2009).

Given the central role of DCs in virus clearance, it is not surprising that HCMV has put in place multiple strategies

to inhibit such process. For instance, HCMV can interfere with MHC-I and -II antigen processing and presentation to avoid detection by $CD8^+$ and $CD4^+$ T cells. This process appears to be mediated by the HCMV-encoded protein US2, capable of degrading both MHC-I and MHC-II proteins through the proteasome (Loureiro and Ploegh, 2006). Likewise, other HCMV proteins such as pp65, pp71, and US2-11 have been implicated in HCMV evasion from T-cell recognition by triggering accumulation and degradation of HLA-DR α-chain in perinuclear vacuoles (Odeberg et al., 2003).

Among HCMV genes hindering APC function, a crucial role is played by the viral interleukin-10 homolog (cmvIL-10), expressed during lytic infection and capable of binding the IL-10 receptor of host cells. Specifically, cmvIL-10 upregulates the HCMV putative receptor DC-SIGN, thus enhancing viral infectivity (Raftery et al., 2004), as well as the expression of hIL-10 by primary blood-derived monocytes, thus modulating existing cellular pathways and the viral immunomodulatory impact during infection (Avdic et al., 2016). In addition, it inhibits a number of DCs functions, including TLR-induced IFN-α/β production in nearby pDCs and CD1-mediated antigen presentation (Raftery et al., 2008; Avdic et al., 2014). This effect is also shared by other viruses, which either upregulate hIL-10 (e.g., HIV and hepatitis C virus) (Reiser et al., 1997; Brockman et al., 2009) or express homologs of this cytokine (e.g., EBV and some cytomegaloviruses) (Slobedman et al., 2009), highlighting the importance of IL-10 signaling in viral escape mechanisms.

An important step of the immune response is the ability of DCs to drift from the infection site to the lymph nodes, a process driven by the chemokines CCL19 and CCL21. Consequently, HCMV has developed strategies to impede DC trafficking in response to lymphoid stimuli and induction of T-cell proliferation (Beck et al., 2003; Moutaftsi et al., 2004). For example, it can prevent CCR5 chemokine receptor from switching to CCR7 in infected mDCs, thus inhibiting CCL19- and CCL21-induced migration of mature mDCs (Moutaftsi et al., 2004). Conversely, in immature mDCs, HCMV does not modulate CCR7, but it affects chemotaxis by internalizing CCR1 and CCR5 (Varani et al., 2005). In this context, UL18, the viral homolog of MHC-I, appears to play a controversial role. Indeed, UL18 has been reported to inhibit CD40L-mediated T-cell proliferation through DC maturation impairment (Wagner et al., 2008), meanwhile stimulating the expression of CD83 on mature mDCs. Moreover, at later times, HCMV downregulates surface but not intracellular CD83 (Wagner et al., 2008). Others have reported that soluble CD83, in turn, inhibits T-cell proliferation (Sénéchal et al., 2004), and that UL18 is also able to reduce RANTES-driven chemotaxis of mDCs (Wagner et al., 2008; **Figure 3**).

Depending on their stage of maturation, $CD34^+$ progenitor cell-derived LCs can be susceptible to HCMV infection. Indeed, immature LCs are poorly supportive of viral replication, whereas LC-derived mature DCs are highly responsive to infection due to HCMV-mediated subversion of the T-cell response through downregulation of several activation markers, such as MHC-I and -II, CD1a, CD80, CD83, CD86, and CD54 (Hertel et al., 2003). This also leads to a substantial loss of dendrites and to impaired dendritic cell migration in response to lymphoid chemokines (Lee et al., 2004; **Figure 3**).

Monocytes and Macrophages

Additional reservoirs for HCMV are represented by monocytes and macrophages. In particular, monocytes have been long involved in HCMV dissemination across the human body and are generally regarded as the main source of latent HCMV in the peripheral blood of seropositive people (Smith et al., 2004). Even though they do not support productive HCMV replication (Sinzger et al., 2008), once fully differentiated into macrophages, they become permissive for viral replication. During this process, a major role for virus reactivation and growth seems to be played by IFN-γ and tumor necrosis factor (TNF)-α, produced by allostimulated T cells (Söderberg-Nauclér et al., 1997). Moreover, monocytes are known to release infectious HCMV directed toward uninfected cells *in vitro* through a not fully defined mechanism (Waldman et al., 1995).

Like DCs, monocyte-derived macrophages play a crucial role in counteracting HCMV spread *in vitro*. In this context, the role of IFN is controversial. Indeed, IFN-I plays an inhibitory role on HCMV replication when macrophages are stimulated by cell-free HCMV. In contrast, upon co-culture of infected cells and macrophages, the antiviral effect appeared to be independent of IFN-γ, TNF-α, and IFN-I (Becker et al., 2018).

Overall, it seems that HCMV has learned how to escape from monocyte antiviral activity and use these cells as "Trojan horses" to achieve viral spread. For instance, infected monocytes display impaired migration and reduced ability to recruit leukocytes and inflammatory mediators, allowing additional "contact time" to transfer HCMV from infected monocytes to uninfected cells (Frascaroli et al., 2006). Furthermore, the observation that purified pUL128 – i.e., a CC chemokine homolog, part of the HCMV pentamer complex (PC) – triggers monocyte migration *in vitro* through a poorly characterized mechanism suggests that HCMV might be able to attract monocytes to the infection site and favor viral dissemination by secreting specific chemokines (Zheng et al., 2012). In addition, pUS2-US11-mediated MHC downregulation in DCs is only partially functional in macrophages, which therefore retain their ability to activate $CD4^+$ and $CD8^+$ T cells (Frascaroli et al., 2018). Lastly, HCMV inhibits the differentiation of both macrophages and DCs from monocytic precursors after stimulation with IL-4 and GM-CSF, impairing immunological functions (Gredmark and Söderberg-Nauclér, 2003). In this context, the main inhibitors of macrophage differentiation are the cell-surface aminopeptidase N/CD13 and HCMV glycoprotein B (gB) (Gredmark et al., 2004; **Figure 3**).

As for DCs, cmvIL-10 can also impair cytokine production of these cells through inhibition of phosphatidylinositol 3-kinase/Akt signaling (Spencer, 2007), with concurrent downmodulation of integrin-like receptor surface expression [i.e., CD11b/CD18 (CR3) and CD11c/CD18 (CR4)], a process that strongly impairs DC phagocytic activity (Gafa et al., 2005). Finally, downregulation of CCR1 and CCR5 is associated with slower cell migration, reorganization of the cytoskeleton and secretion of soluble inhibitors (Frascaroli et al., 2009; **Figure 3**).

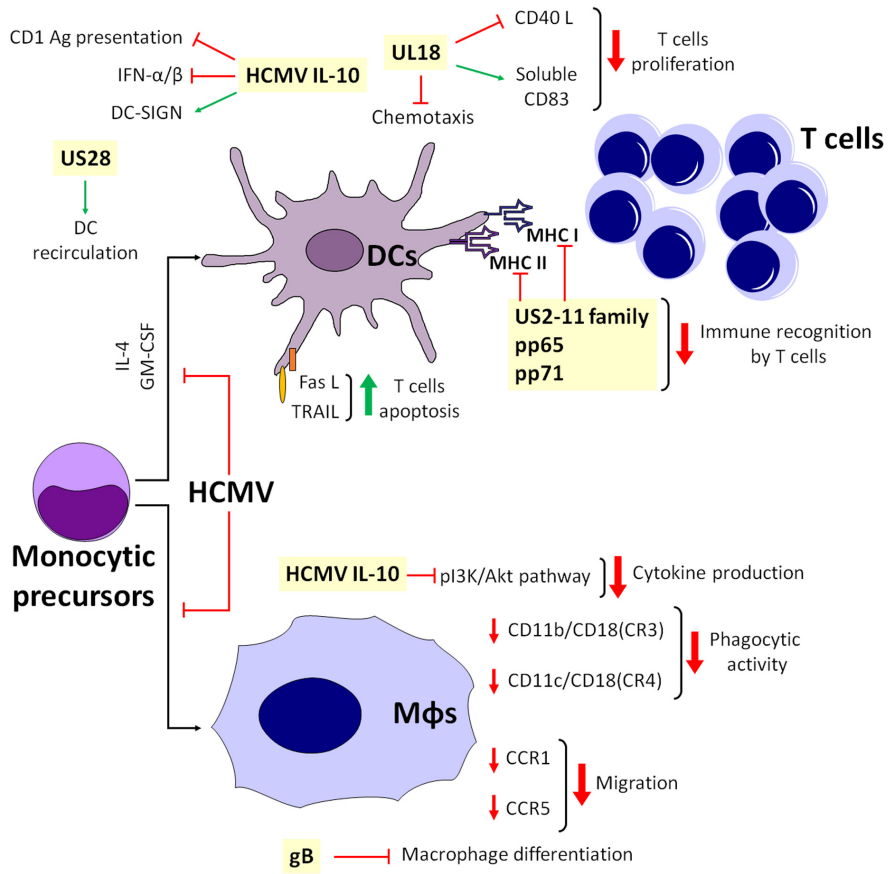

FIGURE 3 | Simplified model depicting the interplay among APCs during HCMV infection.

NK CELLS AND HCMV: A BALANCE OF OPPOSING FORCES

NK cells play crucial role in eliminating HCMV-infected cells through cytotoxicity and secretion of several cytokines and chemokines able to directly impair viral replication (e.g., IFN-g and TNF-a) or to recruit and/or activate other cells of the immune system. However, if on one side there are examples demonstrating the importance of NK cells in controlling HCMV infection, on the other side there is a long list of viral proteins capable of protecting HCMV from NK cell recognition and killing (Brown and Scalzo, 2008; Schmiedel and Mandelboim, 2017; Patel et al., 2018).

The former case is best exemplified by human NK cell primary immunodeficiencies (NKD), which inevitably results in high susceptibility to herpesvirus infections [i.e., HCMV, HSV, EBV, and varicella zoster virus (VZV)] (Biron et al., 1989). In this regard, more than 60% of NKD patients are infected by one of these viruses (Orange, 2013), also in the context of intact CTL functions (Quinnan et al., 1982). The severity of this condition is demonstrated by the fact that nearly half of patients with NKD tend to die prematurely (Orange, 2013; Mace and Orange, 2019).

The antiviral activity of NK cells against HCMV also appears to be mediated by NK cell receptors, whose expression can be to some extent modulated upon viral entry. In particular, HCMV infection can induce the selective expansion of a population of NK cells expressing the activating receptor CD94/NKG2C, giving rise to the so-called "adaptive-like" or "memory-like" NK cells (Gumá et al., 2004). This aspect of NK and HCMV biology is beyond the scope of this review and has already been extensively described in recent reviews (López-Botet et al., 2014, p. 94; O'Sullivan et al., 2015; Rölle and Brodin, 2016).

What is important to point out in this context is that NKG2C receptor skewing is accompanied by other phenotypic, functional and epigenetic modifications, which lead to the generation of a pool of long-living NK cells with increased effector responses upon restimulation. Importantly, Hammer et al. (2018) have recently shown that the triggering event driving NKG2C^{+} NK cell expansion is mediated by an HCMV-encoded peptide derived from the viral protein UL40 and by the NKG2C ligand HLA-E. However, it is worth pointing out that the emergence of NK cell memory in response to HCMV can also occur in individuals lacking expression of NKG2C – i.e., carrying the null allele KLRC2 encoding for NKG2C – (Noyola et al., 2012), suggesting that alternative or compensatory mechanisms may be in place. This mode of activation is nonetheless complex, as HLA-E is also recognized by CD94/NKG2A, the inhibitory counterpart of CD94/NKG2C, with identical peptide specificity

(Braud et al., 1998; Lee et al., 1998; Brooks et al., 1999; Cerboni et al., 2000; Ulbrecht et al., 2000; Tomasec et al., 2005). Stabilization of HLA-E by the UL40-derived peptide can thus have opposite effects on NK cells, depending on which receptor is involved. However, it seems that the $NKG2C^+$ NK cell population expanding in HCMV seropositive individuals lacks the inhibitory NKG2A heterodimer (Hammer et al., 2018). In addition, the peptide repertoire encoded by different HCMV UL40 variants may result in an intermediate state, where peptides able to efficiently inhibit NKG2A and simultaneously trigger suboptimal activation of $NKG2C^+$ NK cells are more prevalent (Hammer et al., 2018).

The important role of NK cells in CMV infection comes also from a plethora of studies conducted in mice. In general, the absence of NK cells – due to genetic or neutralizing/depleting antibody manipulations – results in a significantly diminished, and sometimes lethal, control of MCMV (Bukowski et al., 1984; Brown and Scalzo, 2008). Similarly to HCMV, it has been reported a pathogen-specific recognition mechanism for protection, involving the NK cell-activating Ly49H receptor, which specifically recognizes the MCMV protein m157 (Arase et al., 2002).

Another important strategy for immune escape is the ability of HCMV to manipulate the expression of several ligands of the NKG2D receptor, expressed on all NK cells, $CD8^+$ T cells and other T-lymphocyte subsets (e.g., CD4 + T cells, gd, and NKT cells) (Lanier, 2015; Zingoni et al., 2018). There are eight different NKG2D ligands (i.e., MICA, MICB, and ULBP1-6), all belonging to the MHC class I-like family and possessing two or three αdomains, but not able to bind peptides or β2-microglobulin. These molecules are also known as "stress-induced ligands" or "induced self" as they are rarely expressed on the plasma membrane of healthy cells but can be rapidly up-regulated upon different types of stress, including those triggered by viral infection (Cerboni et al., 2014; Lanier, 2015). In the absence of a specific viral countermeasure, up-regulation of NKG2D ligands (NKG2DLs) would likely result in the killing of infected cells, as it has been observed in some experimental conditions (Cerboni et al., 2000; Wang et al., 2002; Pignoloni et al., 2016). However, *in vitro* studies have shown that this is not always the case since HCMV encodes at least seven different molecules – among which a few identified very recently – able to inhibit NKG2DL expression, thus conferring protection to the infected cells. In particular, MICA seems to be the most frequently targeted ligand, with UL142, UL148a, US9, US18, and US20 viral proteins dedicated to block its expression at different levels, sometimes in an allelic-specific manner (Schmiedel and Mandelboim, 2017; Patel et al., 2018; **Figure 4**). Although the reason for such a high number of HCMV proteins targeting just one ligand is currently unknown, their existence may be ascribed to the fact that, among NKG2D ligands, MICA has the highest affinity for its receptor (Steinle et al., 2001) as well as the largest number of variant alleles, with more than 100 identified thus far[1]. Based on these findings, it is tempting to speculate that the antiviral activity of MICA may have selected viruses able to block MICA expression and the ensuing NKG2D-mediated killing, and that this in turn might have promoted MICA polymorphism.

Among NKG2D ligands, we find MICB, a polymorphic gene with more than 40 allelic variants, and 6 ULBP genes boasting a total of 16 allelic variants[2] (Radosavljevic et al., 2002). MICB expression is inhibited by miR-UL112, the only HCMV-encoded miRNA described to date targeting this ligand (Stern-Ginossar et al., 2012), and by the viral protein UL16, which is a sort of promiscuous immunoevasin since it can also inhibit the expression of ULBP1, ULBP2, and ULBP6 (Cosman et al., 2001; Kubin et al., 2001; Dunn et al., 2003; Rölle et al., 2003; Wu et al., 2003; Eagle et al., 2009). ULBP3 is instead targeted by UL142, also blocking MICA expression (Ashiru et al., 2009; Bennett et al., 2010). The ability to simultaneously evade multiple cellular pathways has also been reported for US18 and US20, capable of inhibiting both MICA and the NKp30 ligand B7-H6 (Charpak-Amikam et al., 2017; Fielding et al., 2017).

Other targets of HCMV include CD155/PVR and CD112/Nectin-2, two adhesion molecules belonging to the Ig-like superfamily able to bind the activating receptor CD226/DNAM-1 expressed on cytotoxic lymphocytes (**Figure 4**; Shibuya et al., 1996; Bottino et al., 2003; Tahara-Hanaoka et al., 2004). Similar to NKG2DLs, DNAM-1 ligands (DNAM-1Ls) are often induced by cellular stresses and can trigger cytotoxicity and cytokine release (Shibuya et al., 1996; Bottino et al., 2003; Iguchi-Manaka et al., 2008). For this reason, DNAM-1Ls are also targeted by HCMV, with UL141 downregulating both of them, alone or in combination with US2 through different mechanisms (Tomasec et al., 2005; Prod'homme et al., 2007; Hsu et al., 2015). Of note, UL141 is also able to downregulate the TRAIL receptors R1 and R2, thus preventing TRAIL-dependent NK-cell killing (Nemčovičová et al., 2013; Smith et al., 2013). UL141 is thus a remarkable immunoevasion protein as it targets at least four different molecules regulating NK cell-mediated cytotoxicity.

Adhesion molecules involved in the formation of NK-target cell conjugates are also affected by HCMV. In particular, UL148 down-regulates CD58/LFA-3, the ligand of the CD2 receptor expressed by different leukocyte populations, including NK and $CD8^+$ T cells. The CD2/CD58 axis promotes cell-to-cell adhesion and immunological synapse formation, providing an important co-stimulatory signal on effectors (Siliciano et al., 1985; Selvaraj et al., 1987; Browne et al., 1990) (Leitner et al., 2015). More recently, CD2 has been shown to play a role in costimulation of adaptive NK cells (Rölle et al., 2003; Liu et al., 2016). Furthermore, inhibition of CD58/LFA-3 expression by the viral protein UL148 has revealed that the CD2/CD58 axis is also needed for the recognition of HCMV-infected cells by NK cells and HCMV-specific CTLs (Wang et al., 2018).

In summary, it appears that there is a steadily increasing number of HCMV-encoded proteins evading NK cell recognition and killing. However, to date there is no single viral protein or RNA able to interfere with all the molecules involved in the anti-viral NK cell response.

