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ORIGINAL RESEARCH

Induction of interferon-λ contributes to TLR3 and RIG-I activation-mediated inhibition of herpes simplex virus type 2 replication in human cervical epithelial cells

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STUDY HYPOTHESIS: Is it possible to immunologically activate human cervical epithelial cells to produce antiviral factors that inhibit herpes simplex virus type 2 (HSV-2) replication?

STUDY FINDING: Our results indicate that human cervical epithelial cells possess a functional TLR3/RIG-I signaling system, the activation of which can mount an Interferon-λ (IFN-λ)-mediated anti-HSV-2 response.

WHAT IS KNOWN ALREADY: There is limited information about the role of cervical epithelial cells in genital innate immunity against HSV-2 infection.

STUDY DESIGN, SAMPLES/MATERIALS, METHODS: We examined the expression of toll-like receptors (TLRs) and retinoic acid-inducible I (RIG-I) in End1/E6E7 cells by real-time PCR. The IFN-λ induced by TLR3 and RIG-I activation of End1/E6E7 cells was also examined by real-time PCR and ELISA. HSV-2 infection of End1/E6E7 cells was evaluated by the real-time PCR detection of HSV-2 gD expression. The antibody to IL-10Rβ was used to determine whether IFN-λ contributes to TLR3/RIG-I mediated HSV-2 inhibition. Expression of interferon regulatory factor 3 (IRF3), IRF7, IFN-stimulated gene 56 (ISG56), 2′-5′-oligoadenylate synthetase I (OAS-1) and myxovirus resistance A (MxA) were determined by the real-time PCR and western blot. End1/E6E7 cells were transfected with shRNA to knockdown the IRF3, IRF7 or RIG-I expression. Student's *t*-test and post Newman–Keuls test were used to analyze stabilized differences in the immunological parameters above between TLR3/RIG-I-activated cells and control cells.

MAIN RESULTS AND THE ROLE OF CHANCE: Human cervical epithelial cells expressed functional TLR3 and RIG-I, which could be activated by poly I:C and 5′ppp double-strand RNAs (5′ppp dsRNA), resulting in the induction of endogenous interferon lambda (IFN-λ). The induced IFN-λ contributed to TLR3/RIG-I-mediated inhibition of HSV-2 replication in human cervical epithelial cells, as an antibody to IL-10Rβ, an IFN-λ receptor subunit, could compromise TLR3/RIG-I-mediated inhibition of HSV-2. Further studies showed that TLR3/RIG-I signaling in the cervical epithelial cells by dsRNA induced the expression of the IFN-stimulated genes (ISGs), ISG56, 2′-5′-oligoadenylate synthetase I (OAS-1) and myxovirus resistance A (MxA), the key antiviral elements in the IFN signaling pathway. In addition, we observed that the topical treatment of genital mucosa with poly I:C could protect mice from genital HSV-2 infection.

LIMITATIONS, REASONS FOR CAUTION: Future prospective studies with primary cells and suitable animal models are needed in order to confirm these outcomes.

WIDER IMPLICATIONS OF THE FINDINGS: The findings provide direct and compelling evidence that there is intracellular expression and regulation of IFN-λ in human cervical epithelial cells, which may have a key role in the innate genital protection against viral infections.

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Key words: herpes simplex virus type 2 / human cervical epithelial cells / interferon-stimulated genes / interferon-λ / Toll-like receptor 3 / retinoic acid-inducible gene I

Introduction

Herpes simplex virus type 2 (HSV-2) is an enveloped, nuclear replicating, double-stranded DNA virus, that belongs to the subgroup of *alphaherpesvirinae*. HSV-2 is among the most common human infectious viral pathogens, causing lifelong diseases with clinical manifestations including cold sores, genital lesions, corneal blindness and encephalitis (Fife et al., 2008; Sperling et al., 2008; Zhang et al., 2009, 2012; Dasgupta and Ben-Mohamed, 2011). Although diseases caused by HSV-2 are generally self-limiting, the virus can cause devastating dissimilated infections and encephalitis in immunocompromised individuals, such as newborns, persons with transplantation, and AIDS-patients (Zhang et al., 2015). Furthermore, HSV-2 infection promotes HIV infection and transmission (Shao et al., 2015; Wen et al., 2015). To date, there is no vaccine available to prevent genital HSV-2 infection (Lin et al., 2015). In addition, current treatment options are limited to the control of virus reactivation and do not eliminate latent virus (Chao et al., 2015; Chiang et al., 2015). Engagement of toll-like receptors (TLRs) activates signaling cascades that culminate in inflammatory and immune defense responses. Among the 10 identified human TLRs, TLR3 has been recognized as one of the major sensors in virus-mediated innate immune response (Barton and Medzhitov, 2002). TLR3 activation induces type I interferon (IFN) expression (Katze et al., 2002; Borden et al., 2007), which is crucial for protection against HSV infection of the target cells in both the genital tract (Gill et al., 2006) and central nervous system (CNS) (Zhou et al., 2009b). In addition, TLR3 activation could induce the expression of type III IFN (IFN-λ), a new member of IFN family (Ank and Paludan, 2009; Li et al., 2012). IFN-λ can be produced by a number of cell types, including non-immune cells (Ank et al., 2006), although the pattern of its expression has not been elucidated. In addition to TLR3, retinoic acid-inducible gene I (RIG-I) is also an important mediator of antiviral immunity, as it has the ability to recognize viral RNAs in cytoplasm and trigger IFN-mediated immune protection against viral infections (Fujita et al., 2007). HSV-2 infection occurs at, and emanates, from mucosal surfaces (Roizman and Taddeo, 2007). The mucosal immunity in the genital tract is considered as an initial and primary innate defense mechanism against HSV-2 infection (Chan et al., 2011). Epithelial cells at the surface of the female reproductive tract participate in the mucosal innate immunity against viral infections. Epithelial cells have been reported to selectively express TLR1, TLR2, TLR3, TLR5 and TLR6 (Fichorova et al., 2002; Hertz et al., 2003; Smith et al., 2003; Schaefer et al., 2004; Funami et al., 2007). However, it remains to be determined whether cervical epithelial cells can contribute to TLR-mediated innate immunity against HSV-2 infection. In the present study, we examined whether human cervical epithelial cells have the ability to mount a TLR3/RIG-I-mediated innate immunity that is effective in the control of HSV-2 infection.

Materials and Methods

Reagents

All culture plasticware was obtained from Corning (Corning, NY, USA). Mouse anti-IL-10Rβ antibody was purchased from R&D system Inc. (Minneapolis, MH, USA) and mouse IgG (control IgG) was from Molecular Probes (Eugene, OR, USA).

Cell culture

Vero cell line (African green monkey kidney epithelial cells) was cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's culture medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco), 2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco). Human End1/E6E7 cell line is a well differentiated endocervical epithelial cell line immortalized by HPV 16 E6/E7 and derived from normal endocervical epithelium (Fichorova et al., 1997), which can be grown as a polarized monolayer (Sathe and Reddy, 2014). The morphological and immunocytochemical characteristics of this immortalized cell line closely resemble the tissue of origin and primary cultures, and differ significantly from the Hela cells (Fichorova et al., 1997). End1/E6E7 cells have been extensively used as a human endocervical epithelial model for *in vitro* studies (Verhoog et al., 2011; Govender et al., 2014; Sathe and Reddy, 2014; Hijazi et al., 2015). The cells respond well to the TLRs and RIG-I ligands even after polarization (Sathe and Reddy, 2014). The cells were cultured in keratinocyte growth medium (Gibco) supplemented with the provided 50 μg/ml bovine pituitary extract, 0.1 ng/ml recombinant epidermal growth factor, 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco).

