

Original Article

Lambda-Interferons Inhibit Herpes Simplex Virus Type 2 Replication in Human Cervical Epithelial Cells by Activating the JAK/STAT Pathway

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SUMMARY: Herpes simplex virus type 2 (HSV-2) is associated with a variety of diseases that are health problems worldwide. Our early study showed that lambda-interferons (IFN- λ s), induced by the activation of the Toll-like receptor 3 and retinoic acid-inducible protein 1 signaling pathways, contribute to inhibition of HSV-2 replication in human cervical epithelial cells. However, anti-HSV-2 mechanisms and specific differences in signaling transduction by different IFN- λ s in human cervical epithelial cells remain unclear. In this study, we demonstrated potent inhibition of HSV-2 replication by IFN- λ s without cytotoxicity. Investigation of the underlying mechanism(s) showed that IFN- λ s induced expression of IFN-stimulated genes (ISGs) and enhanced the expression of several pattern recognition receptors (PRRs). Among the IFN- λ s, IFN- λ 3 induced higher levels of ISG and PRR expression. In addition, IFN- λ s up-regulated a number of genes that encode components of the Janus kinase signal transducers and activators of transcription (JAK/STAT) signaling pathway. Inhibition of the JAK/STAT signaling pathway by a JAK inhibitor abolished IFN- λ -mediated anti-HSV-2 activity and induction of ISGs and PRRs, whereas the induction of ISGs and PRRs by IFN- λ s was not compromised by HSV-2 infection. These findings provide further experimental evidence that IFN- λ s have therapeutic potential for HSV-2 infections.

INTRODUCTION

Interferon lambdas (IFN- λ s) are a class of recently identified members of the IFN family. Collectively, they are considered type III IFNs (1, 2). IFN- λ s functionally resemble type I IFNs (3), inducing type I IFN-like antiviral activity (1, 4–9). IFN- λ -specific signaling occurs through type III IFN receptors, which consist of 2 subunits: IL-10R β and IL-28R α (1,2,10). Unlike type I IFN receptors, which are expressed on most cell types, expression of the IFN- λ receptor (especially subunit IL-28R α) is more cell-specific and has been described mainly on cells of epithelial origin, including epithelial cells, hepatocytes, and fibroblasts (10,11), and some immune cells (10). Thus, IFN- λ s have fewer side effects than type I IFNs (12), and they have the potential to exert an antiviral role in certain cell types (13).

IFN- λ s bind to their own distinctive receptor complex, which activates the intracellular Janus kinase/signal transducers and activators of the transcription (JAK/STAT) signaling pathway, resulting in the phosphorylation of STAT proteins and forming the interferon-stimulated gene factor 3 (ISGF3) complex (1,4,14). This com-

plex binds to the IFN-stimulated response element and induces ISGs, which play important roles in IFN-mediated antiviral activity (1,15). The potential clinical importance of IFN- λ s as novel antiviral therapeutic agents has recently become apparent (1,5, 16–19).

HSV-2 is among the most common human viral pathogens (20,21). Acyclovir and its more current version are still universally used against HSV infection in clinical practice; however, there is no commercial vaccine or specific antiviral treatment for HSV-2. Thus, the need to develop novel efficient therapeutic strategies to counter this virus is urgent. The anti-HSV-2 activity of type I interferons has been observed (22). However, because of the systemic effects of type I interferon, clinical usage is not common. Our group and others have reported that IFN- λ s induced by HSV-2 infection or activation of pattern recognition receptor (PRR) signaling pathways contribute to the inhibition of HSV-2 replication (6,23); however, the anti-viral biological functions of IFN- λ s and mechanisms underlying the effects on HSV-2 replication are not well studied. Furthermore, specific differences in signaling transduction and anti-HSV-2 activity between different members of the IFN- λ family are unclear. In this study, we focused on the effect of IFN- λ s on HSV-2 infection, determined the mechanisms involved in anti-HSV-2 IFN- λ action, and examined the anti-HSV-2 activity of IFN- λ s in human cervical epithelial cells.

MATERIALS AND METHODS

Reagents: Recombinant human IFN- λ 1-3 proteins were purchased from R&D Systems, Inc. (Minneapolis,

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MN, USA). Rabbit antibodies against IFN-stimulated genes (ISGs), including ISG15, ISG56, myxovirus resistance protein A (MxA), 2', 5'-oligoadenylate synthetase 1 and 2 (OAS-1, OAS-2), p-STAT1, and p-STAT3 were purchased from Cell Signaling Technology (Danvers, MA, USA). JAK inhibitor (JAK Inh) I was purchased from EMD Chemicals, Inc. (Gibbstown, NJ, USA) and dissolved in 1% dimethyl sulfoxide (DMSO). An RT² First-Strand Kit and an RT² Profiler JAK/STAT Signaling Pathway PCR Array Kit were purchased from SABiosciences, Qiagen (Hilden, Germany).