It is also important to point out that development, proliferation and effector functions of NK cells are tightly

[1] http://www.ebi.ac.uk/imgt/hla/html

[2] https://www.ebi.ac.uk/ipd/imgt/hla/

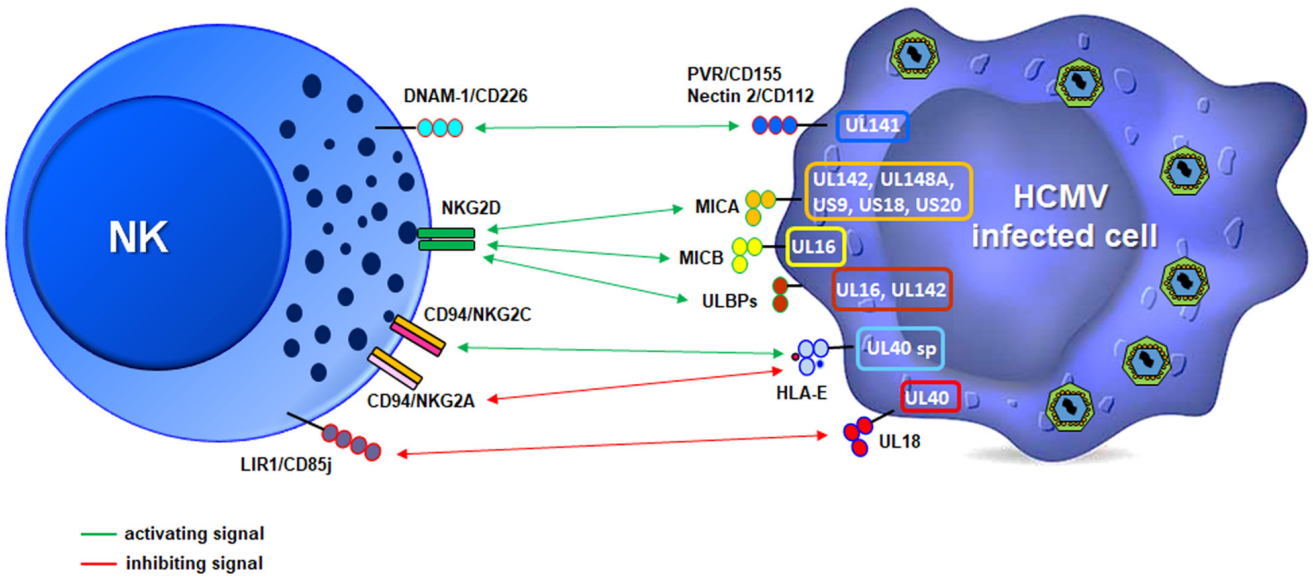

FIGURE 4 | Schematic illustration of the strategies used by HCMV to modulate NK cell receptor ligands. The viral proteins mainly involved are depicted in a representative infected cell (right, color outlines), and activating (green) or inhibitory (red) signals relative to specific receptors on a NK cell (left) are shown.

regulated by both activating and inhibitory receptors, with an outcome that strongly depends on the balance between opposing signals. Inhibition is delivered *via* MHC-I molecules expressed on the surface of target cells. However, HCMV, like many other viruses, negatively affects MHC-I expression in infected cells, as this is a crucial step to avoid cell-mediated killing by viral-specific cytotoxic T cells. In theory, this would render infected cells more susceptible to NK cell recognition due to the absence of inhibitory signals. However, the observation that HCMV-infected cells are resistant to NK lysis *in vitro* seems to suggest otherwise (Cerboni et al., 2000; Wang et al., 2002). What we have in fact described in this section is a plethora of viral molecules evolved by HCMV to escape from NK cell activation, which otherwise would be detrimental for viral fitness.

To complete this picture, HCMV can fully accomplish immunoevasion from NK cells thanks to its own MHC-I surrogate, called UL18. This protein is markedly similar to cellular MHC-I molecules (Beck and Barrell, 1988; Browne et al., 1990) and acts as a viral homolog by binding with high affinity the MHC-I NK cell inhibitory receptor CD85j/LIR1/ILT2, thereby suppressing NK cell functions (Chapman et al., 1999; Cosman et al., 2001; Cerboni et al., 2006; Prod'homme et al., 2007).

In conclusion, HCMV is a driving force in shaping the NK cell receptor repertoire and modes of recognition of infected cells. The virus is not only capable of "hitting the brakes" of NK cells through its own MHC-I surrogate (UL18) or by engaging the CD94/NKG2A inhibitory receptor with UL40, but it can also "block the gas pedal" by inhibiting the expression of several ligands of NK cell activating receptors. The outcome is a million-year-long host-pathogen equilibrium, where neither the host nor the pathogen is at risk of extinction.

HCMV AND APOPTOSIS: "NOT TODAY!"

Apoptosis, or programmed cell death (PCD), is essential for the maintenance of homeostasis and survival of most multi-cellular organisms. Apoptosis occurs predominantly through the following three pathways: (1) extracellular ligand-mediated extrinsic pathway; (2) mitochondria-mediated intrinsic pathway; and (3) ER-mediated pathway. The extrinsic pathway is initiated upon binding of extracellular ligands to death receptors (DRs), leading to the formation of the death-inducing signaling complex (DISC), required for the activation of initiator caspases (i.e., cysteine proteases), caspase-8 and caspase-10. The intrinsic pathway is regulated by B-cell lymphoma 2 (Bcl-2) proteins and is characterized by mitochondrial outer membrane permeabilization (MOMP) (Elmore, 2007). The ER-mediated pathway is instead induced by stress signals, such as excessive unfolded proteins in the ER and triggers the activation of caspases-7, -9, and -12 (Bhat et al., 2017). All these pathways lead to the activation of the executioner caspases-3 and -7 that contribute to the majority of events taking place during apoptosis (Elmore, 2007).

Apoptosis is also one of the main steps of the innate response against viral infections, including HCMV. Also in this case, HCMV has evolved several strategies to subvert host cell apoptotic defenses by targeting key effector molecules in the apoptotic cascade. Upon infection, the slowly replicating HCMV modulates cellular apoptosis pathways in various cell types, such as endothelial cells, fibroblasts and macrophages by encoding numerous death inhibitors to block premature death of host cells, thus favoring its replication (Brune and Andoniou, 2017; Collins-McMillen et al., 2018; **Figure 5**). The following paragraphs will contain a comprehensive review and discussion of some of the

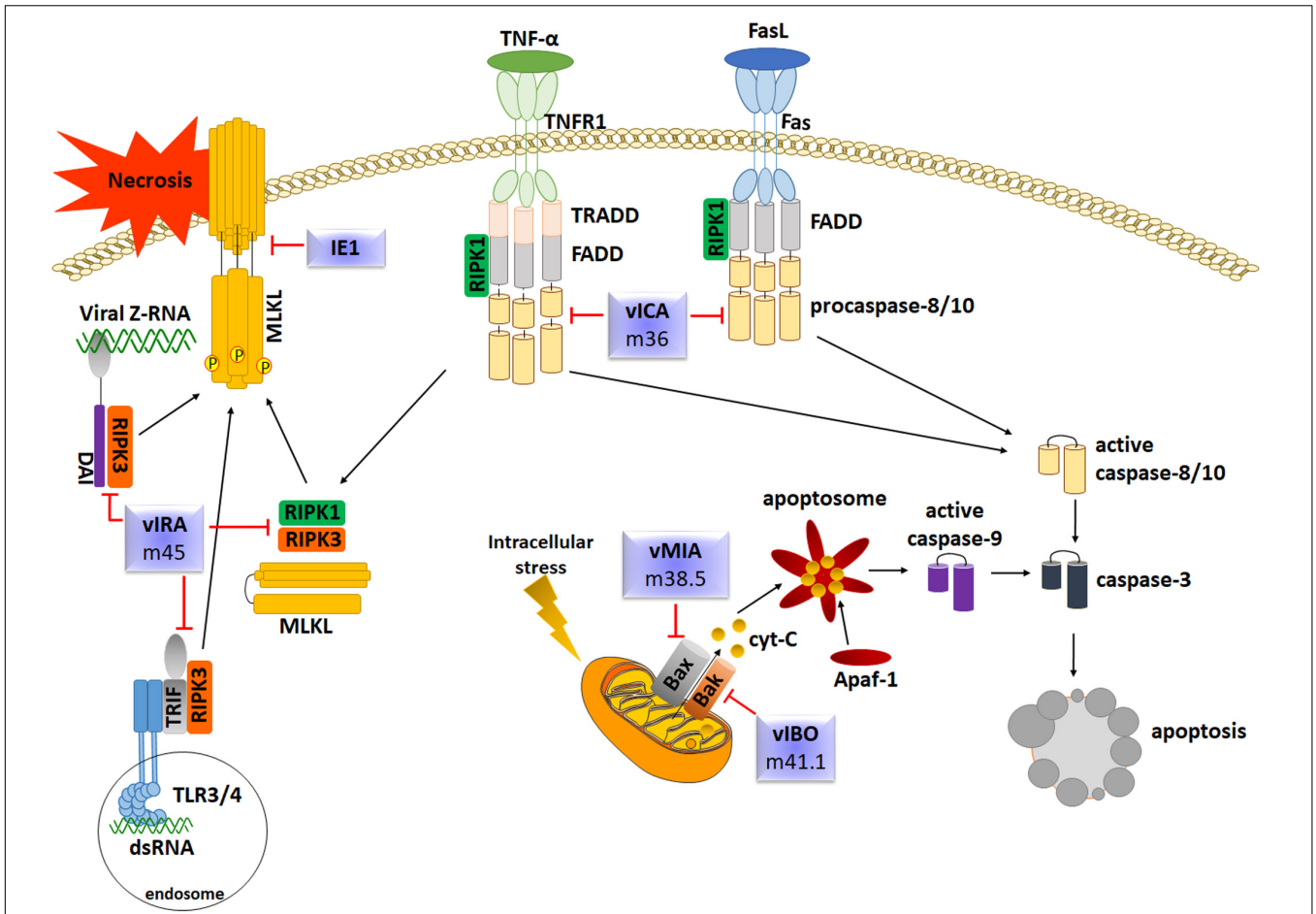

FIGURE 5 | Overview of the main apoptotic pathways and evasion strategies employed by HCMV.

main mechanisms used by HCMV to modulate or prevent the apoptotic pathways of infected host cells.

Inhibition of Extrinsic Apoptosis

Caspase-8 is required for initiation of apoptosis in response to death factors such as Fas-L or TNF-a. Within the Fas-FADD-Caspase-8 complex, also known as DISC, caspase-8 undergoes self-cleavage to convert to the active form. Fully-cleaved caspase-8 is released from DISC to the cytosol to trigger the apoptotic signal to downstream caspase effectors or to cleave the Bcl-2-interacting protein (Bid), which leads to the release of cytochrome c from mitochondria, inducing activation of caspase-9 in a complex with dATP and Apaf-1 (Kruidering and Evan, 2000). To counteract DR- mediated apoptosis and gain a survival advantage, HCMV encodes the viral inhibitor of caspase-8-induced apoptosis vICA/pUL36, which binds the prodomain of procaspase-8, impedes the recruitment of FADD, and prevents the formation of a functional DISC. The fact that homologs of HCMV vICA have been identified in the vast majority of mammalian betaherpesviruses implies that the function of vICA is important and conserved. This is exemplified by M36, the vICA counterpart of MCMV, which also displays an anti-apoptotic activity by interacting with procaspase-8, and that has been shown to be rescued by vICA in order to allow viral replication, confirming the reliability of the murine model (Chaudhry et al., 2017).

Moreover, the replication of UL36-deficient virus can be restored by treatment with the pan-caspase inhibitor z-VAD(OMe)-fluoromethyl ketone (fmk) only in immature but not mature macrophages, suggesting that apoptosis impairs the replication of UL36-deficient virus in defined cell types. However, according to McCormick et al. (2010), it seems that cell death pathways activated by HCMV infection are altered as monocytes differentiate to macrophages. Indeed, early during differentiation, UL36-deficient virus-induced apoptosis is dependent on caspases and can be blocked by z-VAD-fmk, while at later stages of differentiation it appears to be caspase-independent.

Inhibition of Intrinsic Apoptosis

Mitochondria play a pivotal role in the intrinsic apoptosis pathway. Initiation and execution of this pathway is regulated by the Bcl-2 effector proteins Bax (Bcl-2-associated X protein) and Bcl-2 antagonist or killer (Bak) that control MOMP. MOMP prompts the release of proapoptotic intermembrane space (IMS)

proteins that promote the formation of the apoptosome – composed by cytochrome c and Apaf-1 – and activation of caspase-9. Once active, caspase-9 can directly cleave the effector caspases 3 and 7 (Estaquier et al., 2012). HCMV prevents MOMP by encoding the viral mitochondria-localized inhibitor of apoptosis (pUL37x1/vMIA). UL37x1, highly conserved among HCMV strains, is located in a complex transcription unit encoding several transcription variants expressed during the IE phase. Two functionally longer splice variants (i.e., gpUL37 and gp37M) share with pUL37x1 an NH2-terminal 162 aa sequence responsible for inhibiting apoptosis, localize partially to mitochondria and have similar, albeit weaker, anti-apoptotic activities (Goldmacher et al., 1999; Colberg-Poley et al., 2000; Reboredo et al., 2004; Kaarbø et al., 2011). pUL37x1 blocks mitochondria-mediated apoptosis by interacting at the level of the mitochondrial outer membrane (MOM) with Bax, thus preventing cytochrome-c release. It still remains to be clarified whether vMIA can inhibit Bak during infection (Sharon-Friling et al., 2006; Sharon-Friling and Shenk, 2014).

Moreover, by using U251 glioma cells a mechanisms of viral apoptosis inhibition and enhancement of cell proliferation has been shown, relying on the activity of the immediate-early protein IE86 on heterogeneous ribonucleoprotein A2/B1 (hnRNP A2/B1) and consequent alternative splicing of Bcl-x (Zhao et al., 2019).

In addition to the aforementioned strategies, HCMV is also involved in preserving the mitochondrial membrane potential and metabolism to prevent cell death. This is achieved thanks to the production of the long non-coding RNA-lncRNA Beta2.7 that enhances cell survival through interaction with gene associated with retinoid/interferon-induced mortality 19 (GRIM19). This interaction causes the stabilization of mitochondrial membrane functions, thereby preserving ATP production and conserving metabolic activity during stress conditions (Poole et al., 2016).

Inhibition of Necroptosis

Necroptosis is an alternative form of programmed cells death that, despite mimicking features of apoptosis, cannot be prevented by caspase inhibitors. Necroptosis can be triggered following activation of DRs as well as after stimulation with LPS, poly(I:C) or CpG DNA, which are ligands of the pattern recognition receptors (PRRs) TLR3, TLR4, and TLR9, respectively. Many downstream signaling pathways cooperate with a complex formed by the receptor interacting protein kinase 1 (RIPK1), RIPK3 and mixed lineage kinase domain-like (MLKL). Necroptosis and apoptosis are strictly interconnected, as confirmed by the observation that the inhibition of caspase-8, the main mediator of the extrinsic apoptotic pathway, promotes the shift from DR-mediated cells death to necroptosis due to activation of RIPK3 and, consequently, MLKL. Phosphorylation of MLKL generates structural changes allowing its insertion into the inner leaflet of the plasma membrane leading to the disruption of cellular membranes (Green, 2019).

Inhibition of Cellular Stress Response

Disturbances of the normal functions of the ER, causing accumulation of unfolded proteins, trigger an evolutionarily conserved cell stress response, known as unfolded protein response (UPR), which, initially aimed to damage compensation, can eventually lead to cell death to avoid viral spread. HCMV prevents this process, in part, *via* UL38, a multifunctional protein well conserved among different CMV species. In particular, viral DNA replication is severely impaired in viruses lacking UL38 (i.e., AD*dl*UL38), a feature associated with enhanced death of infected cells (Terhune et al., 2007). Moreover, pUL38 itself can inhibit cell death induced by thapsigargin, which perturbs calcium homeostasis followed by ER-mediated cell death, or by a mutant adenovirus lacking the antiapoptotic E1B-19K protein. Of note, pUL38 cannot counteract cell death triggered by anti-Fas antibodies (Xuan et al., 2009).

Overall, the aforementioned findings suggest that pUL38 hampers both intrinsic and ER-mediated cell death, but it only slightly affects extrinsic apoptosis. UL38, expressed both at early and late stages of infection, is localized in a complex transcription unit that also retains the unspliced transcripts of UL36 and several variants of UL37, expressed during the IE phase. Probably, pUL36, pUL37x1 and pUL38 act synergically to inhibit cell death at different times during infection. As described above, while pUL36 inhibits caspase-8 activation, pUL37x1 blocks mitochondria-mediated intrinsic apoptosis. Furthermore, UL38 inhibits c-Jun N-terminal kinase (JNK) signaling through interaction with the activating transcription factor 4 (ATF4), which leads to caspase-12 or caspase-2 activation (Xuan et al., 2009).

More recently, Luganini et al. (2018) have shown that HCMV encodes for a viral-Ca^{2+}-permeable channel, pUS21, able to reduce Ca^{2+} content of intracellular stores and to protect cells from apoptosis. Among the US12 gene family members, which includes a set of 10 contiguous tandemly arranged genes (US12-21), pUS21 shows the highest level of identity with two cellular transmembrane BAX inhibitor motif-containing (TMBIM) proteins: Bax inhibitor-1 and Golgi anti-apoptotic protein, both involved in the regulation of cellular Ca^{2+} homeostasis and adaptive cell responses to stress conditions. Thus, alongside pUL36, pUL37x1 and pUL38, pUS21 contributes to maintaining the viability of the host cell until the virus has completed the infection cycle.

A second mechanism used by CMV to counteract ER stress response involves the downregulation of inositol-requiring enzyme 1 (IRE1) protein levels, an ER stress sensor and cell death executor (Maly and Papa, 2014). Misfolded proteins activate IRE1, which in turn oligomerizes and self-activates its RNase activity, leading to degradation of unfolded proteins and upregulation of ER chaperon to enhance protein folding. IRE1 activation also leads to the recruitment of the TNF receptor associated factor (TRAF)-2 and activation of caspase-12 or JNK. Activated JNK induces cells death by activating proapoptotic BH3 proteins while inhibiting the antiapoptotic Bcl-2. Lastly, both MCMV and HCMV homologs M50 and UL50 enhance IRE1 degradation at later times post-infection, thus preventing all IRE1 signaling events (Stahl et al., 2013).

A second form of stress response induced by HCMV infection is that elicited by DNA damage. To ensure faithful duplication and inheritance of genetic material, cells have

evolved mechanisms – collectively termed the DNA-damage response (DDR) – of DNA damage detection to induce DNA repair or, if the damage is too severe, to induce cell death (Xiaofei and Kowalik, 2014). After cell entry, HCMV capsids travel to the nucleus where the linear genome is released and circularized to serve as a template for transcription and replication by a rolling circle mechanism. This process generates multiple exposed ends that can be recognized as dsDNA by activating ataxia-telangiectasia mutated protein (ATM) and rad-3 related kinases (ATR), which initiate the DNA damage signal transduction pathway by targeting proteins involved in the checkpoint response, such as checkpoint kinase 2 (Chk2). In this regard, recent studies have revealed that HCMV can neutralize host DDR at the level of Chk2. In particular, ATM and ChK2 are mislocalized from the nucleus to the cytoplasm where they colocalize with virion structural proteins, which prevents them from initiating the DNA repair process (Gaspar and Shenk, 2006; Luo et al., 2007).

CONCLUSION

Here, we have provided a comprehensive overview of the main characteristics of HCMV that have allowed this virus to evolve multiple immune evasion strategies and achieve latency and seroprevalence. These include the advanced organization and large size of its genome, restricted host specificity, viral latency and sporadic reactivation.