TLRs and RIG-I activation

Because of the lack of the uptake of poly I:C by the cells, the direct addition of poly I:C to the cell cultures failed to induce IFN-λ induction (Supplementary Fig. S3). Thus, we used a transfection reagent Lyovec (Invivogen, San Diego, CA, USA) for TLR3/RIG-I activation experiments throughout the study. The cells were stimulated with poly I:C (TLR3 ligand), imiquimod (TLR7 ligand), ssRNA 40 (TLR8 ligand), ODN2006 (TLR9 ligand), 5′ppp dsRNA (RIG-I ligand), or 5′ppp dsRNA control using Lyovec transfection reagent (Invivogen). Lyovec-treated cells were used as a vehicle control. The cell culture medium with transfection reagent was replaced with fresh keratinocyte growth medium 12 h post-transfection. Cells were collected for RNA extraction at indicated time points. For disruption of TLR3 function, cells were treated with 100 nM of TLR3/dsRNA Complex Inhibitor (abbreviated as

TCI; EMD Millipore, Inc., Billerica, MA, USA) for 1 h prior to Poly I:C transfection (Cheng *et al.*, 2011).

Cell viability assay

End1/E6E7 cells seeded in 96-well plate (10^4/well) were treated with poly I:C or 5′ppp dsRNA for 72 h. Cells were then exposed to 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (20 μl/well). The formation of soluble formazan from MTT was measured by spectrophotometric determination of absorption at 570 nm using a plate reader (SpectraMax i3, Molecular Devices, Sunnyvale, CA, USA).

Virus propagation and infection

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). Viruses were propagated and purified from Vero cells by the standard sucrose gradient procedure and used for infection studies at a multiplicity of infection of 0.001. After adding the viruses, plates were incubated at 37°C for 90 min followed by washing with DMEM. The cell cultures were then maintained in fresh keratinocyte growth medium for up to 72 h. Cellular DNA was extracted from HSV-2-infected cells with DNA lysis buffer containing 100 mM KCl, 20 mM Tris, pH 8.4, 500 μg/ml proteinase K (Sigma-Aldrich, St. Louis, MO, USA), and 0.2% (v/v) NP-40 (BDH, Poole, UK). Lysates were incubated at 60°C for 2 h followed by 100°C for 15 min. HSV-2 gD was analyzed by real-time PCR using the specific primers 5′-TCTCCGTCC AGTCGTTTAT-3′ (sense) and 5′-ATCCGAACGCAGCCCCGC-3′ (antisense), and was quantified by using serial dilutions of HSV-2 gD standards with known copy numbers.

HSV-2 genital infection of mice

The protocol for the animal experiments was approved by the Institutional Animal Care and Use Committee at the Center for Animal Experiment, Wuhan University. In order to reduce experimental variations due to menstruation difference of female mice, 6-week-old WT Balb/c female mice were pretreated by subcutaneous injection (*s.c.*) of 2 mg Depo-Provera (Pfizer, Morris Plains, NJ, USA), the female hormone progesterone that prevents pregnancy by stopping the oocyte release from ovaries. Five days later, mice were inoculated intravaginally (*i.vag.*) with 30 μl of a half lethal dose (LD_{50}, 3000 pfu) of HSV-2 G strain in DMEM. The mice were placed on their back and maintained for 10 min.

Animals were assessed daily for disease signs (genital pathology) for 15 days after HSV-2 infection (*p.i.*). The survival rate of infected animals was observed for 21 days *p.i.* (Herbst-Kralovetz and Pyles, 2006). Genital pathology was scored daily using a 5-point scale (Kaushic *et al.*, 2003): 0, no pathology; 1, mild vulvar erythema; 2, moderate vulvar erythema and swelling; 3, severe vulvar erythema and swelling and perineal fur loss; 4, perineal ulceration; 5, extension of perineal ulceration and fur loss to surrounding tissue, or death. Mice were euthanized when they reached score 5. To quantify the protection provided by poly I:C treatment, 16 h before or 4 h after intravaginal inoculation of HSV-2, mice were given *i.vag.* poly I:C (25 μg) suspended in 30 μl of Lyovec transfection reagent.

RNA extraction and real-time RT–PCR

Total cellular RNA was extracted from cells using TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA) as described previously (Zhou *et al.*, 2009a, b; Li *et al.*, 2012). Total RNA was subjected to the reverse transcription using reagents obtained from Promega (Madison, WI, USA). The real-time PCR for the quantification of GAPDH, IFN-λ1, IFN-λ2/3, TLR1-10, RIG-I, IRF3, IRF7, ISG56, OAS-1, MxA and PKR mRNA (Table I) was performed with IQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, USA) as described previously (Li *et al.*, 2012). The levels of GAPDH mRNA were used as an endogenous reference to normalize the quantities of target mRNA. The sequences of oligonucleotide primers used are listed in Table I and were synthesized by Invitrogen Inc.

Knockdown of RIG-I, IRF3 or IRF7

Lentiviral plasmids with sequence-verified shRNA for gene silencing of RIG-I was obtained from Dr. Shi Liu (Wuhan University, China) and plasmids for gene silencing of IRF3 and IRF7 were gifts from Dr. Rongtuan Lin (McGill University, Canada). End1/E6E7 cells were plated in a 48-well plates at 2×10^4 per well 24 h before transfection. Cells were then transfected with the plasmids using Lipofectamine 2000 (Invitrogen) at a ratio of 1:2 (μg:μl) prepared in keratinocyte growth medium. After 48 h, cells were stimulated with poly I:C. shRNA control vector pLKO.1 (SHC002; Sigma) was included as a non-target knockdown control in the experiments.

Western blot

Total cell lysates of End1/E6E7 cells treated with poly I:C for 24 h were prepared by using the cell extraction buffer (Invitrogen, Shanghai, China) with 1% protease inhibitor cocktail (Sigma, MO, USA) according to the manufacturer's instructions. Equal amounts of protein lysates (20 μg) were separated on 4–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis precast gels and transfected to an polyvinylidene difluoride membranes (Millipore, Germany). The blots were incubated with primary antibodies in 2% BSA in phosphate-buffered saline with 0.05% Tween 20 (PBST) overnight at 4°C (IRF3, 1:2000; IRF7, 1:2000; ISG56, 1:2000; OAS-1, 1:1000; MxA, 1:1000; GAPDH, 1:5000). The blots were then washed with PBST and further incubated with horseradish peroxidase-conjugated appropriate second antibodies diluted at 1:5000 to 1:8000 in 2% nonfat milk PBST. Blots were developed with SuperSignal West Pico Chemiluminescent Substrated (Thermo Fisher Scientific, Waltham, MA, USA).

ELISA

IFN-λ protein levels in End1/E6E7 culture supernatant were measured with ELISA (Human IL-29/ IFN-λ1: eBioscience, San Diego, CA; Human IL-28A/ IFN-λ2: R&D system Inc., Minneapolis, MH, USA). Assays were performed according to the manufacturer's instructions.

Data analysis

Where appropriate, data were expressed as mean ± Standard deviation (SD) from at least three independent experiments. For comparison of the mean of two groups, statistical significance was measured by Student's *t*-test. To compare the difference between multiple groups, statistical significance was analyzed using a one-way analysis of variance followed by post Newman–Keul's test. Calculations were performed with Graphpad Prism Statistical Software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was defined as $P < 0.05$ or $P < 0.01$.

Results

TLR3 activation induces IFNs

Activation of TLRs triggers intracellular IFN-mediated innate immunity against virus infections, including HSV-2. Thus, we first examined the expression of TLRs in human cervical epithelial cells. As shown in Fig. 1A, End1/E6E7 cells express mRNA for all TLRs except TLR5, however upon HSV-2 infection, only TLR3, 7, 8 and 9, the key sensors for viral infection, were significantly up-regulated (Supplementary Fig. S1). To exam whether these antiviral TLRs are biologically functional in the epithelial cells, we transfected the cells with the ligands (Poly I:C, imiquimod, ssRNA40 and ODN2006) of these TLRs. As shown in Fig. 1B, only

Table 1 Primer sets for real-time RT–PCR.