Cell culture: Human End1/E6E7 cells (ATCC CRL-2615) is a well differentiated endocervical epithelial cell line immortalized by HPV-16 E6/E7, derived from primary normal endocervical epithelium (24), and that can be grown as a polarized monolayer (25). End1/E6E7 cells have been extensively used as a human endocervical epithelial model (22,23,25,26) and were cultured as described previously (23).

Measuring cell viability: The impact of IFN- λ treatment on the viability of End1/E6E7 cells was analyzed by MTS assay. End1/E6E7 cells were treated with 10, 100, or 1,000 ng/mL of IFN- λ 1, IFN- λ 2, or IFN- λ 3 for 72 h. For the MTS assay, 20 μ L of CellTiter 96 AQueous One Solution Reagent was added to each well of a 96-well plate. Four hours after addition of the reagent, the absorbance at 490 nm was measured using a plate reader (SpectraMax i3, Molecular Devices, Sunnyvale, CA, USA).

Virus infection and detection: The HSV-2 G strain was obtained as a gift from Dr. Qinxue Hu (State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, China) (27). Viruses were propagated and purified from Vero cells using the standard sucrose gradient procedure (28) and then used for infection studies at a multiplicity of infection (MOI) of 0.001. After adding the viruses, plates were incubated at 37°C for 90 min, followed by washing with Dulbecco's modified Eagle medium (DMEM) to remove unattached virus. HSV-2 copy numbers were analyzed by real-time PCR as previously described (23).

IFN- λ treatment: Human End1/E6E7 cells were cultivated in DMEM with or without 10 or 100 ng/mL IFN- λ either before (24 h) or after (24 h) HSV-2 G strain infection. In order to determine the role of the JAK/STAT pathway in IFN- λ action against HSV-2, JAK Inh I or DMSO was added to the cell culture 1 h prior to IFN- λ s treatment.

PCR-based gene expression array: The JAK/STAT signaling pathway-focused gene expression array was performed according to the protocol provided by SABiosciences.

RNA extraction and real-time RT-PCR: The methods for total cellular RNA extraction, reverse transcription-PCR, and real-time PCR for quantification have been described previously (23,29,30). The levels of GAPDH mRNA were used as an endogenous reference to normalize target mRNA quantities.