We have also highlighted how the host innate immune response reacts against HCMV infection through different effector cells (e.g., APCs, NK cells, and phagocytes), anti-inflammatory cytokines and IFNs. Briefly, while APCs mediate early immune activation by triggering specific T-cell responses, and cytotoxic NK cells are potent eliminators of HCMV-infected cells, early release of IFN-I and other pro-inflammatory cytokines limit the infection spread through the establishment of the so-called "antiviral state." In addition, several IFN-inducible RFs, which belong to an additional autonomous branch of innate immunity, play a central role in inhibiting viral replication. Lastly, a significant part of the innate immune response is represented by programmed cell death, as apoptotic control greatly contributes to the removal of original population of HCMV-infected cells. Thus, thanks to the presence of multiple innate immune protective mechanisms the host, in most cases, is able to counteract HCMV spread.

However, in the course of host-virus coevolution, as described in this review, HCMV has acquired an extremely wide range of counter-defense mechanisms and manipulation strategies directed against each arm of innate immunity. For instance, HCMV is able to inhibit NK cell activation by encoding numerous proteins targeting multiple host ligands, which are likely to promote viral persistence *in vivo*. The virus is also capable of subverting the immune functions of APCs by reprogramming them as efficient means of viral dissemination, while offsetting their immune surveillance by interfering with MHC-I and MHC-II antigen presentation. Moreover, HCMV can block premature death of infected cells, thereby promoting viral replication. Major interfering with IFN-signaling pathways is also accomplished *via* a wide range of viral proteins that counteract and manipulate IFN production by the host. Thus, there is growing evidence of a highly dynamic and complex interplay between the virus and the IFN system.

From all these data, it is clear that HCMV disease progression depends on the balance between antiviral immune response and viral attempts to manipulate such response to its own advantage. Given the clinical burden of HCMV in immunocompromised patients and congenitally infected infants, there is undoubtedly an urgent and unmet medical need for an effective vaccine against this virus. Significant efforts should also be directed toward the development of more effective therapeutic agents with fewer side effects capable of targeting the virus during both its lytic and latent phases. In this regard, an in-depth analysis of the interplay among HCMV, RFs and INFs resulting in immune evasion should provide potential novel druggable targets.

AUTHOR CONTRIBUTIONS

VD and MD developed the ideas and drafted the manuscript. VD, MB, FG, GGa, and CC wrote sections of the manuscript. GGr, AZ, and SP drew the figures. GGa and MD professionally edited the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Degradation of Herpes Simplex Virus-1 Viral miRNA H11 by Vaccinia Virus Protein VP55 Attenuates Viral Replication

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Among 29 distinct miRNAs expressed by the herpes simplex virus-1 (HSV-1) during lytic infection, miR-H11, together with miR-H1 to miR-H8 are reported to locate in the RNA-induced silencing complex (RISC). miR-H11 is encoded within viral origins of replication and lies entirely within the origins of replication. However, the roles of this miRNA derived from lytic infection with HSV-1 remain unclear. Using the advantage of vaccinia virus protein VP55 (VP55)-mediated degradation of miRNAs, we constructed a recombinant virus expressing VP55 (R5502) to demonstrate that: (1) accumulation of miR-H11 from R5502 was reduced by 540-fold versus that in cells infected with wild-type HSV-1, but miR-H1 to miR-H8 which also located in the RISC were not reduced significantly from R5502 compare with wild-type HSV-1; (2) downregulation of miR-H11 from R5502 infected cells results in markedly lower viral DNA synthesis compared with wild-type HSV-1; and (3) downregulation of miR-H11 also restricted viral spreading, and resulted in low accumulation of representative viral proteins and viral yields. The findings were confirmed through either using of a miR-H11 inhibitor or pre-transfection of a plasmid expressing VP55. These data suggest that miR-H11 plays a currently unidentified role in maintaining sufficient viral DNA synthesis during the course of viral infection.

Keywords: miR-H11, VP55, DNA synthesis, replication, herpes simplex virus-1

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INTRODUCTION

miRNAs are derived from primary transcript (pri-miRNA) and modulated at the levels of transcription and processing (Johnson et al., 2003; Hagan et al., 2009; Heo et al., 2009; Liu and Liu, 2011). Pri-miRNA is recognized by a nuclear microprocessor complex, which includes the RNase III enzyme Drosha, and cleaved into a precursor miRNA (pre-miRNA; Siomi and Siomi, 2010). The pre-miRNA is subsequently cleaved by Dicer to generate a duplex RNA of ∼22 nt (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001), which is subsequently loaded into an RNA-induced silencing complex (RISC; Czech and Hannon, 2011) to produce mature miRNA (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). Vaccinia virus protein VP55 (VP55) has been shown to be both necessary and sufficient for the tailing of RISC-associated host miRNAs (Backes et al., 2012). VP55 adds non-templated adenosines specifically to the miRNAs, which are associated with the RISC; hence, it results in the rapid degradation of those miRNAs (Backes et al., 2012).

Among 29 discovered miRNAs expressed by the herpes simplex virus-1 (HSV-1) during lytic infection with HSV-1, miR-H11 is located in the RISC. Other miRNAs located in the RISC are miR-H1 to miR-H8 (Flores et al., 2013). miR-H11 is encoded within the viral origins of replication (OriL; Jurak et al., 2014; Du et al., 2015) and lies entirely within the OriL (Jurak et al., 2014; Du et al., 2015). The 65 nucleotides at the 5′ terminus of the H11 precursor are complementary to the 65 nucleotides at its 3′ terminus. H11 represents the highest increase observed for the interval between 1 and 12 h after infection with HSV-1 (F) in HEp-2 cells. However, it is not detected in ganglia harboring latent virus or in ganglia incubated for 24 h in medium containing anti-nerve growth factor antibody (Du et al., 2015).

In this study, we constructed an HSV-1 recombinant virus expressing VP55 (R5502) to assess the impact of miR-H11 loss on virus replication. Our data demonstrated that accumulation of miR-H11 was reduced by 540-fold compared with that in wide-type cells infected with HSV-1. In turn, downregulation of miR-H11 resulted in lower viral DNA synthesis, restriction of viral spreading, and low viral yields.

MATERIALS AND METHODS

Cell Lines and Virus

HEp-2 and Vero cells were obtained from the American Type Culture Collection and cultured in Dulbecco's Modified Eagle Medium (DMEM; high glucose content) supplemented with 5% (v/v) fetal bovine serum (FBS), or 5% (v/v) newborn calf serum (NBCS), respectively. HSV-1(F), the prototype HSV-1 strain used in this laboratory, was propagated and titrated using Vero cells.

Antibodies

Antibodies against ICP8 (Rumbaugh Goodwin Institute for Cancer Research, Inc.), ICP0 (Cat No. ab6513; Abcam), ICP4 (Cat No. ab6514; Abcam), ICP27, VP16, and US11 have been described elsewhere (Ackermann et al., 1984; McKnight et al., 1987; Roller and Roizman, 1992). Antibodies against ICP22, VP22, and VP16 were kind gifts of Bernard Roizman (The University of Chicago, United States). Additional antibodies used in this study were anti-green fluorescent protein (anti-GFP) monoclonal antibody (Cat No. KM8009; Sungene Biotech) and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH; Cat No.KM9002; Sungene Biotech).

miRNA Inhibitors

Non-target (NT) inhibitor and miR-H6-5p, H3-3p, and H11 inhibitors were designed and purchased from GenePharma. The sequences were as follows:
NT inhibitor: 5′-CAGUACUUUUGUGUAGUACAA-3′,
miR-H6-5p inhibitor: 5′-UACACCCCCCUGCCUUCCACC-3′,
miR-H3-3p inhibitor: 5′-GUCCCAACCGCACAGUCCCAG-3′,
miR-H11 inhibitor: 5′-GCGUUCGCACUUUGUCCUAA-3′.

Construction of the Plasmid

The plasmid enhanced green fluorescent protein-VP55 (pEGFP-VP55) (p5502) containing VP55 in fusion with EGFP was a kind gift of Benjamin R. (Mount Sinai School of Medicine, United States). The control plasmid pGFP02 was designed to insert a stop codon (TGA) immediately after the VP55 ATG start codon.

Construction of Recombinant Viruses

We constructed the VP55 recombinant virus (R5502) and control virus (RGFP02). The gene encoding VP55 or GFP was inserted into the genes encoding UL3 and UL4 under the cytomegalovirus promoter, respectively. The strategy for the construction of the virus has been previously reported (Ren et al., 2019).

Transfection of Cells

HEp-2 cells were seeded in 12-well plates the day prior to transfection in DMEM containing 5% FBS. The following day, the plasmids or HSV-1 miRNA inhibitors described above were transfected into HEp-2 cells. Lipofectamine 2000 (Invitrogen) was used for transfection according to the protocol provided by the manufacturer. At 24 h post transfection, the cells were infected with 0.1 or 1 plaque-forming unit (PFU) of HSV-1(F) per cell, harvested at an indicated time point post infection or at 48 h post transfection, and lysed according to the protocol for the subsequent analyses.

Immunoblotting Assays

Cell lysates were harvested and lysed with a radioimmunoprecipitation assay lysis buffer (Beyotime) supplemented with 1 mM protease inhibitor phenyl methyl sulfonyl fluoride (Beyotime) and heat denatured, separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Millipore). The proteins were detected through incubation with an appropriate primary antibody, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Invitrogen). Visualization was performed using the enhanced chemiluminescence reagent (Pierce) and film exposure, or the capture of images using the ChemiDoc Touch Imaging System (Bio-Rad) and processed using the Image Lab software. The densities of corresponding bands were quantified using the ImageJ software.

Virus Titration

HEp-2 cells were seeded in a six-well plate at densities of 1×10^6 cells per well. The cells were subsequently exposed to 0.01 PFU of HSV-1(F), VP55 recombinant virus (R5502) and control virus (RGFP02), or 0.1 PFU of HSV-1(F) per cell at 24 h post plasmid transfection. The cells were harvested at 3, 6, 12, 24, 48 and 72 h, or 3, 6, 12 and 24 h post infection. Viral progeny was titrated using Vero cells following three freeze-thaw cycles.

Plaque Assay

Vero cells seeded in six-well plates were exposed to 0.001 PFU of HSV-1(F), R5502, and RGFP02 per cell for 2 h and maintained in 199V medium (Gibco) supplemented with 1% FBS for 48 h. The cells were fixed with 4% (w/v) of paraformaldehyde for 30 min, rinsed thrice with phosphate-buffered saline, and stained with Giemsa stain for 30 min. The images were captured using an inverted Leica microscope.

Viral miRNA Deep Sequencing

HEp-2 cells were infected with 10 PFU of HSV-1(F) and VP55 recombinant virus (R5502) per cell. The cells were harvested 24 h post infection. Small RNAs were isolated and subjected to high-throughput sequencing by Capital Bio Technology to identify HSV-1- and R5502-derived miRNAs.

HSV Genome Labeling and Click Chemistry Study

HEp-2 cells grown on glass coverslips at densities of 1.5×10^4 cells overnight were serum-starved in DMEM containing 0.25% FBS for 24 h to arrest cells at the G_0 stage (Ren et al., 2016). The cells were subsequently infected with HSV-1 (F), R5502, or RGFP02 at 10 PFU per cell for 1 h, the medium was replaced with fresh DMEM containing 1% FBS and 10 μM 5-ethynyl-2′-deoxyuridine (EdU), and the cells were cultured for indicated hours. EdU-labeled DNA was conjugated with Alexa Fluor 647 picolyl azide using the Click-iT Plus EdU imaging kit (Life Technologies). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The images were captured and processed using a confocal laser-scanning microscope (magnification, 40×). The EdU/GFP-positive cells were quantified using the ImageJ software.

RESULTS

VP55 Significantly Downregulates Viral miRNA miR-H11

Construction of the VP55 Plasmid and HSV-1 Expressing the VP55 Recombinant Virus

In this series of studies, we employed a VP55 plasmid and HSV-1 expressing the VP55 recombinant virus to assess the impact of viral miRNA loss on the infection. The structure of the plasmid which expressed EGFP-fused VP55 (p5502) and the control plasmid pGFP02 which expressed EGFP only are shown schematically in **Figure 1A**. In brief, the expression of VP55 was optimized using a human codon (Backes et al., 2012) and fusion was performed with an EGFP (p5502). The control plasmid (pGFP02) was constructed through insertion of a stop codon immediately after the VP55 start codon, resulting in the expression of EGFP only (**Figure 1A**). For the characterization of protein expression, HEp-2 cells were transfected with p5502 or pGFP02 and harvested 48 h later. Cell lysates were prepared and subjected to electrophoresis in denaturing gels, followed by incubation with an anti-GFP antibody. The detected bands were approximately 80 and 30 kDa in the p5502 and pGFP02 plasmid transfection samples, respectively (**Figure 1B**), consistent with the calculated molecular sizes. The effect of degradation of overall host miRNAs by VP55 was confirmed through transfection of p5502 or pGFP02 into HEp-2 cells and subsequent detection of the accumulation of host miRNAs (Let-7a, miR-93, and miR-21). The accumulation of host miRNAs in cells transfected with p5502 was reduced by approximately three-fold. In contrast, there was no reduction of these miRNAs in cells transfected with pGFP02 (**Supplementary Figure S1** and **Supplementary Table S1**). We subsequently constructed the recombinant virus expressing EGFP-fused VP55 (R5502) and the control virus (RGFP02) expressing EGFP only (**Figure 1C**). The protein-coding sequences driven by the cytomegalovirus promoter were inserted into the *UL3* and *UL4* genes. The expression of GFP-fused VP55 from R5502 and GFP from RGFP02 were determined through infection of R5502 and RGFP02 in HEp-2 cells (10 PFU per cell, 12 and 24 h) and subsequent blotting of the cell lysates with anti-GFP antibody (**Figure 1D**).

Deep-Sequencing Analyses of Cells Infected With R5502 Led to the Identification of miR-H11, Which Is Markedly Downregulated by VP55

We investigated the overall expression of viral miRNAs in HEp-2 cells infected with R5502 and HSV-1(F) by performing a microRNA deep-sequencing analysis (**Figure 2A**). Comparative analyses of the miRNAs profiles showed that, among all the viral miRNAs tested, three were present in significantly low amounts in R5502-infected cells (**Figure 2A**). The amounts of miR-H11, H3-3p, and H6-5p were reduced by 540-, 2.1-, and 2.6-fold (**Table 1**). As the key effector of miRNA, miR-H11 is located in the RISC (Flores et al., 2013), which may explain its high degradation by VP55.

Impact of miR-H11 Downregulation on Viral Replication

Downregulation of miR-H11 Results in Lower Viral DNA Synthesis

miR-H11 is encoded within a unique sequence (**Figure 2B**). The 65 nucleotides at the 5′ terminus of the H11 precursor are complementary to the 65 nucleotides at its 3′ terminus (Flores et al., 2014). Interestingly, miR-H11 is encoded within the viral OriL (**Figure 2C**) and could be derived from previously reported transcripts that span the viral origins (Jurak et al., 2014). We used the EdU incorporation method to measure the replication of the R5502 viral genome and to investigate whether reduction of miR-H11 affects viral DNA synthesis. In principle, EdU incorporation remains scarce in serum-starved cells, whereas EdU is incorporated into newly synthesized HSV-1 genomic DNA. In brief, serum-starved HEp-2 cells were exposed to 10 PFU of HSV-1(F), R5502, or RGFP02 per cell for 1 h. The inoculum was replaced with fresh medium which contained 1% FBS and 10 μM EdU conjugated with Alexa Fluor 647. The cells were fixed at 6 and 9 h post infection and stained with DAPI. The cells infected with R5502 or RGFP02 were visualized using the GFP signal (**Figure 3**). Notably, there was single staining of EdU over the F, R5502- and RGFP02-infected cells due to the cells were not fully starved to synchronize the cell cycle. From each image, we have counted all the number of GFP positive cells (green) first, then counted the EdU positive-staining nucleus (red) only from GFP positive

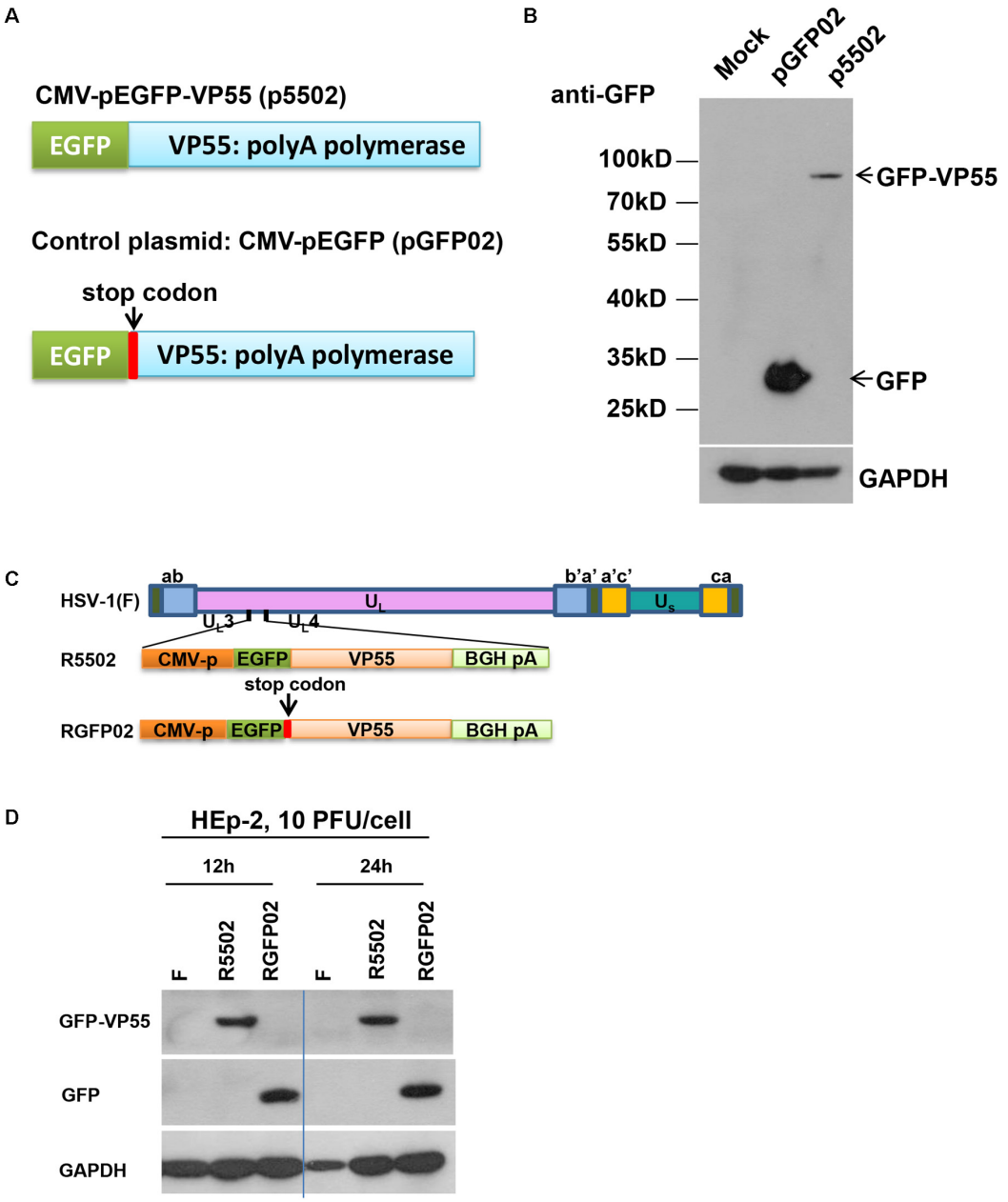

FIGURE 1 | (A) Schematic diagram of the VP55 expression plasmid (p5502) and control plasmid (pGFP02). p5502 was designed to express VP55 in fusion with EGFP based on the pEGFP-C1 plasmid. The VP55 coding sequence was inserted in the C-terminus of EGFP. pGFP02 is the control plasmid, which was constructed through insertion of a TGA stop codon immediately after the VP55 ATG start codon. **(B)** Protein expression levels in cells transfected with the VP55 expression plasmid (p5502) and control plasmid (pGFP02). HEp-2 cells were mock-treated or transfected with 0.75 μg of pGFP02 or p5502 plasmid in a 12-well plate. The cells were harvested 48 h post transfection. Accumulations of GFP and VP55-GFP were measured as described in the "Materials and Methods" section. **(C)** Schematic representation of the parent virus HSV-1(F), VP55-expressing recombinant virus (R5502), or control recombinant virus (RGFP02). R5502, derived from the parent wild-type HSV-1(F), is a recombinant virus expressing VP55 fused with EGFP. RGFP02 is the control recombinant virus, which only expressed EGFP. All constructs were inserted into UL3 and UL4 genes, and the open reading frames (ORFs) were driven by the CMV promoter and tailed with BGH-polyA signal. **(D)** The GFP-VP55 fusion protein or GFP expressed by the recombinant virus was analyzed through infection with 10 PFU of HSV-1(F), R5502, and RGFP02 per cell. The cells were harvested at 12 and 24 h post infection. The accumulations of GFP and VP55-GFP were measured using an immunoblotting assay as described in the "Materials and Methods" section.

cells. In summary, at 6 h post infection, the percentage of EdU and GFP double-stained cells from cells infected with R5502 and RGFP02 was 32 and 66%, respectively. At 9 h post infection, this percentage was 20 and 59%, respectively, indicating that reduction of miR-H11 results in a defect in viral DNA synthesis.