Primer	Accession no.	Orientation	Sequences	Product (bp)
GAPDH	NM_002046	Sense	5′-GGTGGTCTCCTCTGACTTCAACA-3′	127
		Antisense	5′-GTTGCTGTAGCCAAATTCGTTGT-3′	
IFN-λ1	NM_172140	Sense	5′-CTTCCAAGCCCACCCCAACT-3′	142
		Antisense	5′-GGCCTCCAGGACCTTCAGC-3′	
IFN-λ2/3	NM_172138/	Sense	5′-TTTAAGAGGGCCAAAGATGC-3′	267
	NM_172139	Antisense	5′-TGGGGCTGAGGCTGGATACAG-3′	
IRF3	NM_001571	Sense	5′-ACCAGCCGTGGACCAAGAG-3′	60
		Antisense	5′-TACCAAGGCCCTGAGGCAC-3′	
IRF7	NM_001572	Sense	5′-TGGTCCTGGTGAAGCTGGAA-3′	134
		Antisense	5′-GATGTCGTCATAGAGGCTGTTGG-3′	
TLR1	NM_003263	Sense	5′-GCCTATATGCAAAGAGTTTGGC-3′	134
		Antisense	5′-CTCTCCTAAGACCAGCAAGACC-3′	
TLR2	NM_003264	Sense	5′-GGCTTCTCTGTCTTGTGACC-3′	292
		Antisense	5′-GGGCTTGAACCAGGAAGACG-3′	
TLR3	NM_003265	Sense	5′-AGCCACCTGAAGTTGACTCAGG-3′	268
		Antisense	5′-AGCCACCTGAAGTTGACTCAGG-3′	
TLR4	NM_138554	Sense	5′-CAGAGTTTCCTGCAATGGATCA-3′	85
		Antisense	5′-GCTTATCTGAAGGTGTTGCACAT-3′	
TLR5	NM_003268	Sense	5′-AGCCATCTGACTGCATTAAGG-3′	336
		Antisense	5′-GACTTCCTCTTCATCACAACC-3′	
TLR6	NM_006068	Sense	5′-ATTGAAAGCATTCGTGAAGAAG-3′	123
		Antisense	5′-ACGGTGTACAAAGCTGTCTGTG-3′	
TLR7	NM_016562	Sense	5′-AAAATGGTGTTTCCAATGTGG-3′	107
		Antisense	5′-GGCAGAGTTTTAGGAAACCATC-3′	
TLR8	NM_138636	Sense	5′-TTATGTGTTCCAGGAACTCAGAGAA-3′	83
		Antisense	5′-TAATACCCAAGTTGATAGTCGATAAGTTTG-3′	
TLR9	NM_017442	Sense	5′-TACCAACATCCTGATGCTAGACTC-3′	231
		Antisense	5′-TACCAACATCCTGATGCTAGACTC-3′	
TLR10	NM_001017388	Sense	5′-GGCCAGAAACTGTGGTCAAT-3′	205
		Antisense	5′-AAATGACTGCATCCAGGGAG-3′	
RIG-I	AF038963	Sense	5′-CTTGGCATGTTACACAGCTGAC-3′	104
		Antisense	5′-GCTTGGGATGTGGTCTACTCA-3′	
ISG56	NM_001270930	Sense	5′-TTCGGAGAAAGGCATTAGA-3′	85
		Antisense	5′-TCCAGGGCTTCATTCATAT-3′	
OAS-1	NM_001032409	Sense	5′-AGAAGGCAGCTCACGAAACC-3′	71
		Antisense	5′-CCACCACCCAAGTTTCCTGTA-3′	
MxA	NM_001178046	Sense	5′-GCCGGCTGTGGATATGCTA-3′	69
		Antisense	5′-TTTATCGAAACATCTGTGAAAGCAA-3′	
PKR	NM_001139518	Sense	5′-AGAGTAACCGTTGGTGACATAACCT-3′	80
		Antisense	5′-GCAGCCTCTGCAGCTCTATGTT-3′	

poly I:C could significantly induce IFN-λ expression. This poly I:C-mediated induction of IFN-λ was time- and dose-dependent (Fig. 1C–E). In contract, poly I:C had little effect on the induction of IFN-α (Supplementary Fig. S2A). Although poly I:C could induce IFN-β expression in a time- and dose-dependent manner (Supplementary Fig. S2A and B), the degree of the induction was much less than that for IFN-α. HSV-2 infection inhibited poly I:C-mediated IFN-β expression in the cervical epithelial cells (Supplementary Fig. S2C). However, HSV-2 infection had little effect on the induction of IFN-λs (Fig. 1F and G). To determine the uptake efficiency of the cells by different routes of poly I:C delivery, cells were treated with fluorescein labeled poly I:C (FITC-poly I:C; TLR3 ligand) either by directly adding the reagent to the cell culture or by transfection with Lyovec. Interestingly, we found that only the transfection successfully delivered poly I:C into cells (Supplementary Fig. S3).