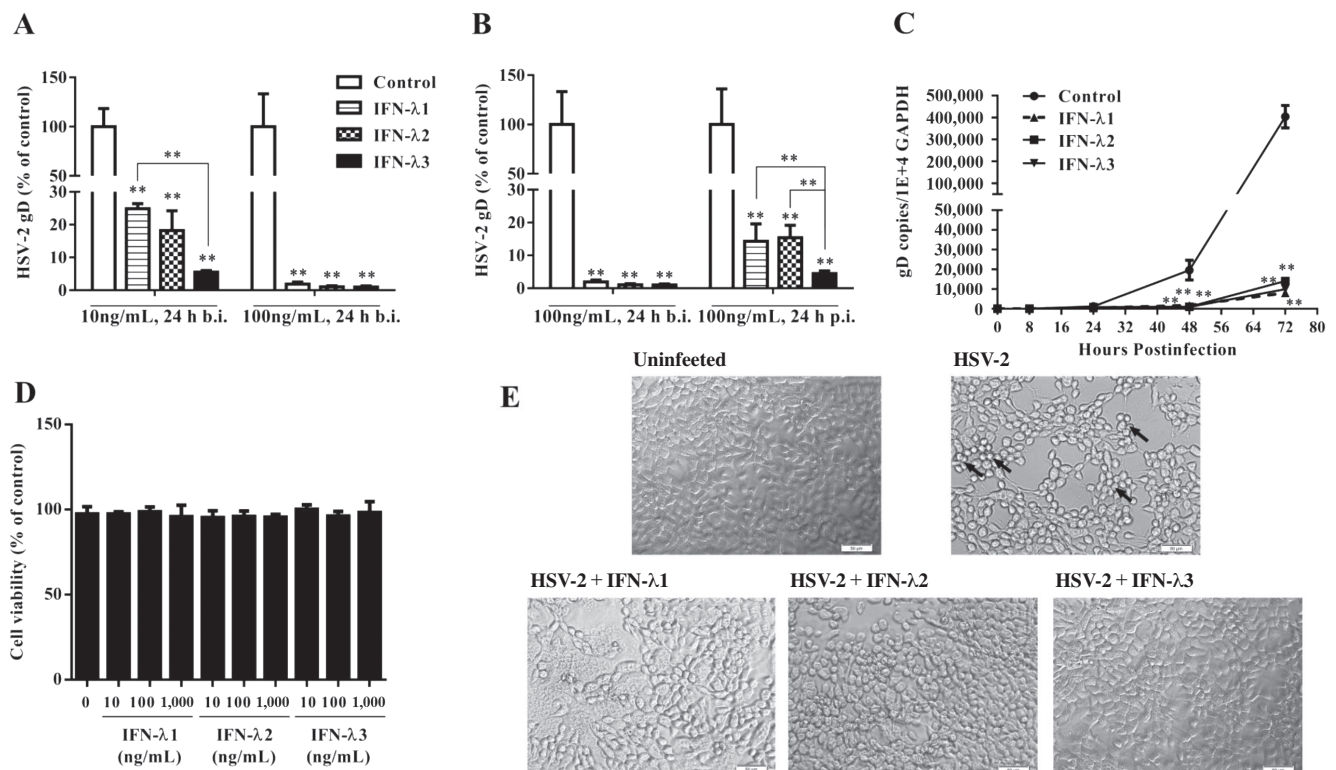


Fig. 1. IFN- λ s inhibit HSV-2 replication in End1/E6E7 cells. A, B: End1/E6E7 cells were treated with or without 10 or 100 ng/mL IFN- λ (IFN- λ 1, IFN- λ 2, or IFN- λ 3) for 24 h before HSV-2 infection (b.i.) or post HSV-2 infection (p.i.). C: End1/E6E7 cells were treated with recombinant IFN- λ 1, IFN- λ 2, or IFN- λ 3 (100 ng/mL) for 24 h before HSV-2 infection (b.i.) (MOI = 0.001). Total DNA was subjected to HSV-2 gD gene expression by real-time PCR. D: End1/E6E7 cells were treated with/without 10 or 100 ng/mL IFN- λ for 72 h. The cells viability was assessed by MTS assay. Data were shown as the absorbance (490 nm) relative to untreated control, which is defined as 1.0. The results were mean \pm SD of triplicate cultures, representative of 3 experiments (IFN- λ vs. control, ** P < 0.01). E: Morphological views of End1/E6E7 cells infected by HSV-2 pretreated with or without IFN- λ (100 ng/mL) observed at 72 h post-infection. Arrows indicate the characteristic cytopathic effect caused by HSV-2.

Western blotting: A western blot analysis was performed, and sample loading was normalized using antibodies against GAPDH. The immunoblots were visualized with a chemiluminescent horseradish peroxidase substrate (Millipore, Billerica, MA, USA), and the stained membranes were analyzed with an LAS-4000 imager (Fujifilm, Tokyo, Japan).

Statistical analysis: Where appropriate, data were expressed as mean \pm standard deviation (SD) from at least 3 independent experiments. Statistical significance was measured by Student's t-test or Bonferroni's multiple comparison test and was defined as $P < 0.05$ or $P < 0.01$.

RESULTS

IFN- λ s inhibit HSV-2 replication: To determine the IFN- λ activity against HSV-2 replication, End1/E6E7 cells were treated with IFN- λ 1, IFN- λ 2, or IFN- λ 3 (10 or 100 ng/mL) under different conditions. End1/E6E7 cells with IFN- λ treatment before or after HSV-2 infection showed a significant concentration-dependent decrease in virus titer (Fig. 1A and B). The antiviral effects of various IFN- λ members were very similar, although IFN- λ 3 was slightly more effective than the others against HSV-2 infection (Fig. 1B). HSV-2 infected End1/E6E7 cells without IFN- λ treatment showed a characteristic morphology (Fig. 1E). As shown in Fig. 1D, IFN- λ was not cytotoxic to End1/E6E7 cells.

IFN- λ s induce IFN- β and antiviral ISG expression: To understand the mechanism(s) of IFN- λ -mediated HSV-2 inhibition, we examined the effects of IFN- λ s on type I IFN expression in End1/E6E7 cells. As shown in Fig. 2A, IFN- λ selectively upregulated the expression of IFN- β . Further, we showed that IFN- λ members could

induce ISG15, ISG56, OAS-1, OAS-2, and MxA expression at both the mRNA (Fig. 2C and D) and protein levels (Fig. 2B). The effects on ISG induction by 100 ng/mL of various IFN- λ members were very similar (Fig. 2B and D), although IFN- λ 3 was more effective in ISG15 and MxA induction in the low concentration treatment group (10 ng/mL) (Fig. 2C).

IFN- λ s regulate the expression of JAK/STAT pathway genes: IFN- β is a key activator of the JAK/STAT pathway (31,32), which is an important pathway for IFN-mediated activation of ISGs. Thus, we explored whether JAK/STAT signaling is involved in the actions of IFN- λ s against HSV-2. As shown in Fig. 3B and C, IFN- λ treatment significantly induced the expression and phosphorylation of STAT-1, while the expression and phosphorylation of STAT-3 were not induced (data not shown). To further evaluate the IFN- λ -mediated modulation of JAK/STAT pathway gene expression, we treated End1/E6E7 cells with 100 ng/mL IFN- λ 1, IFN- λ 2, or IFN- λ 3 for 24 h. Total cellular RNA was extracted for testing with the gene expression array to determine the profiles of IFN- λ -mediated regulation of genes encoding components of the JAK/STAT signaling pathway. We showed that IFN- λ s were able to significantly upregulate the expression of 20 genes in End1/E6E7 cells (Table 1). In addition, the expression of the central elements in the JAK/STAT signaling pathway, including JAK-1, JAK-2, JAK-3, STAT-1, STAT-2, STAT-4, and STAT-6, was enhanced by IFN- λ treatment (Table 1). Furthermore, key regulators in the JAK/STAT signaling pathway, such as interferon regulatory factor 1 (IRF1), IRF3, IRF4, IRF5, and IRF7 were also induced (Table 1). In addition, IFN- λ s also induced suppressors of cytokine signaling (SOCS) 1 and 2 expression (Table 1).