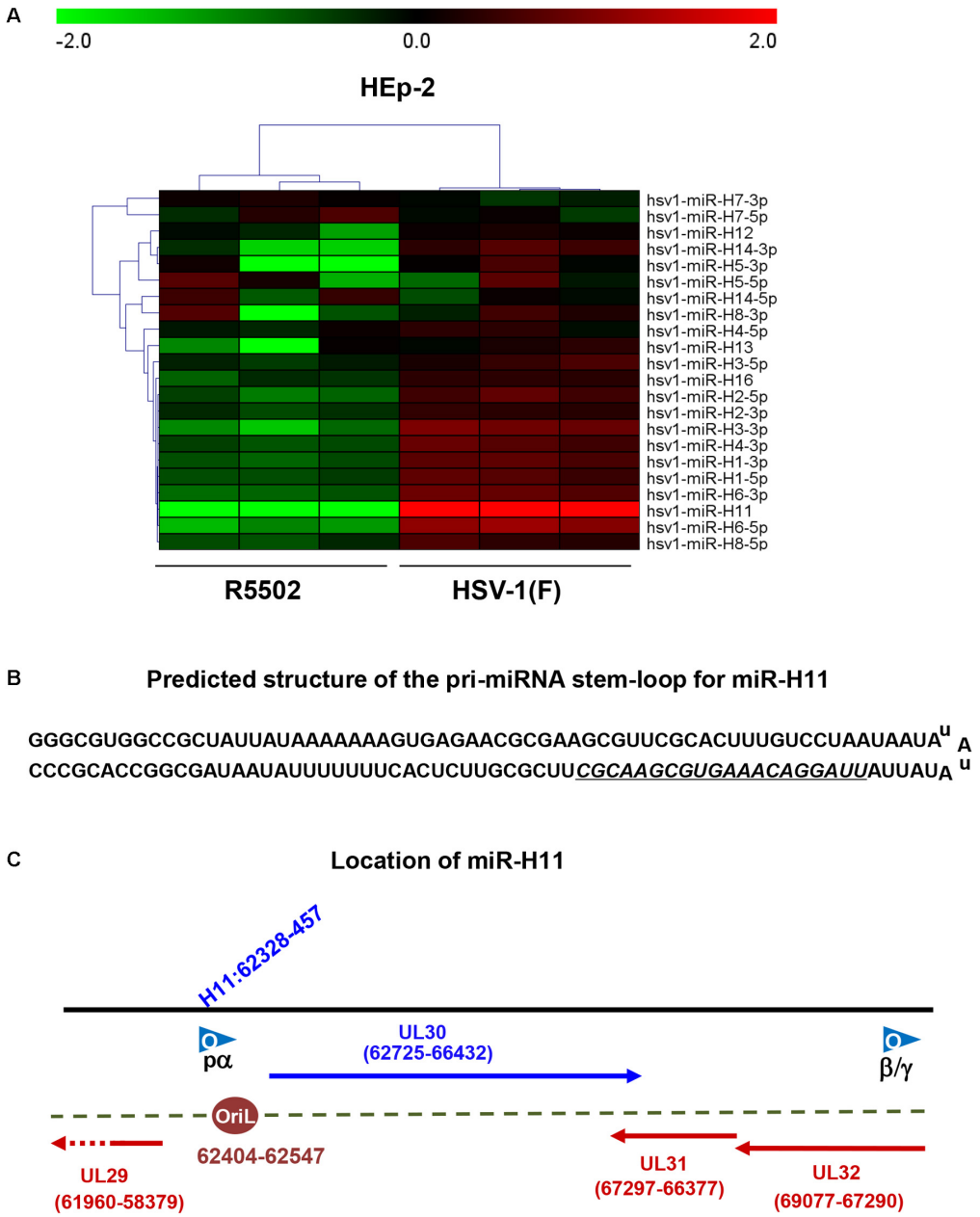

FIGURE 2 | VP55 recombinant virus (R5502) displays decreased expression of most of viral miRNAs, as revealed by miRNA Deep-Seq analyses. **(A)** The heat map depicts the fold-change in the expression of viral miRNAs in HEp-2 cells infected with HSV-1(F) and R5502. HEp-2 cells were exposed to 10 PFU of HSV-1(F) or R5502 per cell for 24 h. The cells were harvested and RNA was extracted for miRNA deep-Seq analyses. Relative expression levels are depicted using different colors: red, upregulation; green, downregulation (n = 3). **(B)** Predicted structure of the pri-miRNA stem-loop for miR-H11, which consists of a perfect 65-nucleotide inverted repeat. Underline indicates the mature miR-H11 sequence. **(C)** The location of miR-H11 in the HSV-1(F) genome, derived from the HSV-1 origin of replication OriL.

TABLE 1 | Viral miRNAs are reduced more than two-fold from R5502 infection versus HSV-1(F) infection.

Cell line	miRNAs	Reduction fold
HEp-2	H3-3p	2.1
	H6-5p	2.6
	H11	540.4

Treatment of HEp-2 Cells With miR-H11 Inhibitor Prior to Infection With F Results in Decreased Accumulation of Viral Proteins

According to the results of the microRNA deep-sequencing analysis shown in **Figure 2A** and **Table 1**, we subsequently investigated the role of H3-3p, H6-5p, and H11 in HSV-1 infection because technically result with R5502 could be a

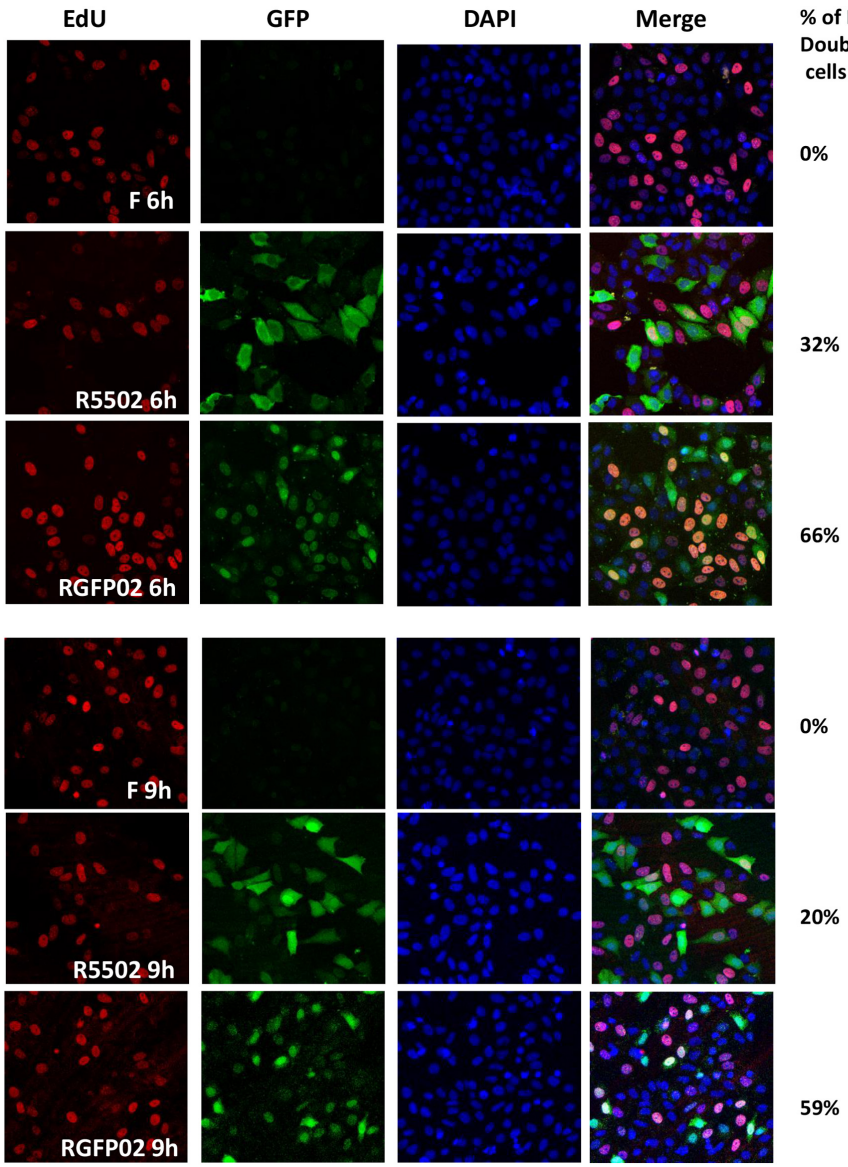

FIGURE 3 | VP55 recombinant virus (R5502) displays defective viral DNA synthesis ability. Serum-starved HEp-2 cells were exposed to 10 PFU of HSV-1(F), R5502, or RGFP02 per cell. After 1 h, the inoculum was replaced with fresh medium containing EdU conjugated with Alexa Fluor 647 (red). The cells were fixed at the indicated time points and stained with DAPI (blue, for nuclei). The images were captured and processed using a confocal laser-scanning microscope (magnification, 40×). The EdU- and GFP-positive cells were quantified using the ImageJ software, and the percentage of EdU and GFP double-stained cells in GFP-stained cells were calculated.

consequence of downregulation of H11, H3-3p or H6-5p. HEp-2 cells were transfected with inhibitors of Non-target inhibitor (NT), H3-3p, H6-5p, and H11. After 24 h, the cells were exposed to 0.1 PFU (**Figure 4A**) or 1 PFU (**Figure 4B**) of HSV-1(F) per cell. The cells were harvested at 8 or 24 h after infection and subjected to electrophoresis in denaturing gels, followed by incubation with antibodies against the α gene products ICP0, ICP4, ICP27, ICP22, antibody against ICP8 (a β gene product), or antibodies against the γ gene products VP16, US11, VP22. GAPDH served as a loading control. The densities of corresponding bands were quantified using the ImageJ software and all relative to GAPDH. In summary, at MOI of 0.1, the accumulations of ICP0, ICP4, ICP27, ICP22, Us11, VP22 are decreased by H11 inhibitor relative to NT and inhibitors of H3-3p, H6-5p at 24 h. The accumulations of these viral proteins by inhibitors of H3-3p, H6-5p are not decreased from those relative to NT (**Figure 4A**, lanes 5, 6, 7, 8). At MOI of 1, there are not much difference between treatment of the three inhibitors and NT treatment at 8 and 24 h (**Figure 4B**). So we conclude that the effect of miR-H11 inhibitor to viral replication is more significant at low multiplicity.

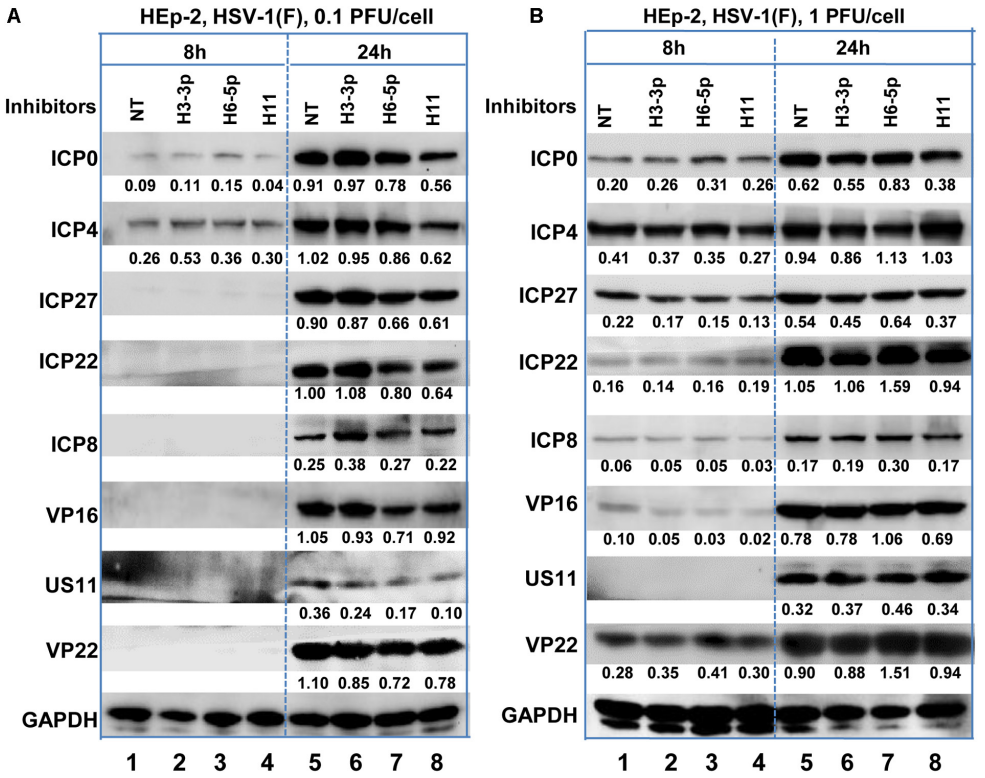

FIGURE 4 | Accumulation of viral protein in HEp-2 cells transfected with viral miRNA inhibitors. Replicate HEp-2 cultures containing 2.5 × 10^5 cells were transfected with 100 nM of non-target (NT), miR-H3-3p, H6-5p, and H11 inhibitors for 24 h, and subsequently exposed to 0.1 **(A)** or 1 PFU **(B)** of HSV-1(F) per cell for 8 and 24 h. The cells were harvested, and the proteins were electrophoretically separated using 10% denaturing gels and incubated with antibodies against ICP0, ICP4, ICP27, ICP22, ICP8, US11, VP16, VP22, or GAPDH.

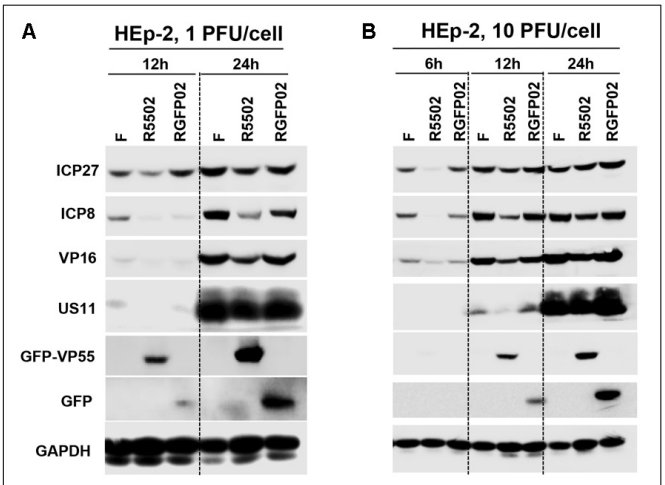

FIGURE 5 | Decreased protein expression following infection with VP55-expressing recombinant virus (R5502). **(A,B)** Accumulation of R5502, RGFP02, and wild-type HSV-1(F) viral proteins in HEp-2 cells. HEp-2 cells were mock-infected or exposed to 1 PFU **(A)** or 10 PFU **(B)** of HSV-1(F), R5502, or RGFP02 per cell in a 12-well plate. The cells were harvested at the indicated time points post infection. The proteins were electrophoretically separated using 10% denaturing gels and incubated with antibodies against ICP27, ICP8, VP16, US11, GFP, or GAPDH.

Properties of the VP55 Plasmid and HSV-1 Expressing the VP55 Recombinant Virus Generated in This Study

VP55 Recombinant Virus (R5502) Showed Defect in Viral Protein Accumulation in HEp-2 Cells

In this series of experiments, replicate cultures of HEp-2 cells were exposed to 1 PFU (**Figure 5A**), or 10 PFU (**Figure 5B**) of HSV-1(F), R5502, or RGFP02 per cell. The cultures were harvested at indicated time points after infection, followed by solubilization, electrophoresis in denaturing gels, and incubation with antibodies against ICP27, ICP8, VP16, US11, GFP, and GAPDH. Of note, ICP27, ICP8, VP16, and US11 represent different kinetic classes of virus replication. GFP is a positive indicator of recombinant virus and GAPDH served as a loading control. The accumulation of ICP27, ICP8 and VP16 from R5502 infected cells at 1 PFU/cell, 12 or 24 h or 10 PFU/cell 6, 12, 24 h post infection are lower than those from either F or RGFP02 infected cells at same time points. The accumulation of US11 from R5502 infected cells at 12 h from 1 or 10 PFU/cell are also lower than what from either F or RGFP02 infected cells, at late infection, there is no difference of US11 accumulation from F, R5502 or RGFP02. There was no difference in viral protein accumulation between HSV-1(F)- and RGFP02-infected cells.