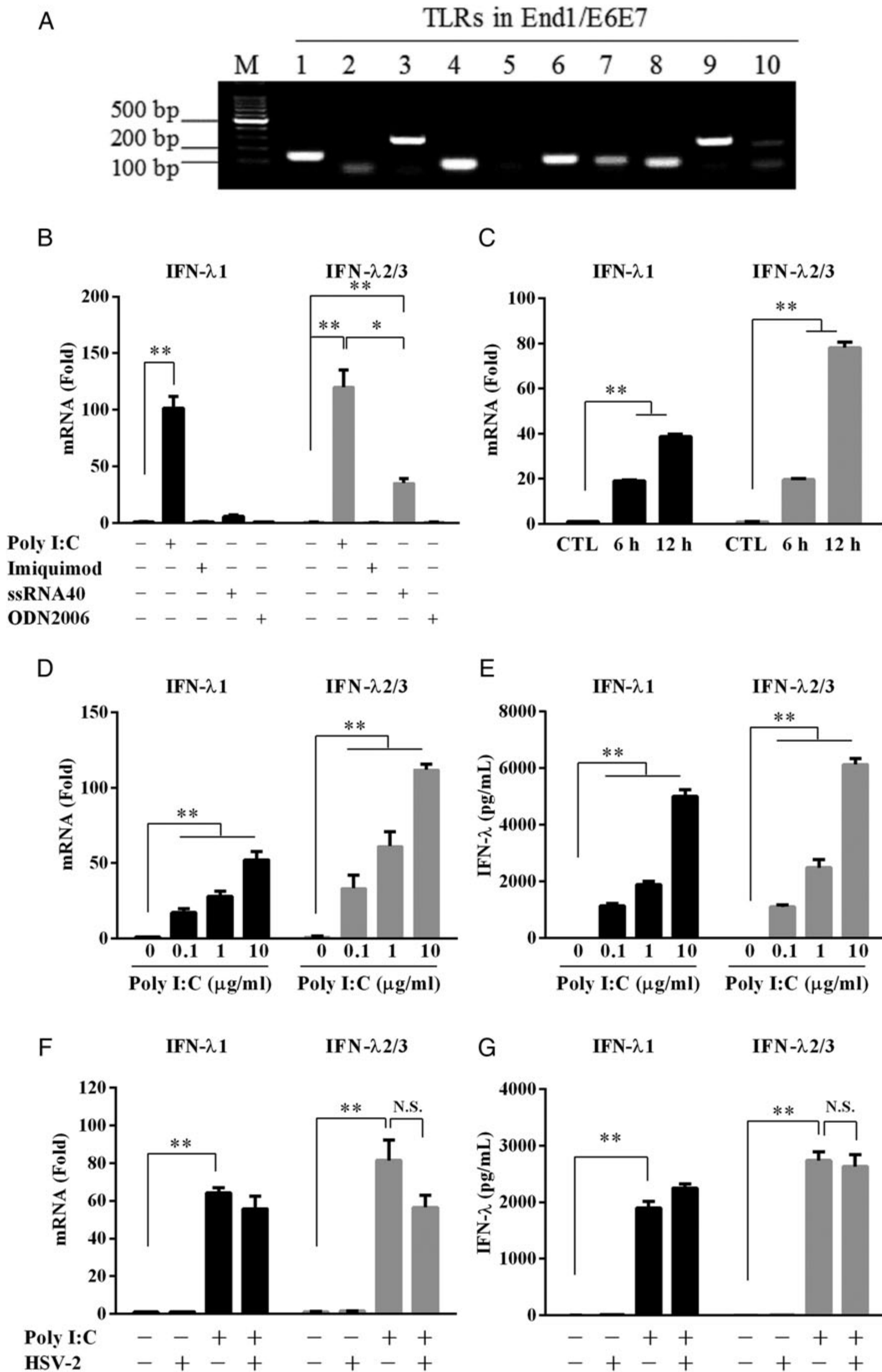

Figure 1 Effect of TLRs activation on IFN-λ expression. (**A**) Expression of TLRs in End1/E6E7 cells. Total cellular RNA extracted from End1/E6E7 cells was subjected to the RT–PCR with the primers specific for toll-like receptors (TLRs) 1–10. Amplified PCR products were displayed on 2% agarose gel. (**B**) End1/E6E7 cells were transfected with poly I:C (10 μg/ml), imiquimod (10 μg/ml), ssRNA40 (10 μg/ml) or ODN2006 (5 μM) for 12 h. (**C**) End1/E6E7 cells were transfected with or without poly I:C (1 μg/ml) for the indicated times. Total cellular RNA was subjected to the real-time RT–PCR for the mRNA levels of IFN-λ1 and IFN-λ2/3 as indicated. (**D** and **E**) End1/E6E7 cells were transfected with or without poly I:C at indicated concentrations (1, 10 μg/ml) for mRNA (D) or protein (E) detection. (**F** and **G**) End1/E6E7 cells infected with or without HSV-2 (MOI = 0.001) for 24 h were then transfected with or without poly I:C (1 μg/ml) for mRNA (F) or for protein (G) detection. The results are the mean ± SD of triplicate cultures, representative of three experiments (*$P < 0.05$, **$P < 0.01$, N.S. as no significance difference).

TLR3 and RIG-I are involved in poly I:C-induced IFN-λ expression

To determine the involvement of TLR3 signaling in poly I:C-mediated induction of IFN-λ, we pretreated End1/E6E7 cells with TLR3/dsRNA complex inhibitor (TCI) that blocks the binding of dsRNA to TLR3. As shown in Fig. 2A, poly I:C-induced expression of IFN-λ was significantly inhibited in TCI-pretreated cells (Fig. 2A). Since poly I:C could induce the expression of TLR3 and RIG-I, which is a key intracellular dsRNA sensor (Fig. 2B), we then examined whether RIG-I activation can induce IFN-λ in human cervical epithelial cells. As shown in Fig. 2C, treatment of End1/E6E7 cells with the 5′ppp dsRNA (RIG-I ligand) up-regulated IFN-λ expression. This 5′ppp dsRNA-induced IFN-λ expression could be compromised by RIG-I shRNA, while the control shRNA had little effect (Fig. 2D).

TLR3/RIG-I activation induces IRF3 and/or IRF7

The IFN regulatory factors (IRFs), particularly IRF3 and IRF7, have been implicated in the control of IFN-λ expression (Li et al., 2012). We thus examined the effect of poly I:C or 5′ppp dsRNA on the expression of IRF3 and IRF7 in the human cervical cells. As shown in Fig. 3A and C, poly I:C in a dose-dependent fashion, induced the expression of IRF3 and IRF7. While 5′ppp dsRNA could also induce the expression of IRF7 (Fig. 3B and D), but it had little effect on IRF3 expression (Fig. 3B and D). To determine the role of IRF3 and/or IRF7 in dsRNA-stimulated IFN-λ induction, we examined whether the knockdown of IRF3 or IRF7 could compromise the induction of IFN-λ. As shown in Fig. 3E, knockdown of IRF3 or IRF7 reduced poly I:C-induced expression of both IFN-λ1 and IFN-λ2/3. In addition, knockdown of IRF7 compromised 5′ppp dsRNA-induced IFN-λ1 and IFN-λ2/3 expression (Fig. 3F).

TLR3/RIG-I activation inhibits HSV-2 replication

To determine whether TLR3/RIG-I signaling of the cervical epithelial cells can inhibit HSV-2 replication, we first examined the cytotoxic effect of poly I:C or 5′ppp dsRNA on the cervical epithelial cells