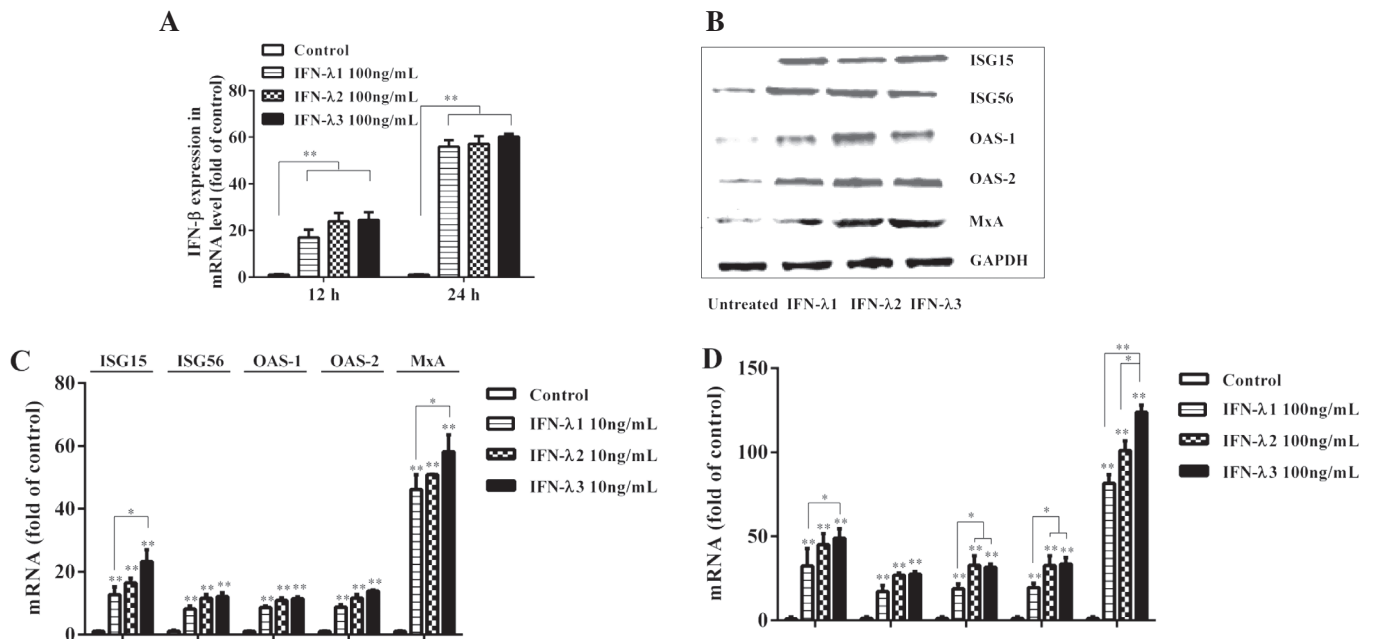


Fig. 2. IFN- λ s induce IFN- β and IFN-stimulated genes (ISGs) expression. End1/E6E7 cells were treated with 10 or 100 ng/mL IFN- λ for 24 h. A, C, and D: For mRNA quantification, cellular RNA was collected at 12 or 24 h post-treatment with/without 10 ng/mL or 100 ng/mL IFN- λ and subjected to the real-time RT-PCR for the mRNA levels of IFN- β (A), ISG15, ISG56, MxA, OAS-1, and OAS-2 (C, D). The data are expressed as mRNA levels for relative (fold) to the control (without treatment, which is defined as 1). The results shown are mean \pm SD of triplicate cultures, representative of 3 experiments (IFN- λ vs. control, * $P < 0.05$, ** $P < 0.01$). B: For protein quantification, cellular proteins (ISG15, ISG56, MxA, OAS-1, and OAS-2) were collected and subjected to Western blot.