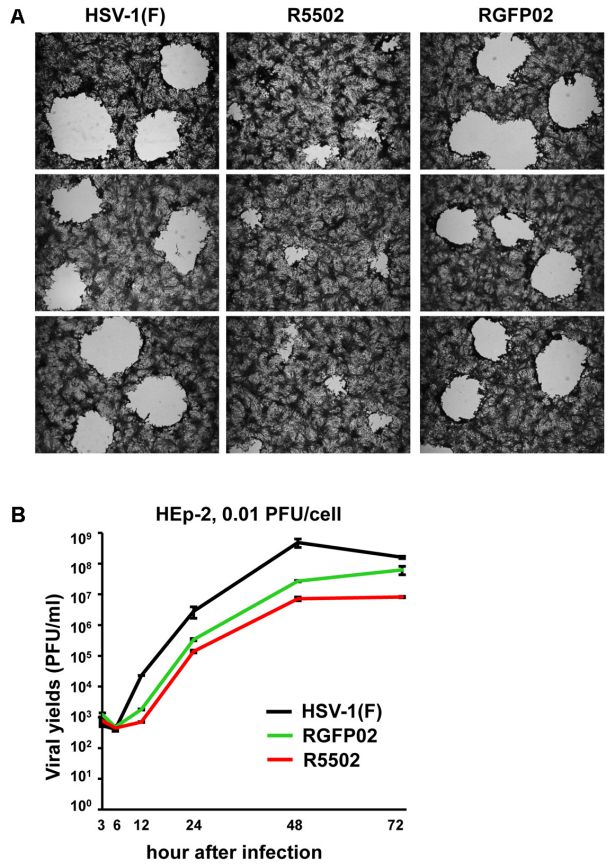

FIGURE 6 | Decreased viral spreading ability and yields of the VP55 recombinant virus (R5502). **(A)** Vero cells grown in six-well plates were exposed to 0.001 PFU of HSV-1(F), R5502, or RGFP02 per cell for 2 h, and subsequently rinsed and overlaid with medium 199V. At 48 h post infection, the cells were fixed with 4% (w/v) paraformaldehyde for 30 min, stained with Giemsa stain for 30 min, and photographed at 5× magnification with the aid of an inverted microscope. **(B)** Viral yields of VP55 recombinant virus (R5502) in HEp-2 cells. HEp-2 cells were exposed to 0.01 PFU of HSV-1(F), RGFP02 or R5502 per cell. After 2 h, the inoculum was replaced with fresh medium. The virus progeny was harvested at he indicated time points and titrated using Vero cells.

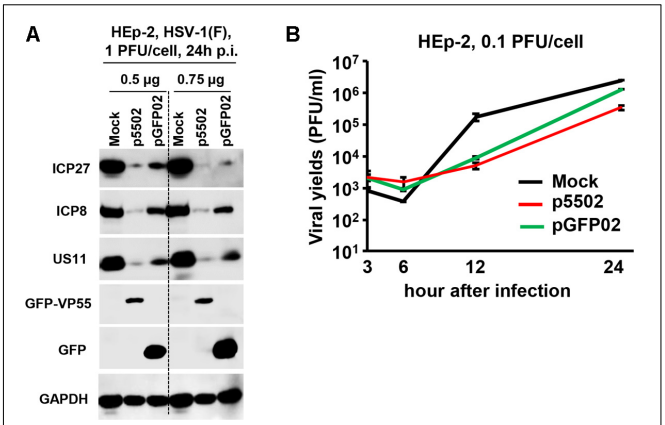

FIGURE 7 | Accumulation of viral proteins and viral yields in cells mock-transfected or transfected with the VP55 expression plasmid (p5502) and control plasmid (pGFP02). **(A)** Viral protein accumulation in HEp-2 cells transfected with p5502 and pGFP02. HEp-2 cells were mock-transfected or transfected with 0.5 or 0.75 μg of p5502 or pGFP02 for 24 h, followed by exposure to 1 PFU of HSV-1(F) per cell in a 12-well-plate. The cells were harvested at 24 h post infection (p.i.). The proteins were electrophoretically separated using 10% denaturing gels and incubated with antibodies against ICP27, ICP8, US11, GFP, or GAPDH. **(B)** Viral yields in p5502- and pGFP02-transfected cells. HEp-2 cells were mock-transfected or transfected with 1.5 μg of p5502 and pGFP02 for 24 h, followed by exposure to 0.1 PFU of HSV-1(F) per cell in six-well plate. After 2 h, the inoculum was replaced with fresh medium. The virus progeny was harvested at the indicated time points and titrated using Vero cells.

Importantly, the VP55-expressing virus R5502 induced a defect in viral protein accumulation.

VP55 Recombinant Virus Showed Limited Spreading Ability in Vero Cells

Vero cells were infected with 0.001 PFU of HSV-1(F), R5502, or RGFP02 per cell. The cultures were fixed and stained with Giemsa stain at 48 h post infection, as described in the "Materials and Methods" section. Representative plaques photographed at the same magnification are shown in **Figure 6A**. The size of plaques formed by R5502 in Vero cells was markedly smaller than that of plaques formed in RGFP02- and HSV-1(F)-infected cells, indicating the spreading of the R5502 virus from cell to cell was markedly impaired. **Figure 6B** shows the yields of R5502, RGFP02 and HSV-1(F) in HEp-2 cells. In this experiment, HEp-2 cells were exposed to 0.01 PFU per cell, and virus progeny was harvested at the indicated time points and titrated in Vero cells. The results shown in **Figure 6B** indicate that the accumulation of virus in cells infected with R5502 was lower than those obtained from cells infected with RGFP02 and HSV-1(F) at 12, 24, 48, and 72 h post infection even though the accumulation of virus in cells infected with RGFP02 was notably lower than those obtained from cells infected with HSV-1(F). The results suggest that down-regulation of miR-H11 by R5502 negatively affects the replication of HSV-1.

Transient Transfection of VP55 Plasmid Results in Decreased Accumulation of Viral Proteins and Reduced Yields of Virus in HEp-2 Cells

Replicate cultures of HEp-2 cells were transfected with 0.5 or 0.75 μg of p5502 or pGFP02 for 24 h and subsequently exposed to 1 PFU of HSV-1(F) per cell. The cultures were harvested following infection, solubilized, subjected to electrophoresis in denaturing gels, and incubated with antibodies against ICP27, ICP8, and US11 representing different kinetic classes of viral replication. GFP is a positive indicator of plasmid transfection and GAPDH served as a loading control (**Figure 7A**). The results showed that the accumulation of viral proteins in cells transfected with the two different doses of p5502 was lower than that observed in cells transfected with pGFP02 (**Figure 7A**). **Figure 7B** shows the yields of HSV-1(F) in p5502- or pGFP02-transfected HEp-2 cells. In this experiment, HEp-2 cells were mock-transfected or transfected with p5502 and pGFP02 for 24 h, followed by exposure to 0.1 PFU of HSV-1(F) per cell.

The virus progeny was harvested at the indicated time points and titrated using Vero cells. The data showed that at 24 h post-infection, viral yields from p5502-transfected cells were four-fold lower than those from pGFP02 and seven-fold lower than from mock-transfected cells. At 12 h of infection, both pGFP02 and p5502-transfected cells shown highly lower virus yield because of DNA transfection overall interferes HSV replication.

DISCUSSION

Accumulating evidence supports the hypothesis that miRNAs play roles in infection by herpes simplex viruses. This evidence suggests a model of infection, in which the production of virus and its virulent effects are tightly controlled to maximize persistence in the host and population (Umbach et al., 2008; Umbach et al., 2010; Sun and Li, 2012; Du et al., 2015; Huang et al., 2019).

Backes et al. reported that the vaccinia virus exploits the cellular miRNA pathway (Backes et al., 2012). Furthermore, they discovered that VP55 is both necessary and sufficient for miRNA polyadenylation to mediate the degradation of miRNAs in mammalian cells. This is achieved by adding non-templated adenosines specifically to the miRNAs associated with the RISC. We wished to determine the global role of HSV-1 viral miRNAs in the cellular response to viral infection. For this purpose, we generated a plasmid expressing VP55 and HSV-1 expressing the VP55 recombinant virus to rapidly eliminate viral miRNA populations. The miRNAs expressed by HSV-1 located in the RISC are miR-H1 to miR-H8 and H11 (Flores et al., 2014). Surprisingly H11, H3-3p, and H6-5p were the only three miRNAs reduced among all the viral miRNAs. Notably, the amount of H11 was reduced by 540-fold, confirming from a different angle that it is truly located in the RISC.

Unlike the other reported 29 miRNAs of HSV-1, the structure of the miR-H11 precursor is unique. This precursor is self-complementary. Its 65 nucleotides at the 5′ terminus are complementary to the 65 nucleotides at its 3′ terminus, and H11 lies entirely within the OriL (Jurak et al., 2014; Du et al., 2015). The HSV genome contains three OriL: OriL is present once in UL and OriS is present twice in the repeated C region. OriL is located between genes encoding replication proteins, ICP8 (UL29), and the catalytic subunit of polymerase (Weller and Coen, 2012). Therefore, it is relatively difficult to investigate the function of H11 through mutation without affecting the OriL to produce recombinant virus. The introduction of VP55 into HSV-1 provides us with an alternative approach. We detected markedly lower viral DNA synthesis versus wild-type HSV-1, as a result of low accumulation of viral proteins and low viral yield. We concluded that degradation of the miRNA H11 by VP55 attenuates viral replication.

In this study, we showed that the yields of viral proteins were reduced in cells infected with R5502 and the VP55-expressing HSV-1. Finally, the results suggested that, in cell cultures infected with R5502, the size of plaques-a direct measure of yields and cell-to-cell viral spreading-is also diminished. Early studies indicated that HSV-1 mutants lacking one or two origins are competent for lytic replication in cell culture (Polvino-Bodnar et al., 1987; Igarashi et al., 1993). OriL plays a role in *in vivo* replication or pathogenesis (Balliet and Schaffer, 2006). However, in this study, degradation of miRNA H11 by VP55 attenuated viral replication and restricted viral spreading from cell cultures. Thus, it is reasonable to suggest that the mutations in OriL altering pathogenicity exert their influence through miR-H11.

miR-H11 is homologous to one of the HSV-2 miRNA prediction termed T-4 by Cui et al. (2006) and Jurak et al. (2010). With regard to potential targets H11 to host cell factors, it cannot be excluded that the attenuation of viral replication through degradation of miR-H11 is attributed to the upregulation of host antiviral genes or downregulation of cellular genes which support viral replication. Lastly, degradation of unknown host miRNAs by VP55 may also contribute to the suppression of viral replication.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the SRA repository. The SRA accession number is PRJNA613860 (https://www.ncbi.nlm.nih.gov/sra/PRJNA613860).

AUTHOR CONTRIBUTIONS

XC and GZ designed the study. WZ, XZ, and LW performed the research. WZ, XZ, XC, and GZ analyzed the data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00717/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Epstein-Barr Virus Early Protein BFRF1 Suppresses IFN-β Activity by Inhibiting the Activation of IRF3

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Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis that is closely associated with several human malignant diseases, while type I interferon (IFN-I) plays an important role against EBV infection. As we all know, EBV can encode some proteins to inhibit the production of IFN-I, but it's not clear whether other proteins also take part in this progress. EBV early lytic protein BFRF1 is shown to be involved in viral maturation, however, whether BFRF1 participates in the host innate immune response is still not well known. In this study, we found BFRF1 could down-regulate sendai virus-induced IFN-β promoter activity and mRNA expression of IFN-β and ISG54 during BFRF1 plasmid transfection and EBV lytic infection, but BFRF1 could not affect the promoter activity of NF-κB or IRF7. Specifically, BFRF1 could co-localize and interact with IKKi. Although BFRF1 did not interfere the interaction between IKKi and IRF3, it could block the kinase activity of IKKi, which finally inhibited the phosphorylation, dimerization, and nuclear translocation of IRF3. Taken together, BFRF1 may play a critical role in disrupting the host innate immunity by suppressing IFN-β activity during EBV lytic cycle.

Keywords: innate immunity, EBV BFRF1, IRF3, IFN-β, ISG54

INTRODUCTION

Innate immunity is the first line of host conservative and rapid defense against pathogen invasion, of which type I interferon (IFN-I) plays an important role in the antiviral immune response (1). Along with viral infection, the pattern recognition receptors such as toll-like receptors (TLRs) (2), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (3, 4), can recognize viral pathogen-associated molecular patterns (PAMPs), including genomic DNA, double-stranded RNA (dsRNA) with 5′-triphosphate end, single-stranded RNA (ssRNA), and viral proteins. RNA helicases retinoic acid inducible gene 1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) are the most vital RLRs and are reported to exert essential roles in the detection of intracellular dsRNA, which signal through IFN promoter stimulator 1 (IPS-1) to activate the kinases TANK-binding kinase 1 (TBK1) and inducible

IκB kinase (IKKi; also known as inhibitor of κB kinase ϵ, IKKϵ), to finally phosphorylate IFN regulatory factor 3 (IRF3), this promotes the nuclear translocation of IRF3 and subsequent induction of IFN-I, proinflammatory cytokines and series interferon-stimulated genes (ISGs) (5–8).

Epstein-Barr virus (EBV), also called human herpesvirus 4 (HHV-4), is associated with the development of a wide spectrum of B-cell lymph proliferative disorders, such as Burkitt's lymphoma (BL), post transplant lymph proliferative disorder (PTLD), Hodgkin and non-Hodgkin lymphomas, as well as epithelial cancers (including nasopharyngeal carcinoma [NPC] and some forms of gastric carcinoma) (9). It's shown that many EBV-encoded gene products are involved in the innate immunity, and some of which can stimulate the production of IFN-I. EBV-encoded small nuclear RNA 1 (EBER1) and EBER2 act as ligand of RIG-I to activate IRF3 (10, 11). The C-terminal activation region (CTAR) of LMP1 also can activate NF-κB and IRF7 upon superinfection (12). Moreover, EBNA2 can stimulate IFN-β expression and ISGF3 activity in BL cell lines (13). However, some EBV-encoded gene products are demonstrated to inhibit the production of IFN-β. BGLF4 interplays with IRF3 to abolish its activity in reactivated EBV-positive cells (14). BZLF1 interacts with IRF7 to inhibit its activity (15). BRLF1 reduces the expression of IRF3 and IRF7, thereby inhibiting IFN-β and promoting viral replication (16). LMP2A and LMP2B abrogate IFN-β signaling cascade by promoting the circulation of I and II IFN receptors IFNAR and IFNGR (17). Furthermore, EBV-encoded RNA miR-BART6-3p also inhibits EBV-triggered IFN-β response and facilitates EBV infection through targeting the 3′UTR of RIG-I mRNA (18).

The herpesviral UL34 family contains herpes simplex virus 1 (HSV-1) UL34, varicella-zoster virus (VZV) ORF24, murine cytomegalovirus (MCMV) M50, Kaposi's sarcoma-associated herpesvirus (KSHV) p29 and EBV BFRF1, etc. Previous studies have shown that the nuclear membrane targeted type II membrane protein HSV-1 UL34 can interact with UL31 to form the heterodimer-nuclear egress complex (NEC), and absence of any one of them will prevent the nuclear egress of viral nucleocapsid (19, 20) and primary envelope (21–23). MCMV M50 and the M53 (homologous to HSV-1 UL31) also form a complex to help the virus nuclear export (24). Besides, KSHV p29 interplays with p33 (homologous to HSV-1 UL31) to co-localize at the nuclear membrane, and p29 is responsible for the hyperphosphorylation and delocalization protein of emerin, which is essential for the maturation of viral nucleocapsid (25).

BFRF1 is an EBV-encoded early lytic protein (26), which can regulate multiple viral and cellular functions, including viral maturation, BFLF2 nuclear membrane targeting, lamin B1 binding, recruitment of ESCRT machinery, and cytoplasmic vesicles formation (27–30). BFRF1 and BFLF2 together can form NEC, which is involved in the early step of EBV nuclear egress. Nevertheless, it's a wonder whether BFRF1 also plays a regulatory role in the host innate immunity. In the present study, we found that BFRF1 inhibited the IFN-β production through its interaction with IKKi to restrain the kinase activity of IKKi and suppressing the activation of IRF3 during BFRF1 plasmid transfection and EBV lytic infection.

MATERIALS AND METHODS

Virus and Cells

Sendai virus (SeV) was propagated in chicken embryo and titered in our lab. HeLa, COS-7, and human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified MEM (DMEM, Gibco-BRL) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco-BRL) at the temperature of 37°C in a humidified incubator with 5% CO_2. Hone1-EBV cells (kindly provided by Prof. Sai Wah Tsao, University of Hong Kong, Hong Kong, China) are EBV positive nasopharyngeal carcinoma cell lines that can be reactivated by a sequence-specific DNA-binding protein, BZLF1 [also called Z, Zebra or EB1, encoded by immediate-early *BZLF1* gene (31, 32)].

Antibodies

Mouse anti-Flag (DYKDDDDK), anti-Myc, and anti-hemagglutinin (HA) monoclonal antibodies (mAbs) were obtained from ABmart. Rabbit anti-Flag mAb and anti-IRF3 polyclonal antibody (pAb) were purchased from Proteintech. Rabbit anti-IKKi and anti-β-actin pAbs were provided by ABclonal. Cy5-conjugated goat anti-rabbit IgG, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, and RBITC-conjugated goat anti-rabbit IgG were bought from BBI Life Sciences. Rabbit phospho-IRF3 (ser396) mAb, alkaline phosphatase (AP) conjugated goat anti-mouse IgG, and goat anti-rabbit IgG were obtained from Cell Signaling Technology.