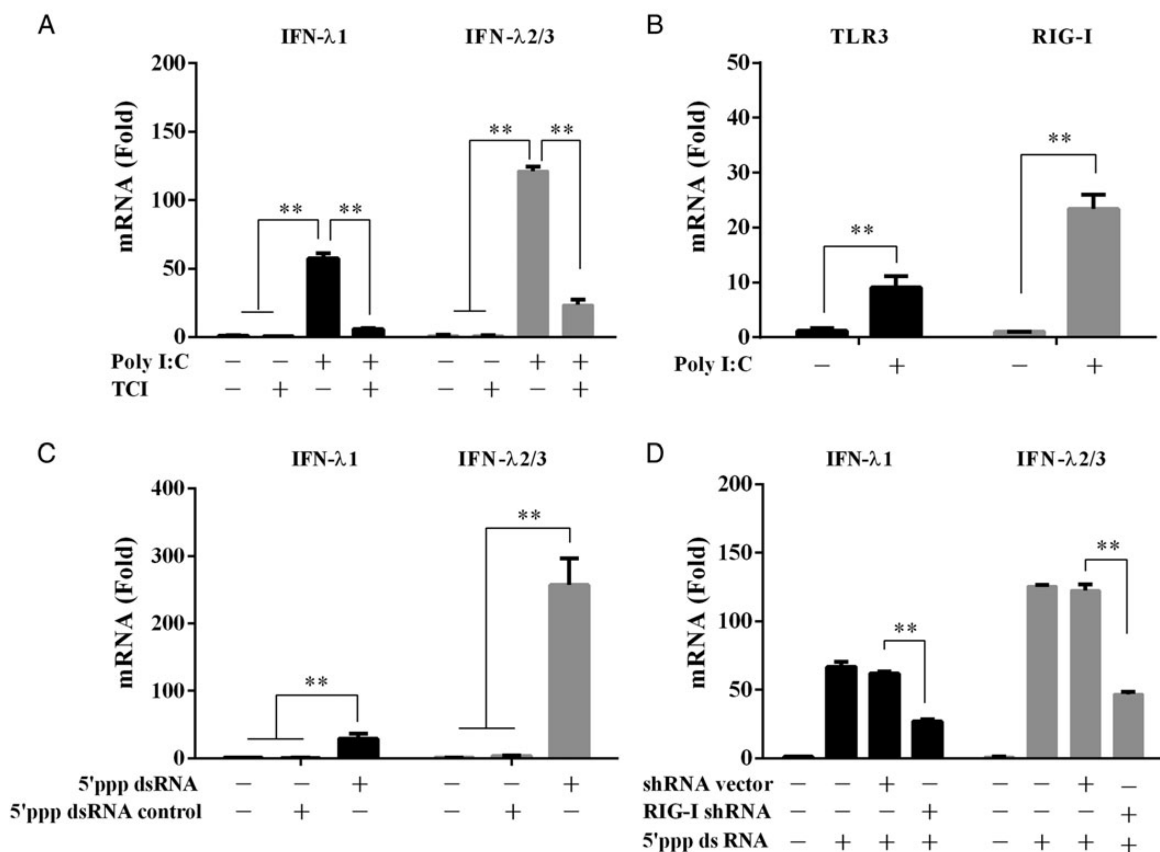

Figure 2 Roles of TLR3 and RIG-I in poly I:C-mediated IFN-λ induction. (**A**) Effect of TLR3/dsRNA complex inhibitor (TCI) on the induction of IFN-λ by poly I:C. End1/E6E7 cells were pretreated with TCI (100 nM) for 1 h prior to poly I:C (1 μg/ml) transfection for 12 h. (**B**) End1/E6E7 cells were stimulated with 1 μg/ml poly I:C for 12 h. (**C**) End1/E6E7 cells were stimulated with 1 μg/ml 5′ppp dsRNA or 5′ppp dsRNA control for 12 h. (**D**) End1/E6E7 cells were pretransfected with RIG-I shRNA or control shRNA for 48 h prior to stimulation with 5′ppp dsRNA (1 μg/ml). Total cellular RNA was subjected to the real-time RT–PCR for the mRNA levels of IFN-λ1 and IFN-λ2/3 (A, C and D), TLR3 and RIG-I (B). The data are expressed as mRNA levels relative (-fold) to control (without poly I:C or 5′ppp dsRNA defined as 1). The results are mean ± SD of triplicate cultures, representative of three experiments (*$P < 0.05$, **$P < 0.01$).

(End1/E6E7 cells). No cytotoxic effect was observed in the cells stimulated with either poly I:C (Fig. 4A) or 5′ppp dsRNA (Fig. 4B). We then examined the impact of poly I:C or 5′ppp dsRNA on HSV-2 infection/replication. As demonstrated in Fig. 4C–F, either poly I:C or 5′ppp dsRNA could dose-dependently inhibit HSV-2 replication in the cervical epithelial cells.

IFN-λ contributes to TLR3/RIG-I-mediated anti-HSV-2 activation

To determine whether TLR3/RIG-I activation-induced IFN-λ is responsible for HSV-2 inhibition in epithelial cells, the cells were treated with or without the antibody to IFN-λ receptor (IL-10Rβ) in the presence of poly I:C or

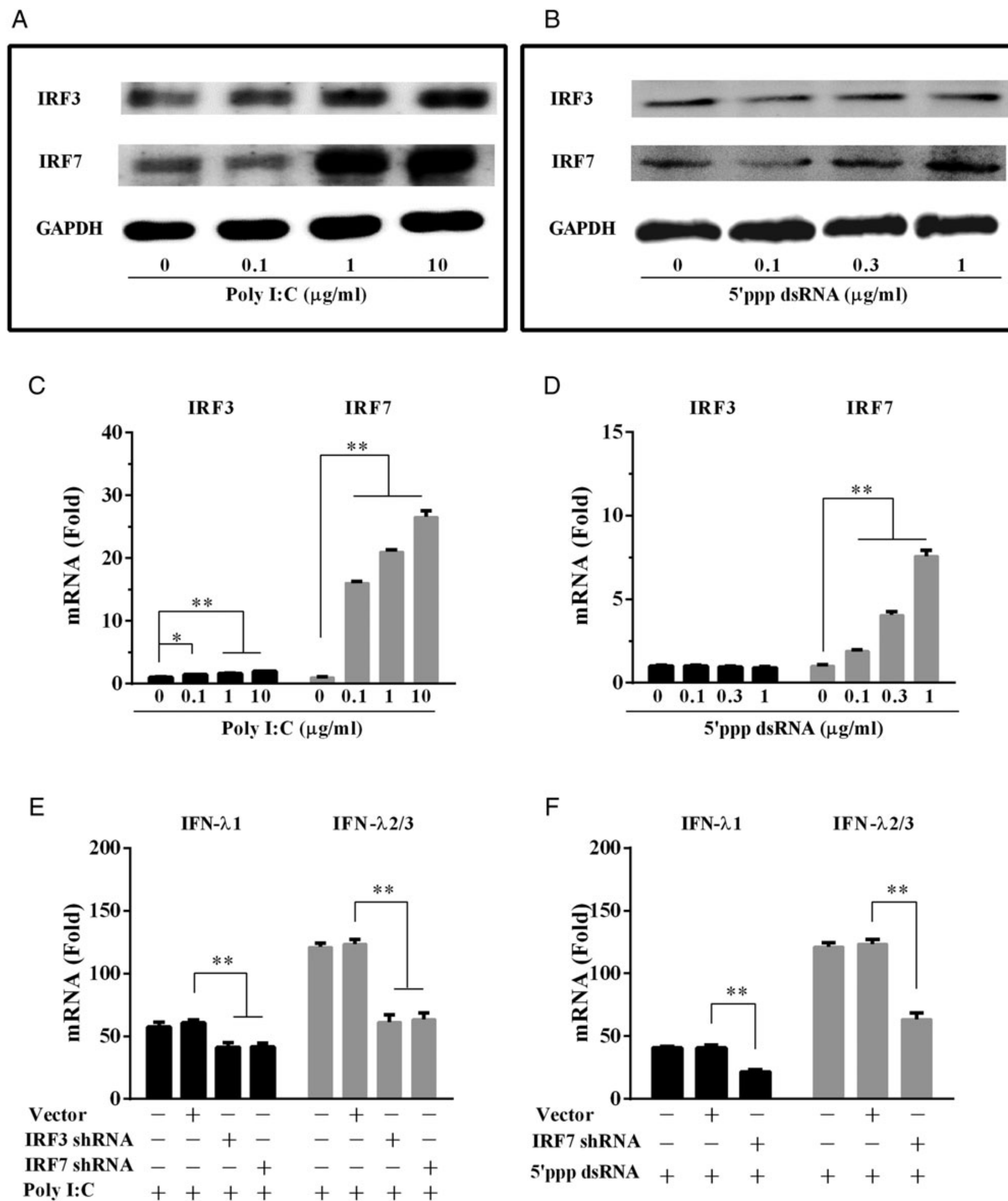

Figure 3 Effect of TLR3/RIG-I activation on IFN regulatory factors (IRFs). (**A** and **B**) End1/E6E7 cells, transfected with or without poly I:C (A) or 5′ppp dsRNA (B) at indicated concentrations for 24 h, were subjected to western blot assay using antibodies to IRF3, IRF7 or GAPDH. GAPDH was used as the loading control. (**C** and **D**) End1/E6E7 cells were transfected with or without poly I:C (C) or 5′ppp dsRNA (D) at indicated concentrations for 12 h. (**E** and **F**) shRNA-mediated knockdown of IRF3 and/or IRF7 impaired IFN-λ expression. End1/E6E7 cells were transfected with or without control vector, IRF3 shRNA or IRF7 shRNA for 48 h. Cells were then transfected with or without poly I:C (E) or 5′ppp dsRNA (F) for additional 12 h. Total cellular RNA extracted from End1/E6E7 cells was subjected to the real-time RT–PCR detection for the mRNA levels of IRF3, IRF7 and IFN-λ. The data are expressed as mRNA levels relative (-fold) to control (without poly I:C or 5′ppp dsRNA stimulation, which is defined as 1). The results are mean ± SD of triplicate cultures, representative of three experiments (*$P < 0.05$, **$P < 0.01$).