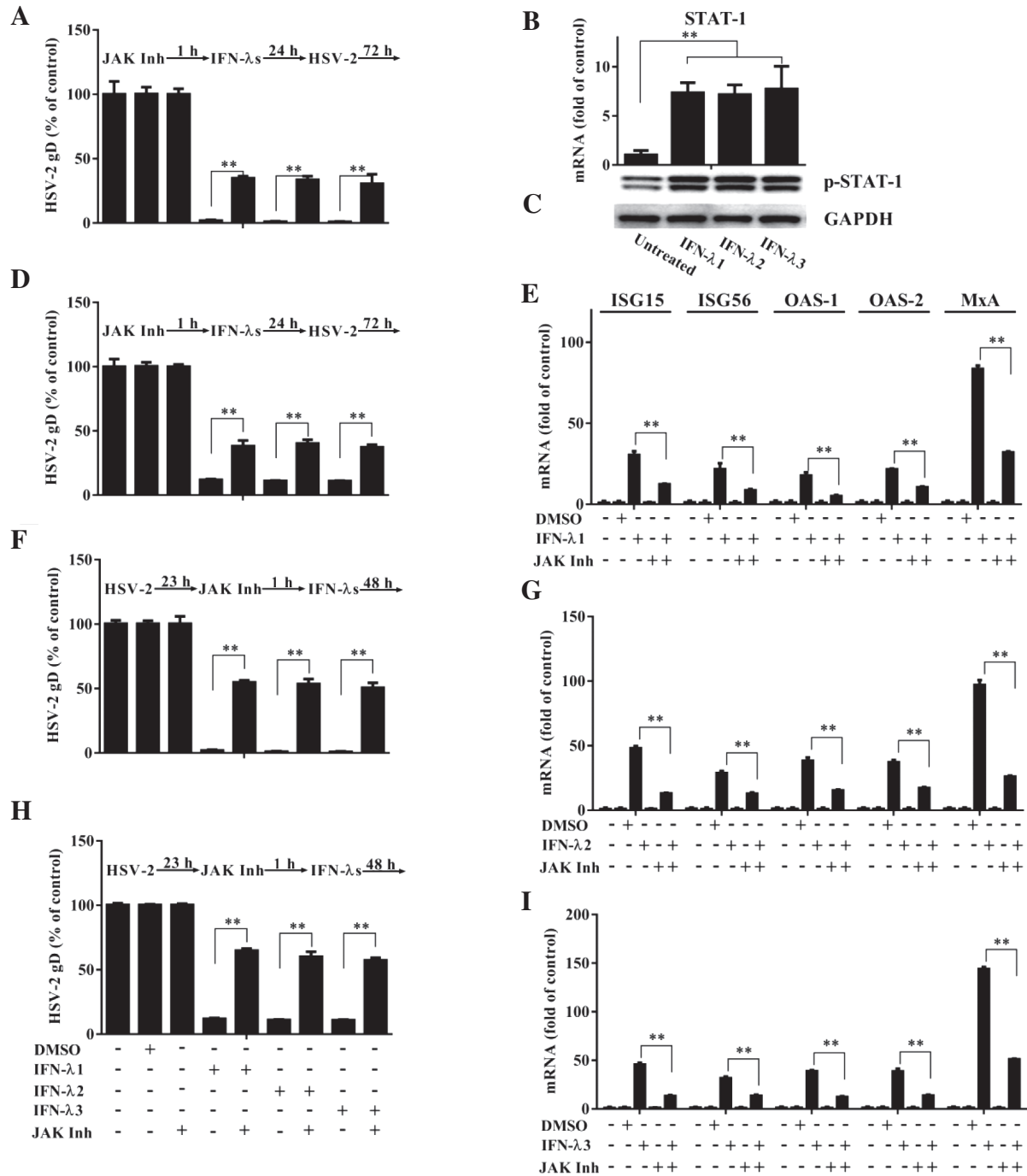


Fig. 3. JAK/STAT pathway inhibitor compromises the IFN- λ s actions against HSV-2 replication. A, D, F, and H: End1/E6E7 cells were treated with 1 μ M JAK inhibitor (JAK Inh) or DMSO for 1 h and then incubated with 100ng/mL IFN- λ (1, 2, or 3) for 24 h before HSV-2 G strain infection (b.i.). A, D: or post HSV-2 infection (p.i.). F, H: Total DNA from cells at 72 h post-infection was subjected to HSV-2 gD gene expression by real-time PCR. B, C: Cellular mRNA and protein were collected and subjected to real-time PCR and Western blot for detection of STAT-1 (mRNA) and p-STAT-1 (protein). E, G, I: End1/E6E7 cells were treated with 1 μ M JAK inhibitor (JAK Inh) or DMSO for 1 h and then incubated with 100ng/mL IFN- λ (1, 2, or 3) for 24 h. Total cellular RNA extracted from cells was subjected to the real-time RT-PCR for the mRNA levels of genes indicated. All the data are expressed as mRNA levels for relative (fold) to the untreated control, which is defined as 1. The results shown are mean \pm SD of triplicate cultures, representative of 3 experiments (IFN- λ vs. control, $**P < 0.01$).