Plasmids Construction

To construct Flag-tagged BFRF1 expression plasmid, the open reading frame of *BFRF1* was polymerase chain reaction (PCR) amplified from the bacterial artificial chromosome (BAC) DNA of B95-8 strain of EBV (174-kb BAC) (33), with forward primer 5′-TTA AGC TTC CGA ATT CAT GGC GAG CCC GGA AGA GAG and reverse primer 5′-TTG CGG CCG CAG GAT CCA AGG TCC ACC TCA GAA ACA TCA G. Then, the purified PCR product was digested with *Eco*RI and *Bam*HI and inserted into the corresponding digested Flag vector (regenerated from pFlag-CMV-2, Sigma) to yield pBFRF1-Flag, as described previously (34–37). pBFRF1-Myc, pIKKi-HA, pBZLF1-HA (Zta-HA), and pBGLF4-HA were also constructed with similar methods, using pMyc-N1 or pHA-N1 vector (regenerated from pEYFP-N1, Clontech). Besides, one pair of oligonucleotide sequences 5′-GGG TCT CTC AAC GGA TGT TGA and 5′-CTC AAC TCA CGT GTC TAG TGT C (38–44) was inserted into the good RNAi product of Oligoengine pSuper-retro-puro (Oligoengine) that can effectively remove off-target of the target gene, to construct RNA interference expression plasmids pSuper-shBFRF1-retro-puro and pSuper-shRandom-retro-puro [a good off-target control (45–48)], respectively. Other gift plasmids were provided by Drs. John Hiscott (IFN-β-Luc) (49), Rongtuan Lin (ISRE-Luc and pIKKi-Flag) (50), Stephan Ludwig (IRF3-Luc) (51), M. Pitha (IRF7-Luc) (52), Takashi Fujita (pRIG-IN-Flag) (53), Takemasa Sakaguchi (pIRF3-HA) (54), Yi-Ling Lin (pIRF3-Flag, pIRF3/5D-Flag, and pIRF7/6D-Flag) (55), Zhengli Shi (pRL-TK and NF-κB-Luc), Chunfu

Zheng (pTBK1-Myc and pRIG-I-Flag), and Jun Cui (pMAVS-Flag, pIKKi-Myc, pTRAF3-Flag, and pTBK1-Flag).

Indirect Immunofluorescent Assays (IFA)

The IFA was carried out as described previously (41, 56–60). In brief, a 14 mm circle microscope cover glass (NEST) was placed in 24-well plate (Corning), then COS-7 or HeLa cells were seeded overnight to 80% confluence and transfected with the indicated plasmids by polyethylenimine (PEI) according to the manufacturer's instructions. At 24 h post-transfection, cells were mock-treated or treated with SeV (100 hemagglutination units [HAU]/ml) for 16 h, then cells were washed three times with PBS and fixed with 4% paraformaldehyde (Beyotime Biotechnology) for 30 min at 37°C, and incubated in 0.1% Triton X-100 (Beyotime Biotechnology) for 10 min. After that, the cells were washed three times with PBS and blocked with 5% BSA for 1 h at 37°C, followed by incubation with primary Abs (anti-Flag, anti-HA, anti-IRF3, or anti-Myc) for 12 h at 4°C, subsequently cells were washed three times with TBST, and incubated with second Ab FITC-conjugated goat anti-mouse IgG, RBITC-conjugated goat anti-rabbit IgG, or Cy5-conjugated goat anti-rabbit IgG for 1 h. Finally, cells were counterstained with Hoechst to visualize the nuclear DNA for 5 to 10 min. The microscope cover glass place microscope slides (biosharp) were obtained with anti-fluorescence quenching reagent (Biosharp) and then fixed with nail polish, and images were eventually captured with 630× of confocal microscope (Leica SP8, 81-933). All scale bars indicate 10 μm.

Dual-Luciferase Reporter (DLR) Assays

The DLR assays were performed as described previously (61). HEK293T cells were seeded on 24-well plate at a density of 1×10^5 cells per well overnight, then cells were co-transfected with 100 ng of promoter reporter expression plasmid, 10 ng of pRL-TK control plasmid and the indicated amounts of expression plasmid. At 24 h post-transfection, cells were mock-infected or infected with 100 HAU/ml SeV for 16 h, followed by washing two times with PBS. Then, cell lysates were collected and luciferase activity was assessed using a luciferase assay kit (Promega). Finally, the data for DLR were detected by Glomax Discover. Data were normalized for transfection efficiency by detecting renilla luciferase activity and firefly luciferase activity, and results were demonstrated as the ratio between firefly and Renilla luciferase. Data were presented as means ± standard deviations (SD) from three independent experiments.

RNA Isolation and Real-Time Quantitative PCR (RT-qPCR)

HEK293T cells seeded on six-well plate (Corning) overnight to 80% confluence were transfected with the indicated plasmids, or Hone1-EBV cells were transfected with the BZLF1 expression plasmid to induce the lytic infection of EBV. After plasmid transfection or EBV induction, cells were mock-treated or treated with 100 HAU/ml SeV for 16 h, then total RNA was extracted with TRIzol reagent (Invitrogen) for reverse transcription to cDNA with RT reagent (YEASEN). The acquired cDNA was used as a template for RT-qPCR, to detect the mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), human IFN-α, IFN-β, and ISG54, with qPCR reagent (YEASEN) using qPCR instrument (BIO-RAD, CFX96). Primers used for GAPDH (forward primer 5′-AGG TCG GTG TGA ACG GAT TTG and reverse primer 5′-TGT AGA CCA TGT AGT TGA GGT CA), IFN-β (forward primer 5′-ATG ACC AAC AAG TGT CTC CTC C and reverse primer 5′-GGA ATC CAA GCA AGT TGT AGC TC), and ISG54 (forward primer 5′-GGA GGG AGA AAA CTC CTT GGA and reverse primer 5′-GGC CAG TAG GTT GCA CAT TGT) were referred to the study of Bing Tian (62). Primers 5′-CAG AGT CAC CCA TCT CAG C (forward primer) and 5′-ATT TGT GCC AGG AGC ATC (reverse primer) were designed to detect the mRNA of IFN-α.

Co-Immunoprecipitation Assays and Western Blot Analysis

The co-immunoprecipitation (Co-IP) and western blot (WB) assays were carried out as described previously (59, 60, 63–69). In brief, HEK293T cells were seeded on 10 cm petri-dish (Corning) and incubated to ~80% confluence, then cells were co-transfected with 20 μg of plasmid combinations tagged with Myc, Flag, or HA. At 24 h post-transfection, cells were harvested and lysed on ice with 800–1,000 μl RIPA lysis buffer (Beyotime Biotechnology) for 30 min. After that, cell lysates were divided into two parts, 10% lysates were directly prepared as the lysates sample, and 90% lysates were incubated with the indicated Ab (anti-Flag or anti-HA) or nonspecific control mouse antibody (IgG) at 4°C for 6 to 12 h, then the antibody-containing lysates were incubated with 50 μl slurry of protein A/G PLUS-Agarose (Santa Cruz) at 4°C overnight. The bead complex was washed at least three times with 1 ml of PBS. Finally, cell lysates and bead protein complex were subjected to WB analysis to detect the potential interaction of virus-host proteins. The original WB results were shown in the section of **Supplementary Material**.

Native PAGE

Native PAGE was carried out as described previously (70). HEK293T cells were seeded on six-well plate overnight to 70% confluence, then cells were transfected with the indicated plasmids. At 24 h post-transfection, cells were mock-treated or treated with 100 HAU/ml SeV for 8 or 16 h, subsequently cells were harvested and lysed with weak RIPA lysis buffer (Beyotime Biotechnology) at 4°C for 30 min. Gels were pre-run with 25 mM Tris and 192 mM glycine (pH 8.4) with 2% deoxycholate (DOC) in a cathode chamber for 30 min at 75 V. Samples in native sample buffer (1 M Tris-Hcl [pH 6.8], 15% glycerol, and 2% bromophenol blue) were then size fractionated by electrophoresis for 120 min at 75 V and transferred to nitrocellulose membrane (BBI Life Sciences) for WB analysis. For analyzing the BFRF1 inhibitory effect of IRF3 dimerization during EBV lytic infection, Hone1-EBV cells were co-transfected with the BZLF1 expression plasmid and pSuper-shBFRF1-retro-puro expression plasmid or pSuper-shRandom-retro-puro expression plasmid, then other experimental procedures were performed as described above.

Statistical Analysis

All data were analyzed with Student's T tests in GraphPad Prism 5 software. Here, ns indicates not significant; * indicates *P* value <0.05; ** indicates *P* value <0.01; *** indicates *P* value <0.001; and **** indicates *P* value <0.0001. A *P* value <0.05 was considered significant.

RESULTS

BFRF1 Suppresses SeV-Mediated IFN-β Transcriptional Activation

IFN-α/β play an essential role in antiviral innate immunity (71, 72). Here, to investigate whether BFRF1 can regulate IFN-β transcriptional activity, expression plasmid BFRF1-Flag, BGLF4-HA, or vector was co-transfected with reporter genes IFN-β-Luc and pRL-TK into HEK293T cells. At 24 h post-transfection, cells were treated with 100 HAU/ml SeV for 16 h, and DLR assays were performed. As a result, the activity of IFN-β promoter was obviously induced by SeV, which was significantly inhibited by the ectopic expression of BFRF1 (**Figure 1A**), with similar inhibitory effect to the positive control BGLF4 (14). Furthermore, to explore whether BFRF1 regulate IFN-induced gene expression, the activation of interferon-stimulated response element (ISRE) promoter was detected by DLR assays. As shown in **Figure 1B**, the ISRE promoter activity was also inhibited by BFRF1. To validate these results, expression plasmid BFRF1-Flag, BGLF4-HA, or vector was transfected into HEK293T cells to see whether BFRF1 can regulate the mRNA expression of IFN-β and its downstream gene, such as ISG54. The results showed BFRF1 could reduce SeV-induced mRNA expressions of IFN-β and ISG54 when compared to BGLF4 (**Figure 1C**), confirming BFRF1 could suppress SeV-mediated IFN-β transcriptional activation. Besides, BFRF1 also could restrain SeV-mediated IFN-α transcriptional activation (**Figure 1C**).

Knockdown of BFRF1 Enhances IFN-β Transcriptional Activity During EBV Infection

To further confirm the physiological function of BFRF1, the expression of BFRF1 was firstly knocked down in EBV-positive Hone1 cells co-transfected with reporter IFN-β-Luc, BZLF1-HA, and pSuper-shBFRF1-retro-puro expression plasmid or pSuper-shRandom-retro-puro expression plasmid, and DLR assays showed that the SeV-induced IFN-β promoter activity was inhibited after EBV was reactivated by BZLF1, but this inhibition is weakened when knockdown the expression of BFRF1 in EBV-positive Hone1 cells (**Figure 2A**). To further verify this result, RT-qPCR was carried out and showed that the mRNA expressions of IFN-β and ISG54 were reduced after EBV was reactivated, while these mRNA expressions were up-regulated when BFRF1 was knocked down in EBV-positive Hone1 cells (**Figure 2B**). These data indicated that BFRF1 could down-regulate IFN-β and downstream ISG54 during EBV infection.

BFRF1 Restrains the Promoter Activation of IRF3, but Not IRF7 or NF-κB

The transcription activation of IFN-β depends on the synergistic interactions among IRFs, NF-κB, and other transcription factors that bind to distinct regulatory domains of the IFN-β promoter (73). Here, to explore whether the activation of NF-κB and IRFs were inhibited by BFRF1, different concentrations of BFRF1-Flag expression plasmid or vector was co-transfected with reporter genes pRL-TK and NF-κB-Luc, IRF3-Luc or IRF7-Luc into HEK293T cells. At 24 h post-transfection, cells were treated with 100 HAU/ml SeV for 16 h, and DLR assays were performed. As shown in **Figure 3**, the promoter activities of IRF3, IRF7 and NF-κB were obviously induced by SeV infection. However, ectopic expression of BFRF1 could dose-dependent inhibit SeV-mediated IRF3 promoter activity (**Figure 3A**), but not IRF7 (**Figure 3B**) or NF-κB (**Figures 3C, D**) promoter activity. Accordingly, these results demonstrated that BFRF1 significantly inhibited the transcriptional activation of IFN-β through IRF3, but not IRF7 or NF-κB.

BFRF1 Represses IFN-β Promoter Activity at the Level of IKKi

In order to examine at which level BFRF1 inhibits the production of IFN-β, different stimuli were used in HEK293T cells to induce the IFN-β reporter activity. RIG-IN (a constitutively active variant containing only the amino-terminal CARD of RIG-I), IPS-1, TBK1, IKKi, IRF-3/5D (a phosphorylated form of IRF-3), or IRF-7/6D (a phosphorylated form of IRF-7) was overexpressed to analyze the IFN-β reporter activity in the presence of various concentrations of BFRF1 expression plasmid. As results, overexpression of signaling components RIG-IN, IPS-1, TBK1, and IKKi activated the IFN-β promoter, which was significantly inhibited by BFRF1 in a dose-dependent manner (**Figures 4A–D**). However, IRF-3/5D and IRF-7/6D induced IFN-β promoter activity were not affected by BFRF1 (**Figures 4E, F**). Collectively, these results suggested that BFRF1 likely acted at the level of IKKi to inhibit the production of IFN-β.

BFRF1 Co-Localizes and Interacts With IKKi

In order to probe the inhibition mechanism of IFN-β transcriptional activation by BFRF1, IFA and Co-IP assays were carried out to determine whether BFRF1 could co-localize and interact with IKKi. COS-7 cells, with the cytoplasm and nucleus relatively larger than that of HEK293T cells for observing the subcellular localizations of specific proteins in different cell compartments, were transfected with the expression plasmid pIKKi-HA or pBFRF1-Flag, or co-transfected with plasmids combination pIKKi-HA and pBFRF1-Flag, then IFA was performed and showed that BFRF1 and IKKi co-localized at the perinuclear region (**Figure 5A**). Besides, HEK293T cells, a common cell model for analyzing the protein-protein interaction, were transfected with the expression plasmid pBFRF1-Flag, or

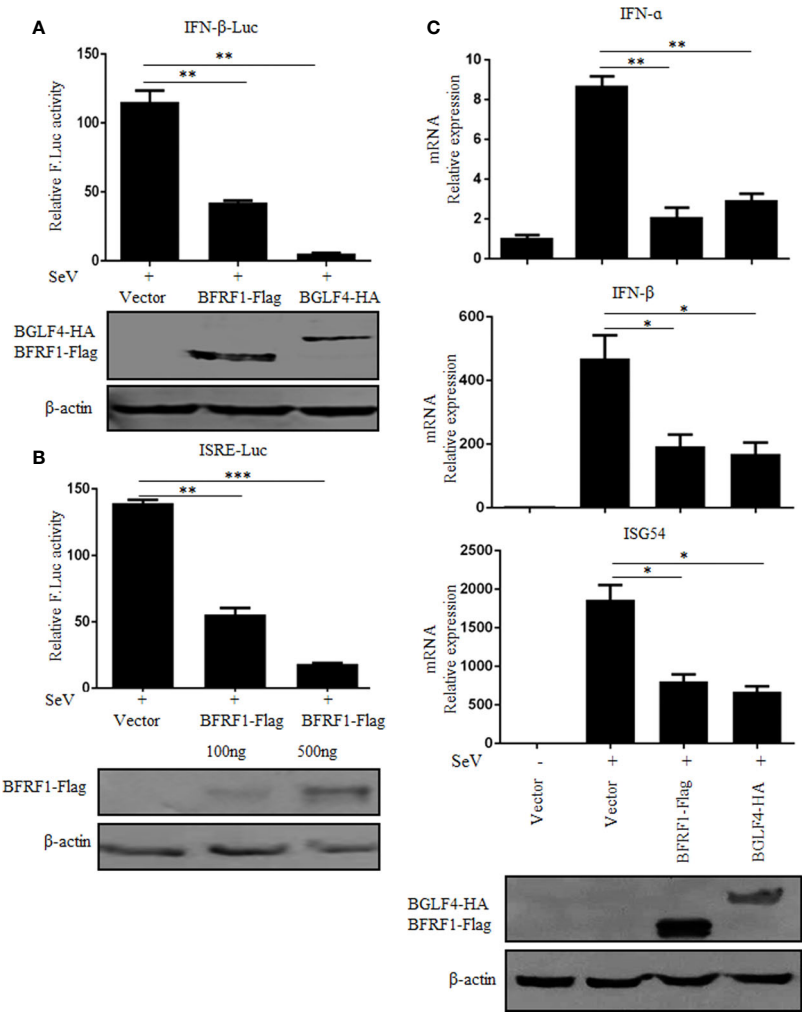

FIGURE 1 | BFRF1 suppresses SeV-mediated activation of IFN-β and ISRE promoters. Vector, BFRF1-Flag, or BGLF4-HA expression plasmid was co-transfected with pRL-TK control plasmid and IFN-β-Luc **(A)** or ISRE-Luc **(B)** reporter plasmid into HEK293T cells. At 24 h post-transfection, cells were infected with 100 HAU/ml of SeV for 16 h. Cell lysates were then collected, and luciferase activity was measured by DLR. The expression of BFRF1 or BGLF4 protein was also detected by WB using anti-Flag or anti-HA mAb, and β-actin was used to verify equal loading of protein in each lane. **(C)** Expression plasmid BFRF1-Flag, BGLF4-HA or vector was transfected into HEK293T cells, at 24 h post-transfection, cells were mock-infected or infected with 100 HAU/ml SeV for 16 h. Cells were then lysed and RNA was extracted for RT-qPCR analysis for IFN-α,IFN-β and ISG54. The expression of BFRF1 or BGLF4 protein was also detected by WB using anti-Flag or anti-HA mAb, and β-actin was used to verify equal loading of protein in each lane. Statistical analysis was performed using student's t test. ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

plasmids combination pIKKi-Myc and pBFRF1-Flag, then cells lysates were immunoprecipitated with anti-Flag mAb or mouse nonspecific IgG, and results demonstrated that BFRF1 interacted with both the overexpressed (**Figure 5B**) and endogenous IKKi (**Figure 5C**).

To investigate whether BFRF1 also can interact with the signal protein(s) of the RLR signal pathway to inhibit the transcriptional activation of IFN-β, HEK293T cells were co-transfected with BFRF1 and RIG-I, IPS-1, TBK1, TRAF3, or IRF3 expression plasmid, then cell lysates were immunoprecipitated with anti-Flag mAb or mouse nonspecific IgG. As results, BFRF1 could not associate with the adaptor protein of RLR signal pathway mentioned above (**Figures 5D–H**). These results revealed that BFRF1 might affect the IFN-β production *via* only interacting with IKKi.