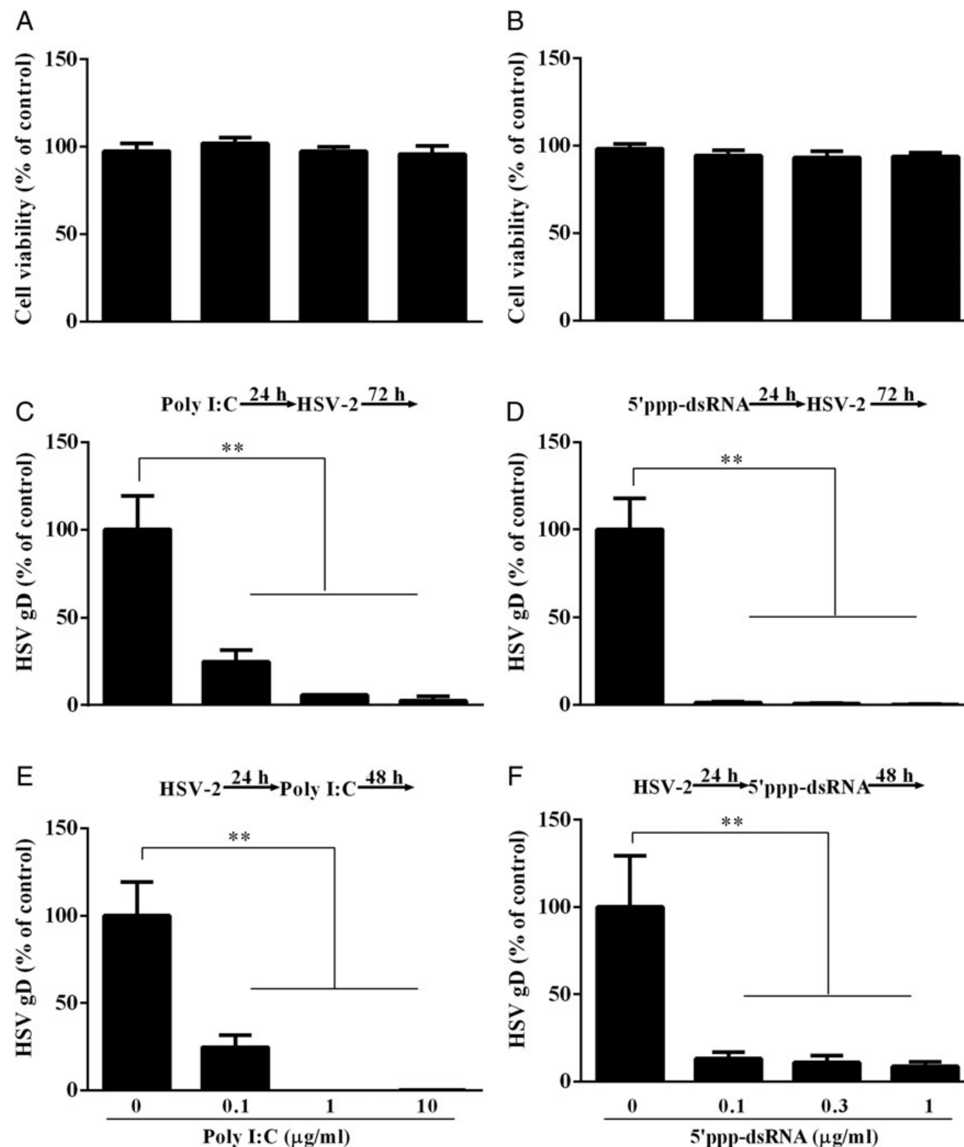

Figure 4 Effect of TLR3, RIG-I activation on HSV-2 replication. (**A** and **B**) MTT assay of End1/E6E7 cells transfected with poly I:C or 5′ppp dsRNA. End1/E6E7 cells were transfected with poly I:C (A) or 5′ppp dsRNA (B) at the concentrations indicated at the bottom of the figure. Cells viability was assessed by MTT assay 72 h post transfection. The results are mean ± SD of triplicate cultures, representative of three experiments. (**C** and **E**) End1/E6E7 cells were transfected with poly I:C at the concentrations indicated at the bottom of the figure for 24 h prior to HSV-2 infection (MOI = 0.001) (C) or transfected with poly I:C at 24 h post-infection (E). (**D** and **F**) End1/E6E7 cells were transfected with 5′ppp dsRNA at concentrations indicated at the bottom of the figure for 24 h prior to HSV-2 infection (MOI = 0.001) (D) or transfected with 5′ppp dsRNA at 24 h post-infection (F). Genomic DNA extracted from the cell cultures at 72 h post-infection was subjected to the real-time PCR for HSV-2 quantification. The data are expressed as HSV-2 gD gene levels relative (%) to the control (without poly I:C treatment, which is defined as 100). The results are mean ± SD of triplicate cultures, representing three independent experiments ($^{**}P < 0.01$).

5′ppp dsRNA. As shown in Fig. 5A and B, the anti-HSV-2 effects of poly I:C or 5′ppp dsRNA were partially reversed in the cells treated with the antibody to IL-10Rβ. The anti-HSV-2 effect of endogenous IFN-λ was also confirmed in experiments using recombinant IFN-λ1 or IFN-λ2 (Fig. 5C).

TLR3/RIG-I activation induces ISGs

It is known that IFN activation triggers an antiviral state, which is characterized by the induction of multiple IFN-stimulated genes (ISGs) (Katze *et al.*, 2002). We thus examined the effect of poly I:C or 5′ppp dsRNA on the expression of several key antiviral ISGs (ISG56, OAS-1, MxA and PKR) in epithelial cells. As shown in Fig. 6, poly I:C or 5′ppp dsRNA dose-dependently induced the expression of ISG56, OAS-1 and MxA, but not PKR, in the cells.

Poly I:C treatment reduces genital HSV-2 infection of mice

To determine whether the *in vitro* anti-HSV-2 activity mediated by TLR3/RIG-I signaling of the cervical epithelial cells has *in vivo* significance, we

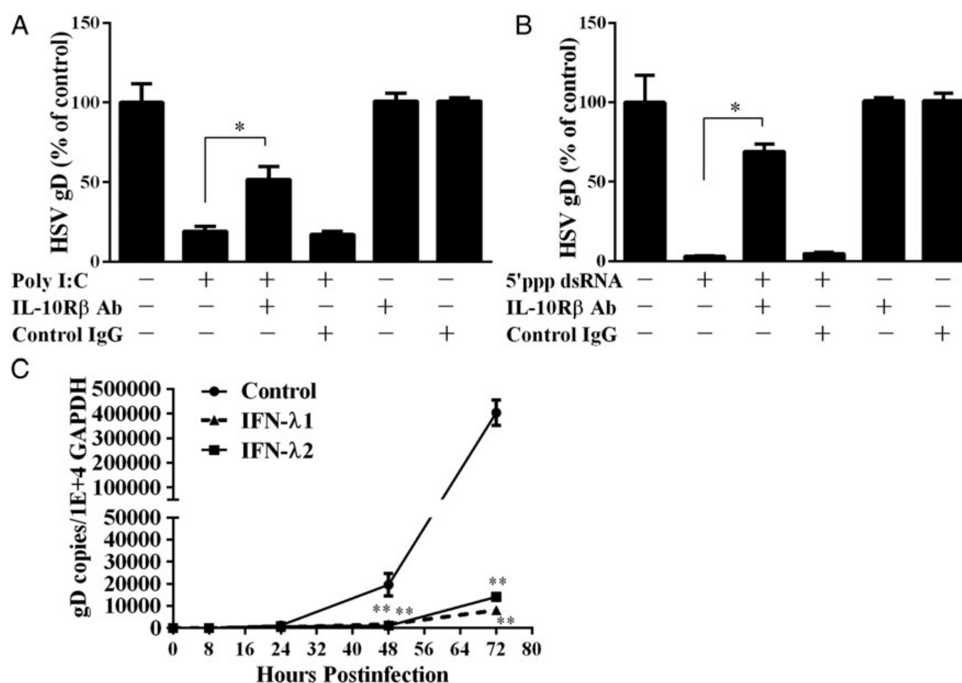

Figure 5 The role of IFN-λ in TLR3/RIG-I activation-mediated HSV-2 inhibition. (**A** and **B**) End1/E6E7 cells were treated with poly I:C (1 μg/ml) (A) or 5′ppp dsRNA (B) and anti-IL-10Rβ antibody or control IgG at the concentration of 5 μg/ml for 24 h prior to HSV-2 infection. HSV-2 replication was quantified 72 h post-infection. (**C**) End1/E6E7 cells were treated with recombinant IFN-λ1 or IFN-λ2 (100 ng/ml) for 24 h and then infected with HSV-2 (MOI = 0.001). Genomic DNA extracted from the cell cultures at the indicated time points post-infection was subjected to the real-time PCR for HSV-2 quantification by using serial dilutions of HSV-2 gD standards with known copy numbers. The results are mean ± SD of triplicate cultures, representative of three experiments (*$P < 0.05$, **$P < 0.01$).

investigated the preventive effect of poly I:C topical treatment on genital mucosa of mice. Prophylactic poly I:C treatment (16 h prior to HSV-2 infection) significantly reduced HSV-2 infection-mediated pathological damage in genital mucosa and completely protected the mice from death due to HSV-2 infection (Fig. 7 and Table II). The treatment of mice with poly I:C 4 h after HSV-2 infection also reduced the pathological damage and death by HSV-2 infection (Fig. 7 and Table II).