Inhibition of the JAK/STAT pathway compromises the anti-HSV-2 activity of IFN- λ s: To investigate whether IFN- λ -mediated induction of ISGs and inhibition of HSV-2 are associated with JAK/STAT signaling, we examined the effect of the JAK/STAT pathway inhibitor JAK Inh on the actions of IFN- λ s. In the presence of JAK Inh (1 μ M), IFN- λ -induced expression of ISGs was inhibited (Fig. 3C, E, and G). In addition, JAK

Inh compromised IFN- λ treatment-mediated anti-HSV-2 activity when it was administered either before or after HSV-2 infection (Fig. 3A, D, F, and H).

IFN- λ s induce PRR expression: To further investigate the intracellular mechanisms of IFN- λ treatment-mediated HSV-2 inhibition in cervical epithelial cells, we examined whether IFN- λ s regulated different PRRs that play important roles during HSV-2 infection.

Table 1. Upregulated genes in JAK/STAT pathway by IFN- λ

Gene	Description	Fold of control		
		$\lambda 1$	$\lambda 2$	$\lambda 3$
CXCL9	Chemokine (C-X-C motif) ligand 9	5.14	4.98	5.54
IL-20	Interleukin-20	2.54	2.17	4.38
IRF1	Interferon regulatory factor 1	12.47	11.72	15.24
IRF3	Interferon regulatory factor 3	3.41	3.27	4.13
IRF4	Interferon regulatory factor 4	2.95	3.04	4.57
IRF5	Interferon regulatory factor 5	4.10	4.31	5.15
IRF7	Interferon regulatory factor 7	15.47	14.87	18.31
JAK-1	Janus kinase-1	1.53	1.49	1.71
JAK-2	Janus kinase-2	1.61	1.57	2.01
JAK-3	Janus kinase-3	2.41	2.68	3.04
JUNB	Jun B proto-oncogene	1.74	1.62	2.21
SOCS1	Suppressor of cytokine signaling 1	3.47	3.38	3.97
SOCS2	Suppressor of cytokine signaling 2	1.97	2.41	2.86
SP1	Sp1 transcription factor	1.87	1.68	1.95
STAT-1	Signal transducer and activator of transcription-1	7.14	6.92	7.58
STAT-2	Signal transducer and activator of transcription-2	3.41	3.38	3.93
STAT-4	Signal transducer and activator of transcription-4	1.61	1.54	2.34
STAT-6	Signal transducer and activator of transcription-6	1.97	1.89	2.52
TYK2	Tyrosine kinase 2	2.06	1.94	2.62
USF1	Upstream transcription factor 1	1.88	1.67	1.94

Human cervical epithelial cells (End1/E6E7 cells) were treated with IFN- $\lambda 1$, $\lambda 2$, or $\lambda 3$ (100 ng/mL) for 24 h. Total RNA was collected and subjected to the gene array using RT² Profiler PCR Array System (SABiosciences, QIAGEN). Data analysis was performed using Data Analysis Template from SABiosciences. The genes that displayed no significantly relative to the control were omitted.

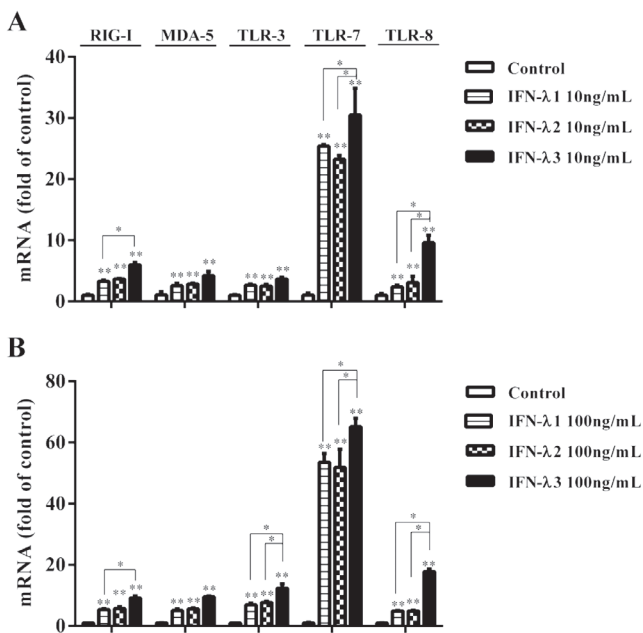


Fig. 4. IFN- λ s induce pattern recognition receptors (PPRs) expression. End1/E6E7 cells were treated with 10 ng/mL (A) or 100 ng/mL (B) IFN- λ for 24 h. Cellular RNA was subjected to the real-time RT-PCR for the mRNA levels of RIG-I, MDA5, TLR3, TLR7, TLR8, and GAPDH. The data are expressed as mRNA levels for relative (fold) to untreated control, which is defined as 1. The results shown are mean \pm SD of triplicate cultures, representative of 3 experiments (IFN- λ vs. control, * P < 0.05, ** P < 0.01).