BFRF1 Cannot Inhibit the IKKi and IRF3 Interaction but Impede the Kinase Activity of IKKi

The association of BFRF1 with IKKi raised the possibility that BFRF1 may disturb the interaction between IKKi and IRF3. Alternatively, the binding of BFRF1 to IKKi may act as a pseudosubstrate for IKKi to inhibit its kinase activity. To address these possibilities, HEK293T cells were co-transfected with plasmids combination of pIKKi-HA/pIRF3-Flag or pIKKi-HA/pIRF3-Flag/pBFRF1-Myc, then Co-IP assays were

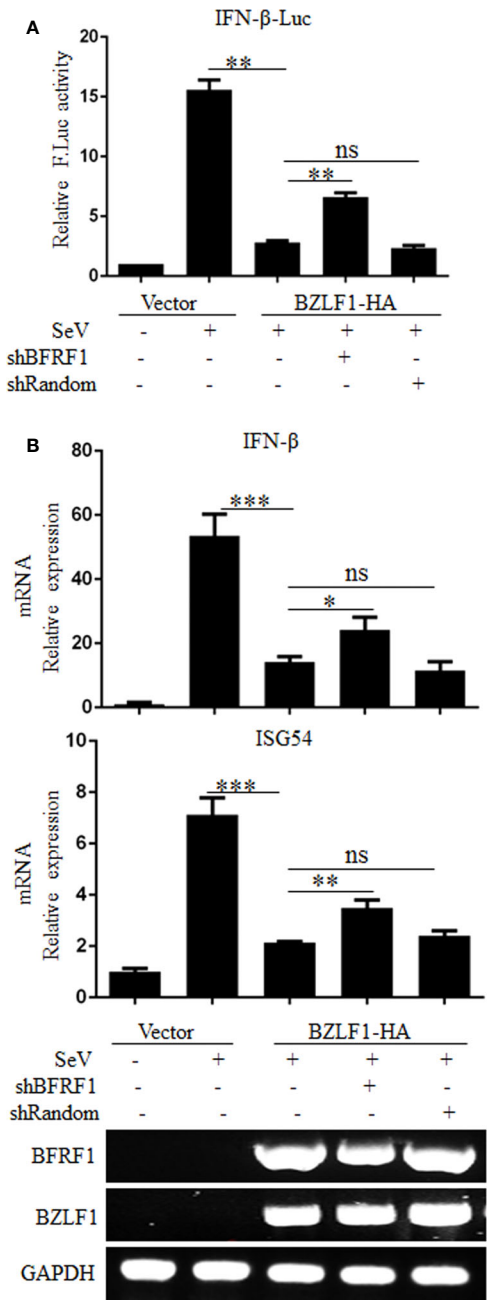

FIGURE 2 | Knockdown of BFRF1 during EBV lytic infection increases the transcriptional activations of IFN-β and ISG. **(A)** Hone1-EBV cells were co-transfected with reporter IFN-β-Luc, BZLF1-HA expression plasmid and pSuper-shBFRF1-retro-puro or pSuper-shRandom-retro-puro expression plasmid. At 24 h post-transfection, cells were mock-infected or infected with 100 HAU/ml SeV for 16 h. Cell lysates were then collected, and luciferase activity was measured by DLR. **(B)** Hone1-EBV cells were co-transfected with BZLF1-HA expression plasmid and pSuper-shBFRF1-retro-puro or pSuper-shRandom-retro-puro expression plasmid. At 24 h post-transfection, cells were mock-infected or infected with 100 HAU/ml SeV for 16 h. Cells were then lysed and RNA was extracted for reverse transcription into cDNA and qPCR analysis for the mRNA levels of IFN-β and ISG54, and RT-PCR analysis for the mRNA levels of BFRF1, BZLF1, and GAPDH. Statistical analysis was performed using Student's t test. ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

carried out, and results found that BFRF1 could not affect IKKi and IRF3 interaction (**Figure 6A**). To further detect whether the BFRF1 and IKKi association influence the kinase activity of IKKi, HEK293T cells were transfected with the expression plasmid of BFRF1-Flag or IKKi-Myc, or co-transfected with plasmids combination pIKKi-Myc/pBFRF1-Flag, and results showed that BFRF1 could inhibit the kinase activity of IKKi, since the IKKi-mediated phosphorylation of IRF3 was restrained (**Figure 6B**). Accordingly, these data disclosed the engagement of BFRF1 could not affect the interaction of IKKi and IRF3 but block the catalytic activity of IKKi.

BFRF1 Inhibits the Activation of IRF3

Generally, IRF3 Ser396 is targeted for phosphorylation following virus infection, which plays an essential role in IRF3 activation (74, 75). Therefore, to dissect whether IRF3 phosphorylation is inhibited by BFRF1, HEK293T cells were transfected with BFRF1-Flag expression plasmid or vector, then cells were mock-treated or treated with 100 HAU/ml SeV for 8 or 16 h, and cells were collected for WB analysis using phospho-IRF3 (Ser396) Ab. As results, SeV infection for 8 or 16 h could induce the accumulation of IRF3 Ser396, while this phosphorylation was significantly inhibited by BFRF1 when SeV infection for 16 h (**Figure 7A**). Since IRF3 dimer formation is a consequence of IRF3 phosphorylation, we continued to test whether BFRF1 could inhibit SeV-induced IRF3 dimerization, and result showed that IRF3 dimerization was also obviously reduced by the expression of BFRF1 when cells were treated with SeV for 16 h (**Figure 7B**). To continue verify the BFRF1 inhibitory effect of IRF3 phosphorylation and dimerization during EBV lytic infection, Hone1-EBV cells were co-transfected with the BZLF1 expression plasmid and pSuper-shBFRF1-retro-puro or pSuper-shRandom-retro-puro expression plasmid. Compared with lane 4 of **Figures 7C, D**, the phosphorylation of IRF3 Ser396 (**Figure 7C**, lane 5) and dimerization of IRF3 (**Figure 7D**, lane 5) could be up-regulated by knocking down the expression of BFRF1 after inducing EBV into lytic infection in Hone1 cells, verifying the experimental results at the transfection level of **Figures 7A, B**. These results demonstrated that BFRF1 could effectively prevent the phosphorylation and dimerization of IRF3 during EBV lytic infection, which was also consistent with the results of **Figures 2A, B**, again confirming EBV lytic infection could inhibit the production of IFN-β and downstream ISG, and BFRF1 played a certain important role in this process.

Upon phosphorylation, IRF3 dimerizes and translocates into the nucleus, where it can form a complex with CBP/p300 and act as a transcriptional factor. The holocomplex then binds to IRF3 sites in the IFN-β promoter, and eventually activates the transcription of IFN-β (76). As the aforementioned results indicated that BFRF1 repressed the phosphorylation and dimerization of IRF3, we subsequently investigated whether BFRF1 inhibited SeV-induced IRF3 nuclear translocation. HeLa cells were transfected with BFRF1-Myc expression plasmid or vector, then cells were mock-treated or treated with 100 HAU/ml SeV for 16 h, and IFA was performed using confocal microscope. As shown in **Figure 7E** and statistical analysis of the subcellular localization in **Table 1** that is widely

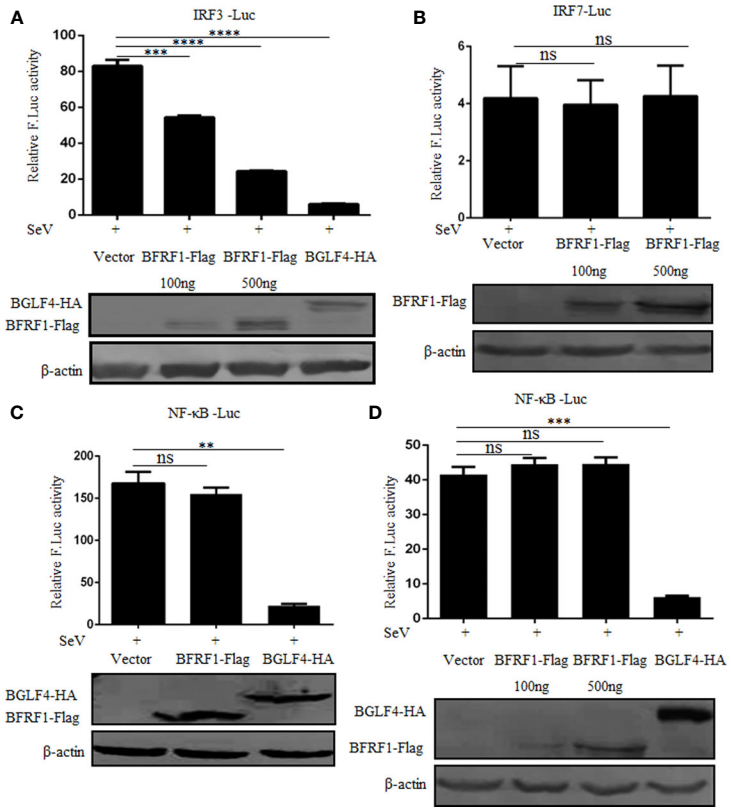

FIGURE 3 | BFRF1 restrains the activation of IRF3 promoter, but not IRF7 or NF-κB promoter. Vector, BFRF1-Flag (100 or 500 ng) or BGLF4-HA (500 ng) expression plasmid was co-transfected with pRL-TK control plasmid and IRF3-Luc **(A)**, IRF7-Luc **(B)**, or NF-κB-Luc **(C, D)** reporter plasmid into HEK293T cells. At 24 h post-transfection, cells were infected with 100 HAU/ml SeV for 16 h. Cell lysates were then collected, and luciferase activity was measured by DLR. The expression of BFRF1 or BGLF4 protein was also detected by WB using anti-Flag or anti-HA mAb, and β-actin was used to verify equal loading of protein in each lane. Statistical analysis was performed using student's t test. ns, not significant; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

applied in many studies (70, 77–80), IRF3 was localized exclusively in the cytoplasm in mock-infected HeLa cells, while most of IRF3 translocated into the nucleus after SeV stimulation. However, IRF3 was restricted in the cytoplasm in BFRF1 expressing cells. These results indicated that SeV-induced IRF3 nuclear translocation was conspicuously inhibited by BFRF1.

DISCUSSION

Innate immunity is an ancient and conserved defense that rapidly responds to pathogen invasion. However, viruses have evolved diverse strategies to overcome the host antiviral responses for their survivals. EBV is the first identified human cancer virus, which has developed a series of elaborate and sophisticated strategies to escape host immune system (12, 81–85). In addition to the EBV-encoded products (BGLF4, BZLF1, BRLF1, LMP2A, LMP2B, and miR-BART6-3p) described in the introduction, EBV-encoded BILF4 (LF2) also can bind to IRF7 to restrain its activity and subsequent IFN promoter activation (86), EBNA2 can inhibit IFN-I signaling by reducing or abolishing the expression of distinct ISGs (87). Besides, EBV-induced host miR-146 can target TRAF6, IRAK1, and IRAK2 to attenuate IFN-I production in macrophages (88).

To further explore whether there are other EBV-encoded proteins can inhibit RLR-mediated IFN-β production, a screening of EBV proteins for their abilities to block SeV-induced activation of IFN-β promoter was performed. Interestingly, EBV early lytic protein BFRF1 was found to significantly inhibit SeV-stimulated IFN-β production. While previous study demonstrates that BFRF1 is essential for the efficient primary viral envelopment and egress (28), it's not known that BFRF1 is involved in the regulation of IFN-I signaling pathway. In the present study, we showed that BFRF1 blocked the activation of IRF3 promoter (but not IRF7 or NF-κB) through specifically targeted the IKK-related kinase IKKi (but not TBK1) and affected its kinase activity but did not alter the interaction of IKKi and IRF3, which finally inhibited RLR-induced phosphorylation, dimerization, and nuclear translocation of IRF3. These results are similar with previous reports that viral proteins can inhibit the RLR pathway at the level of IRF3 upstream but cannot restrain the downstream

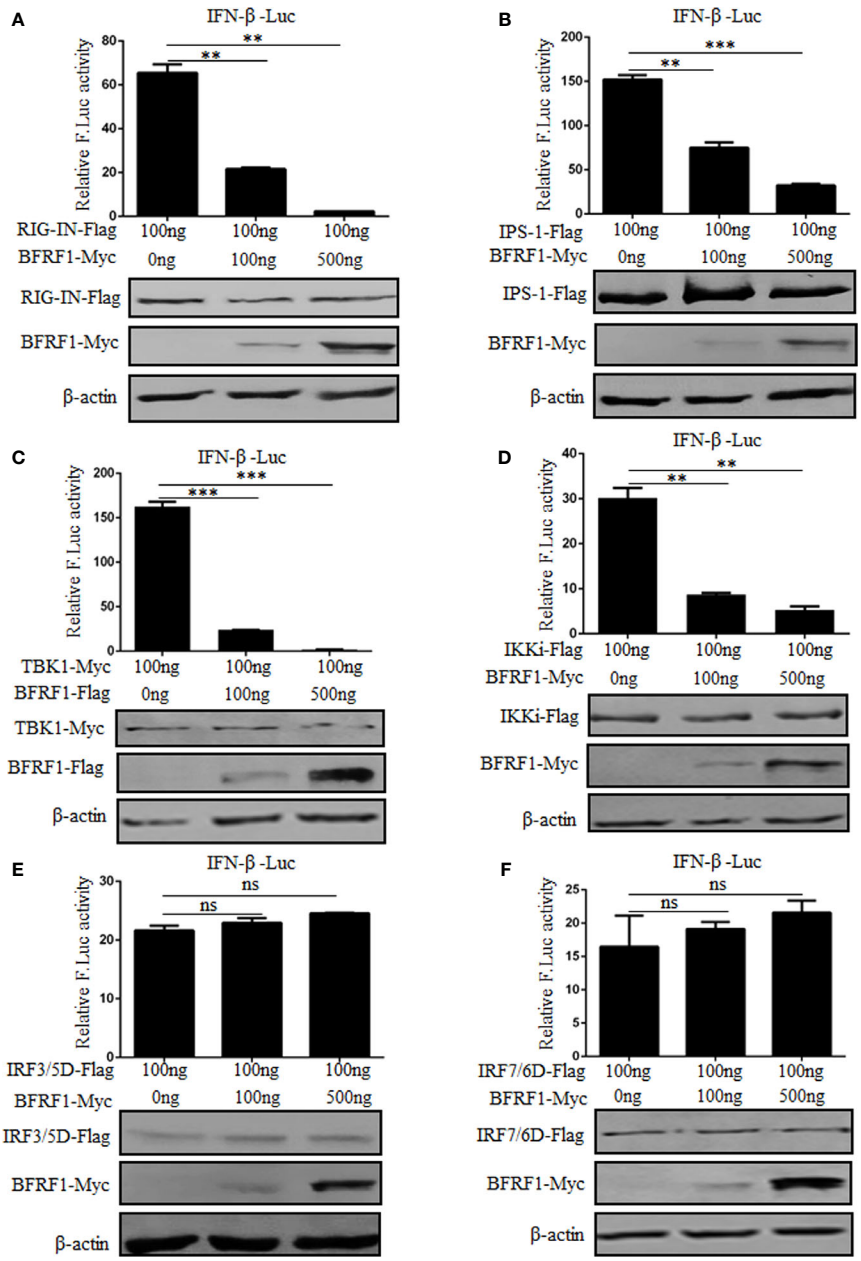

FIGURE 4 | BFRF1 dose-dependent represses IFN-β promoter activity at the level of IKKi. IFN-β-Luc reporter and pRL-TK control plasmid were co-transfected with RIG-IN **(A)**, IPS-1 **(B)**, TBK1 **(C)**, IKKi **(D)**, IRF3/5D **(E)**, or IRF7/6D **(F)** expression plasmid into HEK293T cells, together with the indicated amounts of BFRF1 expression plasmid for 24 h, then luciferase activity was analyzed by DLR. The expressions of related adaptor and viral proteins were also detected by WB using specific tag Abs, and β-actin was used to verify equal loading of protein in each lane. Statistical analysis was performed using Student's t test. ns, not significant; $^{**}P < 0.01$; $^{***}P < 0.001$.

adaptor mediated-promoter activity (89–91). Taken together (as shown in **Figure 8**), these data indicated that BFRF1 abrogated IFN-β production by blocking IRF3 activation, which may be important for viral maturation and nuclear egress.

The result that BFRF1 targets to IKKi but not TBK1 was unpredictable, because TBK1 takes a predominant role in the production of IFN-I in response to dsRNA and viral stimulation (92–94) However, the IFN-I production was not influenced in TBK1-deficient macrophages (93), suggesting IKKi is also essential for the optimal induction of IFN-I. Therefore, the roles of TBK1 and IKKi indicate that IRF3 phosphorylation in cells involves a complicated requiring of both kinases (95). Here, we speculate that BFRF1 may bind to the kinase domain of IKKi and intercept its autocatalytic activity to phosphorylate IRF3, or BFRF1 may sequester IKKi into an inactive complex (95, 96). The interaction of BFRF1 with IKKi may also compete for the

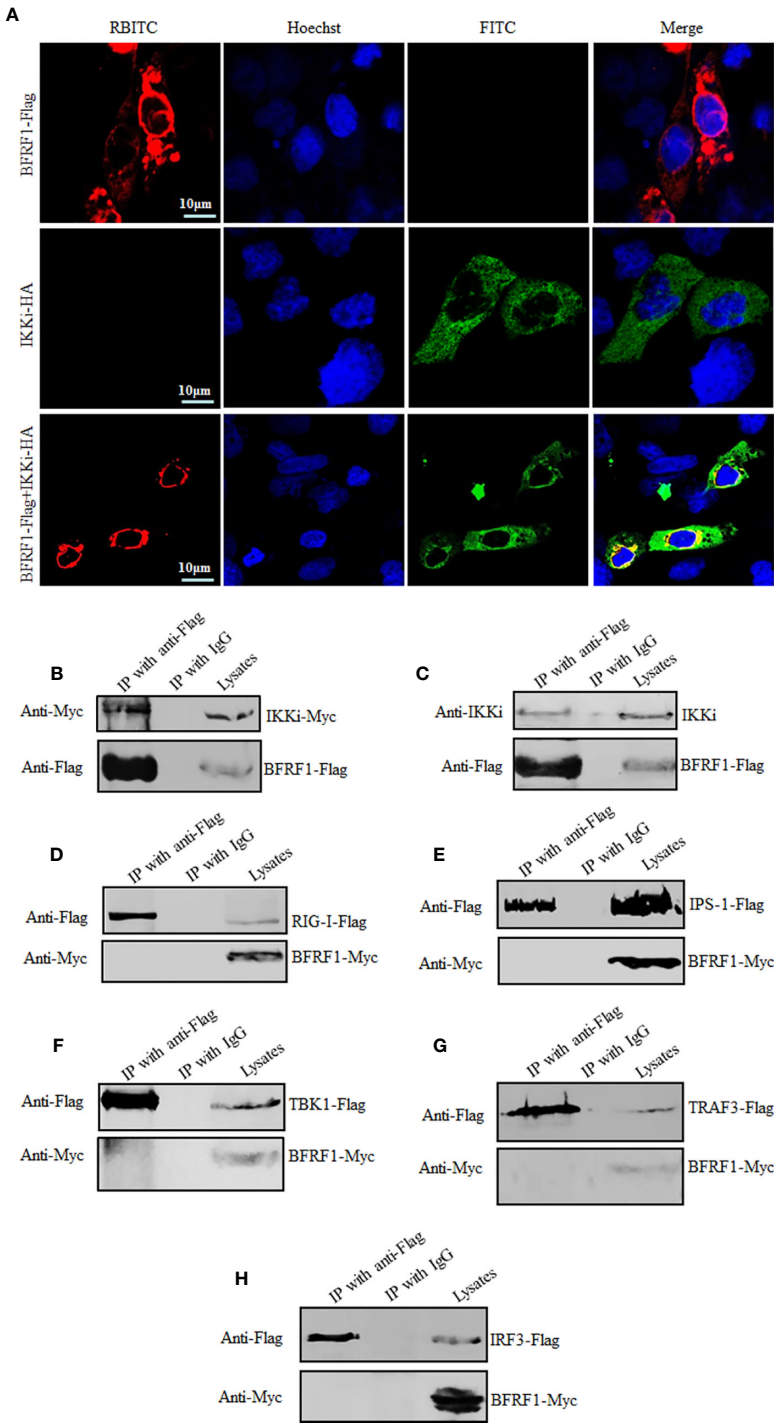

FIGURE 5 | BFRF1 co-localizes and interacts with IKKi. **(A)** COS-7 cells were transfected with expression plasmid pIKKi-HA or BFRF1-Flag, or co-transfected with plasmids combination pIKKi-HA and BFRF1-Flag. At 24 h post-transfection, IFA analysis was performed with primary Abs anti-HA and anti-Flag mAb, and their corresponding fluorescent secondary Abs FITC-conjugated goat anti-mouse IgG (green) and RBITC-conjugated goat anti-rabbit IgG (red), respectively. Cells were counterstained with Hoechst to visualize the nuclear DNA (blue) for 5 to 10 min. Images were obtained by confocal microscopy using a 63× lens objective. All scale bars indicate 10 μm. **(B, C)** HEK293T cells were transfected with plasmid BFRF1-Flag **(C)** or co-transfected with plasmids combination BFRF1-Flag and pIKKi-Myc **(B)**. At 24 h post-transfection, cells were lysed and immunoprecipitated with anti-Flag mAb or mouse nonspecific IgG, then WB analysis was performed using the indicated Abs. **(D–H)** HEK293T cells were co-transfected with plasmids combination of pRIG-I-Flag/pBFRF1-Myc **(D)**, pIPS-1-Flag/pBFRF1-Myc **(E)**, pTBK1-Flag/pBFRF1-Myc **(F)**, pTRAF3-Flag/pBFRF1-Myc **(G)**, or pIRF3-Flag/pBFRF1-Myc **(H)**. At 24 h post-transfection, cells were lysed and immunoprecipitated with anti-Flag mAb or mouse nonspecific IgG, then WB analysis was performed using the indicated Abs.