Discussion

As the first line of cells in contact with invading pathogens, cervical epithelial cells play an essential role in mucosal innate immunity against viral infections (Wira *et al.*, 2005; Drannik *et al.*, 2013; Cannella *et al.*, 2014). Human cervical epithelial cells express functional TLR3 as well as other TLRs (Ashkar *et al.*, 2004; Sathe and Reddy, 2014). The ligands for TLRs 3, 5, or 9 can protect genital epithelial cells from HSV-2 genital infection (Janeway and Medzhitov, 2002; Herbst-Kralovetz and Pyles, 2006; Nazli *et al.*, 2009). In addition, the mucosal delivery of TLR3 or TLR9 ligands can protect mice from HSV-2 genital infection (Ashkar *et al.*, 2004; Gill *et al.*, 2006). Compared with TLR5 or TLR9 ligand, the TLR3 ligand poly I:C has selective advantages, including less local inflammation in the genital mucosa (Ashkar *et al.*, 2004) and no splenomegaly (Alexopoulou *et al.*, 2001; Azulay-Debby *et al.*, 2007; Gill *et al.*, 2008). These advantages are clinically significant and relevant. Therefore, the present study focused on poly I:C-mediated anti-HSV-2 effects in genital epithelial cells. We demonstrated that the activation of TLR3 by poly I:C significantly enhanced the expression of all three members of the IFN-λ family (Fig. 1B), which provided a sound explanation for the poly I:C-mediated anti-HSV-2 effect. Interestingly, TLR7 or TLR9 stimulation with their ligands had little impact on IFN-λ induction (Fig. 1B), suggesting that TLR3 is a key player in the induction of IFN-λ in human cervical epithelial cells. An early study showed that there was differential induction of innate antiviral responses by TLR ligands against HSV-2 infection in primary genital epithelium of women (Nazli *et al.*, 2009).

To determine whether the TLR3 signaling pathway plays a major role in poly I:C-mediated IFN-λ induction in human cervical epithelial cells, we used TCI, an inhibitor of the TLR3 signaling pathway, to treat End1/E6E7 cells prior to poly I:C stimulation. The observation that TCI treatment could largely block the action of poly I:C on the induction of IFN-λ (Fig. 2A) supports the key role of TLR3 activation in IFN-λ induction. In addition to TLR3 signaling, the RIG-I signaling of human cervical epithelial cells also induced the expression of IFN-λ by 5′ppp dsRNA (Fig. 2C). Our further investigation of the mechanisms for the induction of IFN-λ by human cervical epithelial cells showed that the induction of IRF3 and IRF7 by poly I:C or 5′ppp dsRNA (Fig. 3A–D). The contributing role of IRF3 or IRF7 in TLR3 or RIG-I-mediated IFN-λ induction was confirmed by the knockdown experiments (Fig. 3E and F).

IFN-λ has been shown to have antiviral activities against a number of viruses (Ank *et al.*, 2006; Doyle *et al.*, 2006; Hou *et al.*, 2009; Cannella *et al.*, 2014), including HSV-2. IFN-λ can be induced by the low-risk human papillomavirus (HPV) infection, in human cervical epithelial cells

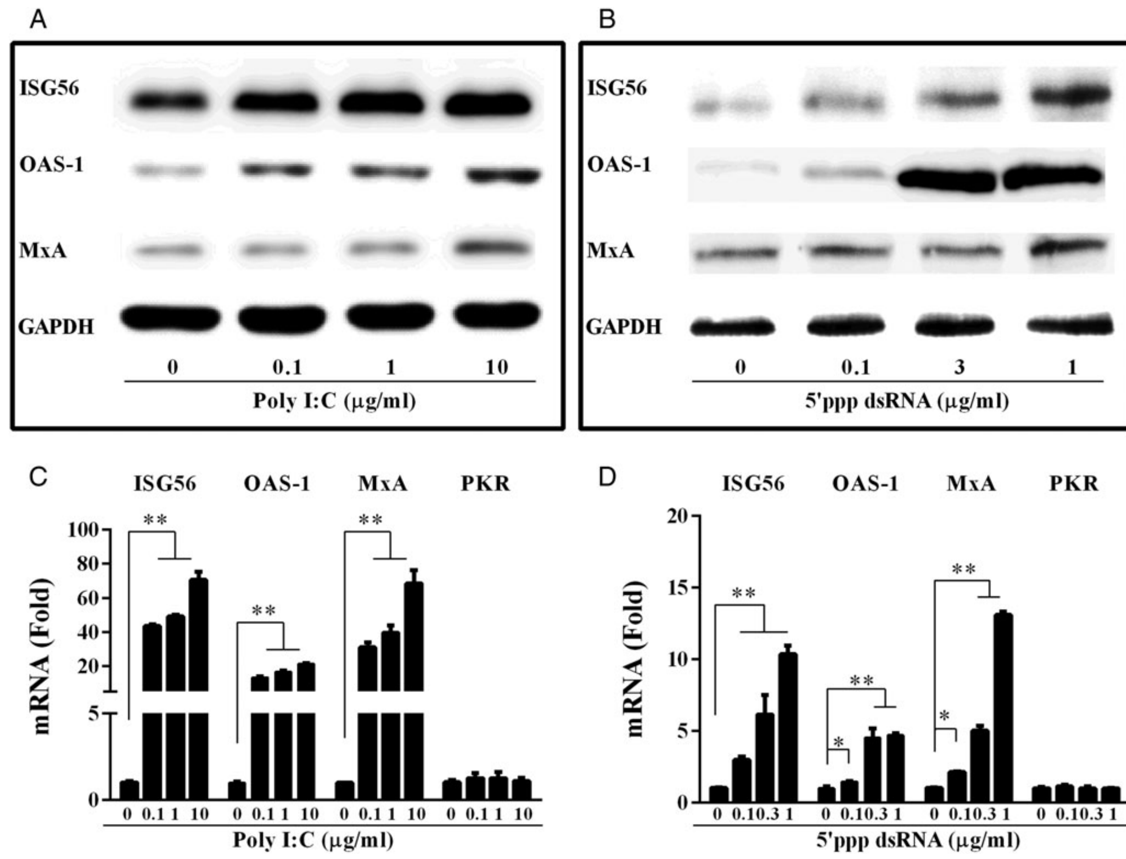

Figure 6 Effect of poly I:C and 5′ppp dsRNA on the expression of IFN-stimulated genes (ISGs). (**A** and **B**) Total proteins extracted from End1/E6E7 cells transfected with or without poly I:C (A) or 5′ppp dsRNA (B) at indicated concentrations for 24 h were subjected to western blot assay using antibodies to ISG56, MxA, OAS-1 or GAPDH. (**C** and **D**) End1/E6E7 cells were transfected with or without poly I:C (C) or 5′ ppp dsRNA (D) at indicated concentrations for 12 h. Total cellular RNA extracted from End1/E6E7 cells was subjected to the real-time RT–PCR detection for the mRNA levels of ISG56, OAS-1 MxA and PKR. The data are expressed as mRNA levels relative (-fold) to control (without treatment, defined as 1). The results are mean ± SD of triplicate cultures, representative of three experiments (*$P < 0.05$, **$P < 0.01$).