We showed that IFN- λ treatment significantly increased the expression of retinoic acid-inducible protein I (RIG-I), melanoma differentiation-associated protein 5 (MDA-5), Toll-like receptor 3 (TLR3), TLR7, and TLR8 (Fig. 4A and B). The potent induction of these PRRs by IFN- $\lambda 3$ was more than that of IFN- $\lambda 1$ or IFN- $\lambda 2$ (Fig.

4A and B).

IFN- λ s induce ISG and PRR expression in HSV-2 infection of End1/E6E7 cells: As indicated, IFN- λ treatment before HSV-2 infection was more potent in HSV-2 inhibition than IFN- λ treatment after HSV-2 infection. We also investigated whether IFN- λ s could induce the expression of antiviral ISGs and PRRs in HSV-2-infected End1/E6E7 cells. As shown in Fig. 5, IFN- λ -induced ISG and PRR expression was not compromised by HSV-2-infection in End1/E6E7 cells.

DISCUSSION

Because epithelial cells are IFN- λ 's main target (11), IFN- λ has less side effects than type I IFN in epithelial cells and can inhibit virus replication in them. In this study, we compared the ability of IFN- $\lambda 1$ -3 to inhibit HSV-2 replication without cytotoxicity (Fig. 1D). The inhibitory effect of IFN- λ s on HSV-2 was dose-dependent and highly effective (Fig. 1A, C, and E). This significant inhibitory effect of IFN- λ s was observed whether cells were treated with IFN- λ before or after HSV-2 infection (Fig. 1B). Furthermore, the inhibitory effect of IFN- $\lambda 3$ on HSV-2 was more potent than that of IFN- $\lambda 1$ and IFN- $\lambda 2$ (Fig. 1A and B), which is consistent with previous publications (33,34).

To further investigate the mechanisms by which IFN- λ s inhibit HSV-2, we compared the effect of IFN- λ s on type I IFN and antiviral ISG expression in End1/E6E7 cells. The results showed that IFN- λ treatment induced the expression of IFN- β (Fig. 2A) and multiple ISGs (Fig. 2B–D), which is in line with its anti-HSV-2 action (Fig. 1). IFN- $\lambda 3$ induced higher expression of ISG15 and MxA than IFN- $\lambda 1$ or IFN- $\lambda 2$ did in the low concentration treatment group (10 ng/mL) (Fig. 2C). These results suggest that the different antiviral properties of IFN- λ s may be related to differential regulation of ISG