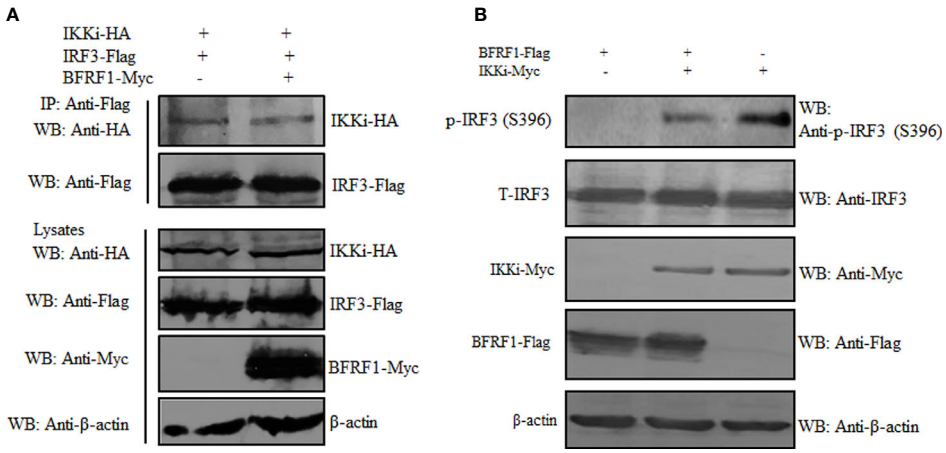

FIGURE 6 | BFRF1 cannot affect the interaction between IKKi and IRF3 but inhibit the kinase activity of IKKi. **(A)** HEK293T cells were co-transfected with plasmids combination of pIKKi-HA/pIRF3-Flag or pIKKi-HA/pIRF3-Flag/pBFRF1-Myc, at 24 h post-transfection, cells were lysed and immunoprecipitated with anti-Flag mAb, then WB analysis was performed using the indicated Abs. **(B)** HEK293T cells was transfected with expression plasmid of BFRF1-Flag or IKKi-Myc, or co-transfected with plasmids combination of pIKKi-Myc/pBFRF1-Flag, at 24 h post-transfection, cells were lysed and WB analysis was performed using the indicated Abs.

association of IKKi to IRF3 (but not IRF7) to inhibit IRF3 (but not IRF7) binding to its corresponding binding site on the IFN-β promoter (97). It is conceivable that BFRF1 interacting with IKKi may also disturb the function of TBK1. After further investigation (**Figure 6**), we confirmed that the association of BFRF1 with IKKi did not disturb the binding of IKKi to IRF3, but BFRF1 could block the catalytic activity of IKKi. Accordingly, the specificity of BFRF1 for IKKi would be favorable for persistent EBV to circumvent the host innate immunity.

IRF3 is an essential transcriptional factor in antiviral process, which is required for the expression of IFN-I and many other genes. Previous works have suggested a dual phosphorylation-dependent mechanism regulates the activation of IRF3. Specifically, Ser 385 and 386 comprise "site 1," and Ser 396, 398, 402, 405, and Thr 404 comprise "site 2" (94, 98). The phosphorylation of both site 1 and site 2 are required for the full activation of IRF3 (99). Upon viral infection, cellular TBK1- and IKKi-mediated phosphorylation of serines 385 and 386 and the serine/threonine cluster between amino acids 396 and 405 of IRF3 lead to its conformational change and activation (100–102). Here, the Ser 396 is essential for IRF3 activation, especially after viral infection. Therefore, we investigated whether the phosphorylation of IRF3 (Ser 396) was inhibited by BFRF1 during ectopic expression and EBV lytic infection, and our results confirmed this speculation. Of course, it does not exclude the possibility that the IRF3 phosphorylation of other sites are also inhibited.

Since IRF3 plays a central role in the innate immune response, it's not surprising many viral proteins can disrupt IRF3 activation. HSV-1 VP24 protein binds IRF3 to prevent TBK1/IRF3 interaction and block the phosphorylation and dimerization of IRF3 during viral infection (103). Encephalomyocarditis virus 3C protein disrupts the TANK-TBK1-IKKi-IRF3 tetramer formation and decreases TBK1- and IKKi-mediated IRF3 phosphorylation and IFN-I production (104). Heartland virus NSs protein interacts with TBK1 and blocks TBK1/IRF3 interaction to constrain the activation of IRF3 (105). Paramyxovirus V protein interacts with IKKi/TBK1 to act as their substrates to inhibit IRF3 phosphorylation and its activation (106). Thrombocytopenia syndrome bunyavirus nonstructural protein NSs can interact with TBK1 to sequester the IKK complex to restrict phosphorylated IRF3 translocates into the nucleus (107). While in this study, BFRF1 was proved to interact with IKKi, but may not RIG-I, IPS-1, TBK1, TRAF3, or IRF3 (since we cannot completely rule out that the antibodies used in this study may not work well in the Co-IP experiments), to abrogate IFN-β production by blocking IRF3 phosphorylation, dimerization, and nuclear translocation.

It's believed that analyzing the transcriptional expression level of BFRF1 in plasmid transfected cells and EBV lytic infected cells induced from EBV latent cells can help us to confirm whether the plasmid transfected BFRF1 has similar inhibitory effect of IFN-β production with that of BFRF1 during EBV infection. Although we did not have EBV-positive Akata cell line (EBV latent B cells), EBV-positive Hone1 cell line was selected as a representative cellular model because it grow better and is suitable for related *in vitro* and *in vivo* experiments (108), which is also widely used in the related studies of EBV lytic infection from latency (109–115). Simultaneously, studies have shown that the expression levels of BFRF1 at different time points in plasmid transfected cells are consistent with that of the EBV lytic infected cells induced from EBV latent cells (116, 117). Therefore, it can be concluded that the inhibitory effect of BFRF1 on IFN-β production during plasmid transfection is not an illusion, and BFRF1 also has similar inhibitory effect on IFN-β during EBV lytic infection, which was confirmed in EBV-positive Hone1 cells of our study (**Figures 2**, **7**).

As mentioned above, BFRF1 is reported to take a very important role in the nucleocapsid release and viral maturation of EBV, and deletion of BFRF1 will lead to a serious decrease in

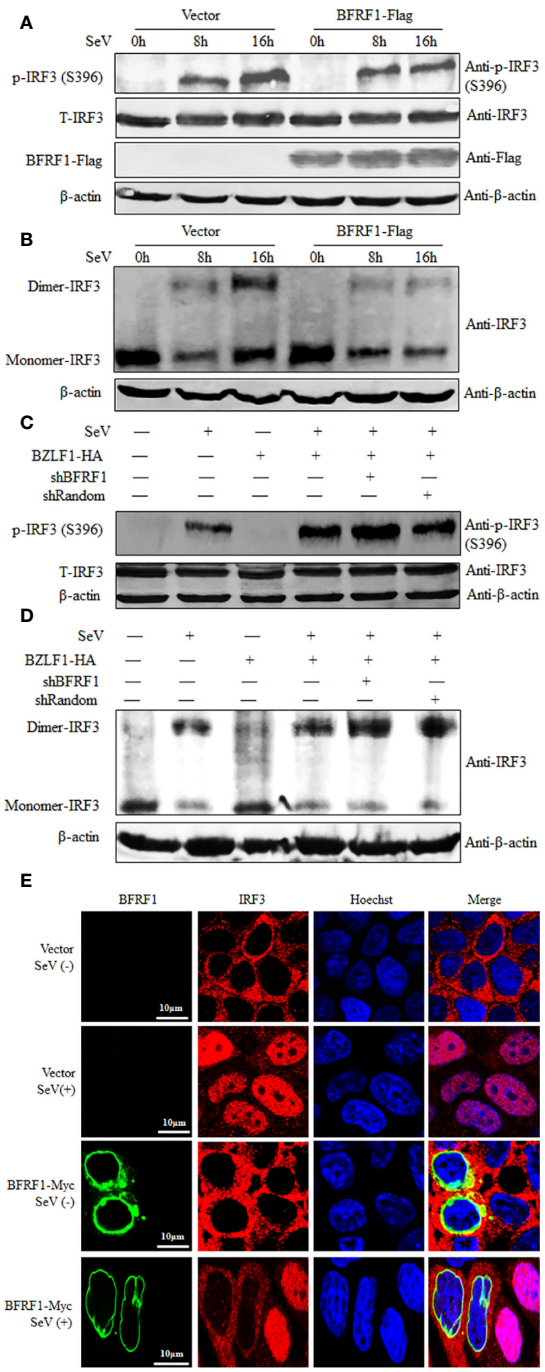

FIGURE 7 | BFRF1 blocks the SeV-induced phosphorylation, dimerization, and nuclear translocation of IRF3. **(A, B)** HEK293T cells were transfected with vector or Flag-tagged BFRF1 expression plasmid. At 24 h post-transfection, cells were mock-infected or infected with 100 HAU/ml SeV for 8 or 16 h. Then, whole-cell extracts were prepared and subjected to IRF3 phosphorylation analysis **(A)** for phosphorylated IRF3 (Ser396), total IRF3, β-actin, BFRF1-Flag, and native PAGE analysis **(B)** for IRF3 dimerization, using related Abs as indicated. **(C, D)** Hone1-EBV cells were co-transfected with BZLF1-HA expression plasmid and pSuper-shBFRF1-retro-puro or pSuper-shRandom-retro-puro expression plasmid. At 24 h post-transfection, cells were mock-infected or infected with 100 HAU/ml SeV for 16 h. Then, whole-cell extracts were prepared and subjected to IRF3 phosphorylation analysis **(C)** and native PAGE analysis **(D)**, as indicated in **(A, B)**, respectively. **(E)** HeLa cells were transfected with vector or Myc-tagged BFRF1 expression plasmid, at 24 h post-transfection, cells were mock-infected or infected with 100 HAU/ml SeV for 16 h. Cells were then probed with primary Abs mouse anti-Myc mAb and rabbit anti-IRF3 pAb, and secondary Abs FITC-conjugated goat anti-mouse IgG (green) and Cy5-conjugated goat anti-rabbit IgG (red), respectively. Cells were counterstained with Hoechst to visualize the nuclear DNA (blue). Images were obtained by confocal microscopy using a 63× lens objective. All scale bars indicate 10 μm. Statistical analysis of the subcellular localization of IRF3 in the absence or presence of BFRF1 was shown in **Table 1**.

TABLE 1 | Subcellular localization of IRF3 in the presence of EBV BFRF1.

Host protein	Cells transfected with vector or BFRF1 expression plasmid	Cells treated with SeV	Total number of cells transfected with vector or BFRF1 expression plasmid	Subcellular localization pattern of IRF3 in cells transfected with vector or BFRF1 expression plasmid	Number of subcellular localization change of IRF3 in cells transfected with vector or BFRF1 expression plasmid	Percentage of subcellular localization change of IRF3 in cells transfected with vector or BFRF1 expression plasmid
IRF3	Vector	–	20	Pan-cytoplasmic	0	0
IRF3	Vector	+	20	Nuclear	1	5
IRF3	BFRF1-Myc	–	20	Pan-cytoplasmic	0	0
IRF3	BFRF1-Myc	+	20	Pan-cytoplasmic or pan-cellular	15	75

HeLa cells were transfected with Myc vector or BFRF1-Myc expression plasmid. At 24 h post-transfection, cells were treated with or without 100 HAU/ml SeV for 16 h. Then, cells were examined for the subcellular localization pattern of IRF3 by IFA.

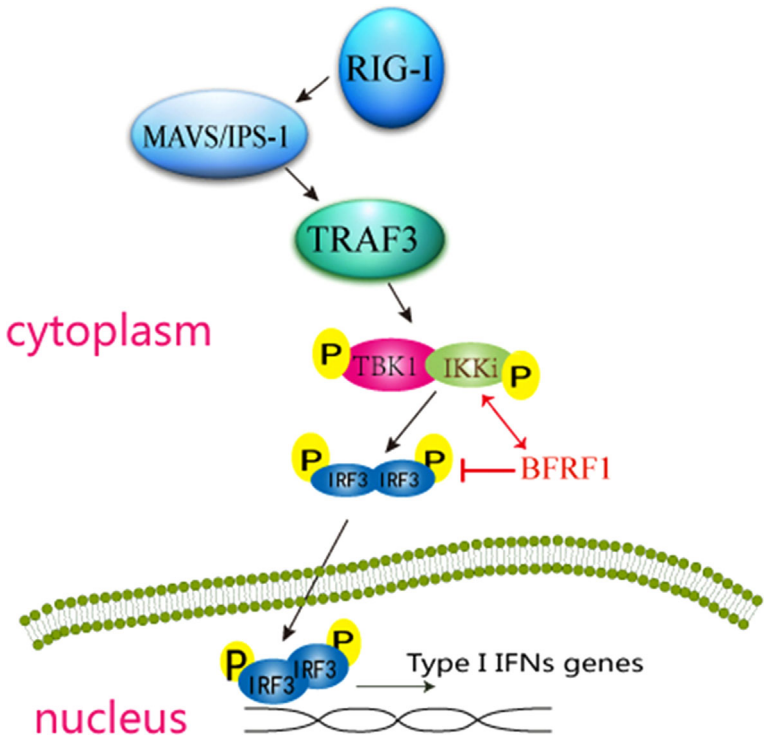

FIGURE 8 | Schematic diagram of EBV early protein BFRF1 inhibiting RLR-mediated IFN-β signaling pathway. RNA helicase RIG-I is activated by upstream stimulation, which signals through IPS-1 to activate the kinases TBK1 and IKKi, then TBK1/IKKi complex phosphorylates IRF3, this leads to the induction of phosphorylation, dimerization, and the nuclear translocation of IRF3 and subsequent IFN-I production. In this study, EBV-encoded early lytic protein BFRF1 is shown to repress IFN-β transcriptional activity by interacting with IKKi to inhibit the phosphorylation, dimerization, and nuclear accumulation of IRF3. The red line (↕) shows that BFRF1 interacts with IKKi, and red line (T) shows that BFRF1 suppresses the phosphorylation, dimerization, and nuclear translocation of IRF3.

viral production (27, 28). In addition, our study found that overexpression of BFRF1 could down-regulate IFN-β production, and knocking down the expression of BFRF1 in EBV lytic infected Hone1 cells could increase IFN-β production, which is speculated inevitably inhibit the proliferation of EBV. Therefore, if the cells are infected with wild-type EBV and BFRF1 knocked-out EBV, or BFRF1 is knocked down in EBV lytic infection cells induced from EBV latent Hone1 cells, it cannot directly make a conclusion that whether the decrease of EBV production is directly caused by the BFRF1 inhibitory effect of IFN-β, because BFRF1 knockout or knockdown itself will limit the release and maturation of EBV nucleocapsid, which finally reduces the viral production.

To date, six EBV-encoded proteins (BZLF1, BGLF4, LMP2A, LMP2B, LF2, and BRLF1) and three EBV-related RNAs (EBNA2, miR-146, and miR-BART-3p) have been implicated in inhibiting the production of IFN-I. In this study, the EBV early lytic protein BFRF1 was demonstrated to be a novel antagonist of IFN-β production, with evidence that BFRF1 regulated the interferon antiviral response by inhibiting IRF3 activation. This finding will lead to a better understanding of the mechanisms employed by EBV to dampen host antiviral signaling and provide information

for the development of therapeutic interventions to modulate EBV pathogenesis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MSC and MLL conceived and designed the experiments. PW, YXD, YJG, ZX, YWL, XWO, LX, MJL, JYZ, BLL, LH, and SYD performed the experiments. MLL, MSC, and PW analyzed the data. TP consulted and advised on the research. PW, MSC, and MLL wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.513383/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Original western blot results of Fig. 1.

SUPPLEMENTARY FIGURE 2 | Original western blot results of Fig. 3.

SUPPLEMENTARY FIGURE 3 | Original western blot results of Fig. 4A-B.

SUPPLEMENTARY FIGURE 4 | Original western blot results of Fig. 4C-D.

SUPPLEMENTARY FIGURE 5 | Original western blot results of Fig. 4E-F.

SUPPLEMENTARY FIGURE 6 | Original western blot results of Fig. 5B-C.

SUPPLEMENTARY FIGURE 7 | Original western blot results of Fig. 5D-H.

SUPPLEMENTARY FIGURE 8 | Original western blot results of Fig. 6A.

SUPPLEMENTARY FIGURE 9 | Original western blot results of Fig. 6B.

SUPPLEMENTARY FIGURE 10 | Original western blot results of Fig. 7A-B.

SUPPLEMENTARY FIGURE 11 | Original western blot results of Fig. 7C-D.

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