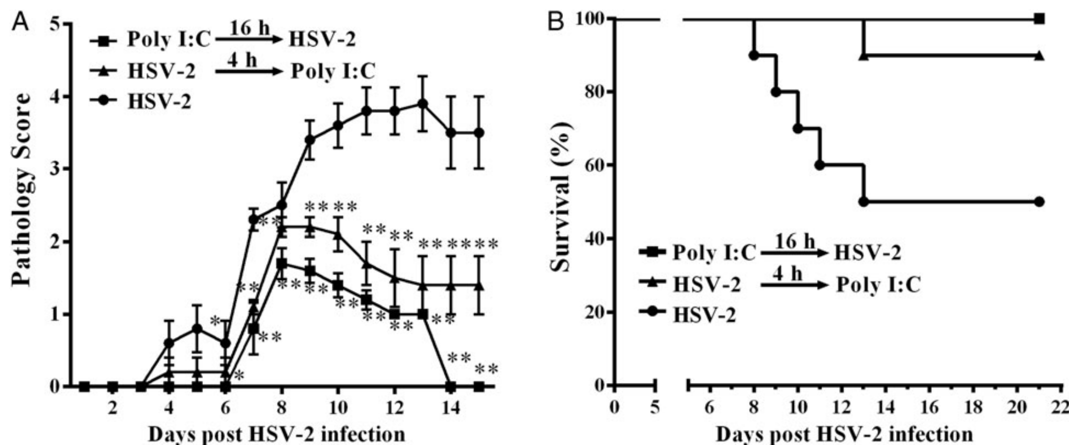

Figure 7 Effect of disease progression in HSV-2 infected mice. Poly I:C 25 μg suspended in 30 μl Lyovec transfection reagent was delivered to each mice prophylactically ($n = 10$) or therapeutically ($n = 10$), 16 h before or 4 h after, intravaginal inoculation of HSV-2 (3000 pfu/mouse). Mice were then monitored daily for genital pathology (**A**) and survival rate (**B**). Groups were compared for statistical significance relative to HSV-2 infection group ($n = 10$) by analysis of variance (*$P < 0.05$, **$P < 0.01$).

Table II Poly I:C treatment reduced HSV-2 infection-mediated pathological damage in genital mucosa.

Treatment[a]	Incidence[b] (%)	Time to symptoms[c] (days)	Survival time[c] (days)	Survival[d] (%)
Poly I:C/Lyovec, 16 h prior	60	7.6 ± 0.5	21	100
Poly I:C/Lyovec, 4 h after	80	6.7 ± 0.9	20.1 ± 2.7	90
PBS alone	100	5.9 ± 1.4	16.4 ± 4.9	50

[a]Intravaginal application of Poly I:C/Lyovec was delivered prophylactically ($n = 10$, 16 h prior to challenge) or therapeutically ($n = 10$, 4 h after challenge) relative to viral challenge with 3000 pfu.
[b]Incidence of disease is measured by the development of hair loss and erythema and is expressed as the number of mice that develop symptoms/number of the animals in the group.
[c]Mean for mice with disease signs with 15 days *p.i.* or mean survival time within 21 days *p.i.*
[d]Survival is presented as number of mice alive 21 days *p.i.*/number of animals in the group.

(Cannella *et al.*, 2014), although the mechanism(s) of the induction of IFN-λ remain to be determined. The induction of IFN-λ expression in the cervical epithelial cells is at least partially responsible for the TLR3/RIG-I activation-mediated anti-HSV-2 activity, as an antibody to IL-10Rβ (one of subunits of IFN-λ receptor) could at least partially reverse the inhibitory effect of poly I:C (Fig. 5A) or 5′ppp dsRNA (Fig. 5B) on HSV-2 replication in the cervical epithelial cells. Due to the lack of a commercial antibody to IL-28Rα (a subunit of IFN-λ receptor), we were unable to demonstrate whether IFN-λ is a sole contributor of TLR3/RIG-I activation-mediated HSV-2 inhibition. Nevertheless, we did not expect a complete blockage of the poly I:C effect on HSV-2 by the antibody to IFN-λ receptor, as TLR3 activation also induced IFN-β expression, that could also contribute to the suppression of HSV-2 (Nazli *et al.*, 2009).

Like many viruses that can establish persistent infections (Finlay and McFadden, 2006), HSV-2 has evolved several mechanisms to subvert and repress the type I IFN-mediated antiviral immunity (Harle *et al.*, 2002; Kotenko *et al.*, 2003; Murphy *et al.*, 2003; Pletnev *et al.*, 2003; Duerst and Morrison, 2004; Melroe *et al.*, 2004). We demonstrated that the ability of poly I:C to induce IFN-β was compromised in HSV-2-infected human cervical epithelial cells (Supplementary Fig. 2C). This finding is in the line with studies by others showing that HSV-2 inhibits intracellular IFN-β expression in human embryonic kidney cells and human vaginal epithelial cells (Yao and Rosenthal, 2011; Xing *et al.*, 2013). Interestingly, HSV-2 infection had little effect on poly I:C-mediated induction of IFN-λ at both mRNA and protein levels (Fig. 1F and G). This finding suggests that IFN-λ-mediated antiviral immunity is critical in the protection of cervical epithelial cells from HSV-2 infection, as it is not impaired by HSV-2 infection. Therefore, the induction of IFN-λ and its associated antiviral factors by TLR3/RIG-I activation in cervical epithelial cells is therapeutically important for people infected with HSV-2. This statement is supported by our *in vitro* as well as *in vivo* studies. We demonstrated that TLR3/RIG-I activation in cervical epithelial cells inhibited HSV-2 infection/replication (Fig. 4). The protective effect of poly I:C or 5′ppp dsRNA on human cervical epithelial cells was observed even after HSV-2 infection had taken place (Fig. 4E and F). These findings are supported by the previous reports (Herbst-Kralovetz and Pyles, 2006; Zhou *et al.*, 2009b; Li *et al.*, 2012) showing that TLR3 activation results in the inhibition of HSV-2 infection of both murine and human cervical epithelial cells. In addition, we showed that the topical treatment of genital mucosa with poly I:C could protect the study mice from genital HSV-2 infection (Fig. 7). Because 5′ppp dsRNA failed to induce type I IFNs in a mouse macrophage line (data not shown), we did not examine the *in vivo* impact of 5′ppp dsRNA on the prevention of HSV-2 infection.

Taken together, we have, for the first time, provided the experimental evidence that human cervical epithelial cells possess a functional TLR3/RIG-I signaling system, the activation of which can mount an IFN-λ-mediated anti-HSV-2 response. The ability to mount an effective innate immune response to HSV-2 infection by cervical epithelial cells is clinically significant as it has potential in clinical use for genital protection from HSV-2 infection. However, due to the limitation of using the cervical epithelial cell line in this study, more studies with primary cells and suitable animal models are necessary to determine whether the TLR3/RIG-I signaling in cervical epithelial cells is clinically beneficial for anti-HSV-2 mucosal immunity in the female reproductive tract. These studies will be critical for the design and development of TLR3/RIG-I signaling-based intervention and treatment strategies for the control of HSV-2 transmission and infection.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

L.Z. and W.-Z.H. conceived and designed the experiments; L.Z., J.-L.L. and Y.Z. performed the experiments; L.Z., K.Z., M.S. and W.-Z.H. analyzed the data; J.-B.L. S.L., J.-G.W. and J.-F.G. contributed to the reagents, materials and analysis tools; L.Z., J.-L.L. and W.-Z.H. wrote the paper.

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Conflict of interest

The authors declare no competing financial interests.

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