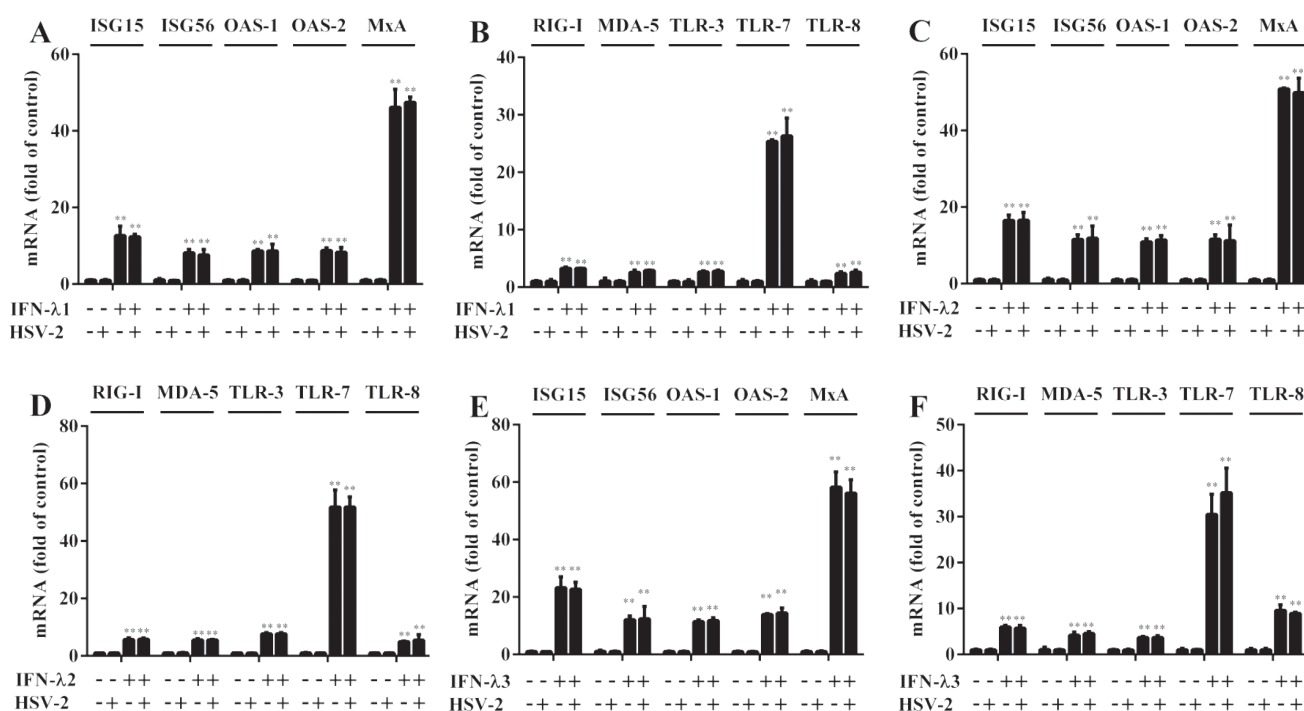


Fig.5. Effect of HSV-2 infection on IFN- λ -induced ISGs and PPRs expression. End1/E6E7 cells infected with or without HSV-2 for 24 h were treated with 100 ng/mL IFN- λ for 24 h. Cellular RNA was subjected to the real-time RT-PCR for the detection of ISG15, ISG56, OAS-1, OAS-2, MxA, RIG-I, MDA-5, TLR-3, TLR-7, TLR8, and GAPDH. The data are expressed as mRNA levels for relative (fold) to the control (without treatment, which is defined as 1). The results shown are mean \pm SD of triplicate cultures, representative of 3 experiments (IFN- λ vs. control, ** P < 0.01).

expression by various members of the IFN- λ family, as ISGs have been reported to play important roles in IFN-mediated antiviral activity (35,36).

Because activation of the JAK/STAT signaling pathway is crucial to antiviral action, we examined whether the JAK/STAT signaling pathway plays a major role in IFN- λ -mediated anti-HSV-2 activity. We used JAK Inh to treat End1/E6E7 cells prior to IFN- λ treatment, and we found that JAK Inh blocked the induction of ISGs (Fig. 3E, G, and I), largely compromised the action of IFN- λ s against HSV-2 infection (Fig. 3A, D, F, and H). Then, we performed a PCR-based gene array assay to determine JAK/STAT signaling pathway gene expression profiles in IFN- λ s-treated End1/E6E7 cells. We showed that all 3 members of the IFN- λ family were able to upregulate expression of 20 genes in End1/E6E7 cells (Table 1). In addition, expression of the key elements in the JAK/STAT signaling pathway was enhanced by IFN- λ treatment (Table 1) (37). Furthermore, central regulators in the JAK/STAT signaling pathway, such as IRF-1 and ISGF3G (IRF-9) (38), were also induced by IFN- λ treatment (Table 1).

Induction of IFN-based antiviral innate immunity depends on several TLRs and RIG-I-like receptors (RLR). TLR-3, TLR-7, and TLR-8 recognize viral RNA, triggering IFN signaling (39,40). In addition to TLRs, RIG-I and MDA-5, which belong to the RLR family, recognize viral RNA during infections (39,40). Here, we showed that IFN- λ s induce TLR-3, TLR-7, TLR-8, RIG-I, and MDA-5 expression in End1/E6E7 cells and that the induction levels were higher in the IFN- λ 3 treatment group (Fig. 4). These findings indicate a sound mechanism to explain the IFN- λ -mediated anti-HSV-2 effects

in End1/E6E7 cells, as signaling through these PRRs plays crucial roles in early innate immune response to viral infections.

Viruses evolve strategies to evade host immune response, including inhibition of intracellular innate immunity, to establish persistent infections in target cells. However, until recently, few studies had examined whether HSV-2 replication inhibits anti-viral ISG and PRR expression induced by IFN- λ treatment in cervical epithelial cells. In this study, we showed that the IFN- λ -mediated inhibition of HSV-2 replication was less potent after HSV-2 infection than before it (Fig. 1B). This phenomenon may be because rapid replication of HSV-2 occurs before the IFN- λ -mediated antiviral immune response. When we treated cells infected with HSV-2 24 h post-infection with IFN- λ , IFN- λ -mediated antiviral ISG expression was similar to that in uninfected cells; TLR-3, TLR-7, TLR-8, RIG-I, and MDA-5 expression were also similar to that in the control (Fig. 5). These findings suggest that IFN- λ -mediated antiviral immunity is critical to the inhibition of HSV-2 in cervical epithelial cells. IFN- λ 4 has been associated with impaired clearance of hepatitis C virus (HCV) and has shown anti-HCV activity (2). Because commercial IFN- λ 4 was unavailable, we were unable to test its anti-HSV-2 properties. In the absence of a specific anti-HSV-2 vaccine or other treatments, novel inhibitors of HSV-2 replication to kill or inactivate HSV-2 infectious particles are needed. Our study suggests that IFN- λ 1-3 should be explored as an anti-HSV-2 candidate in clinical trials. These trials will be critical for the design and development of IFN- λ 1-3-based interventions and treatment strategies to control HSV-2 transmission.

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Conflict of interest None to declare.

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