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Human Cervical Epithelial Cells Release Antiviral Factors and Inhibit HIV Replication in Macrophages

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Keywords

Human cervical epithelial cells · Toll-like receptor 3 · Macrophages · Interferon-stimulated genes · HIV

Abstract

The female reproductive tract is a major site of HIV sexual transmission. We here examined whether human cervical epithelial cells (HCEs) can be immunologically activated and produce antiviral factors against HIV. We demonstrated that HCEs (End1/E6E7 cells) possess the functional toll-like receptor (TLR)3 signaling system, which could be activated by Poly I:C and induce multiple cellular HIV restriction factors. The treatment of primary human macrophages with supernatant (SN) from TLR3-activated End1/E6E7 cell cultures resulted in HIV inhibition. This SN-mediated HIV inhibition was mainly through the induction of interferons (IFN)-β and IFN-λs, as the antibodies to IFN-β or IFN-λs receptor could effectively block the SN-mediated anti-HIV effect. Further studies showed that the incubation of macrophages with SN from the activated cervical epithelial cell cultures induced the expression of a number of IFN-stimulated genes (ISGs), including IFN-stimulated gene (ISG15), ISG56, 2′, 5′-oligoadenylate synthetase 1 (OAS 1), OAS 2, Myxovirus Resistance A (MxA), MxB, and Guanylate-binding protein 5 (GBP5). In addition, TLR3-activated cells produced the CC chemokines [regulated on activation, normal T cell expressed and secreted (RANTES), Human macrophage inflammatory protein 1 alpha (MIP-1α), MIP-1β] the ligands of HIV entry co-receptor CCR5. These observations support further studies on HCEs as potentially crucial and alternative targets for immunological intervention to control and prevent HIV sexual transmission.

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Introduction

In the absence of a protective vaccine or a cure for HIV infection, HIV continues to spread globally with an average of 2.5 million new infections per year, most of which are through sexual transmission [1]. Today, women worldwide are more likely to be infected with HIV than

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men, and transmission to women through the female reproductive tract (FRT) accounts roughly for one third of all HIV transmission events. Among these cases, more than 90% of these events occur by unprotected sexual intercourses [2]. During sexual intercourse, HIV in male semen traverses the female genital epithelium [2, 3], establishing infection in underlying CD4$^+$ target cells, including T cells, dendritic cells, Langerhans cells, and macrophages [3]. Studies have shown that semen contains an HIV-enhancing factor that can facilitate HIV replication up to a thousand-fold [4]. As the first layer cells in the FRT, the mucosal epithelium is in the direct contact with HIV or infected cells. Thus, the interaction of HIV with the mucosal epithelium in the FRT should have a role in HIV sexual transmission.

Physical barriers of the FRT are composed of the vulva and cervix, the epithelial surface of the mucosa, the basement membrane of ovarian follicles, and the zona pellucid of the oocyte. Among these components, epithelial cells are essential in host defense against pathogens [5–8], as they can regulate the movement of molecules across the epithelium and prevent the entry of microbes [9]. Immune cells, such as T cells, DCs, and macrophages are present beneath and between epithelial cells layer [10]. The absence of the superficial epithelium results in weakly joined cells and may allow pathogens to penetrate the epithelial layer. HIV infection is initiated primarily at cervical epithelium as the portal of entry through sexual transmission [8]. The high HIV transmission efficiency could be due to the fact that the cervical mucosal surface is mainly composed of a single layer of epithelial cells [10].

It has been reported that the human cervical epithelial cells (HCEs) could secrete specific mucosal proteins, cytokines, CC chemokines, proinflammatory mediators, and adhesion molecules [11–13]. Importantly, the cervical cells could recognize pathogen-associated molecular patterns on microbes through pattern recognition receptors, including the families of toll-like receptors (TLRs) and RIG-I-like receptors [9]. Several studies including ours [14–17] have shown that HCEs execute immunological functions against viral infections by activation of pattern recognition receptors such as TLRs and RIG-I-like receptors, which can mount a type I interferons (IFNs) and/or type III IFNs (IFN-λs)-mediated antiviral responses. These findings indicate that HCEs have a crucial role in the FRT immunity against pathogen invasion. However, there is little information about whether HCEs can produce antiviral factors against HIV infection. In this study, we examined the ability of HCEs to mount TLR3 activation-mediated innate immune response and produce HIV restriction factors that suppress HIV replication in human macrophages. We also explored the cellular and molecular mechanisms underlying the HCEs-mediated action on HIV infection of macrophages.

Materials and Methods

Cells Culture

The HCE line (End1/E6E7 cells) has been extensively studied and well established as a human cervical epithelial model [18–20]. The cells were cultured in keratinocyte growth medium (Gibco, Grand Island, NY, USA) supplemented with the provided recombinant epidermal growth factor (0.1 ng/mL) and bovine pituitary extract (50 μg/mL). Monocytes from human peripheral blood were obtained from Human Immunology Core at the University of Pennsylvania (Philadelphia, PA, USA). The Core has the Institutional Review Board approval for blood collection from healthy donors. Freshly isolated monocytes were cultured in the 48-well plate at a density of 2.5×10^5 cells/well in complete DMEM containing 10% FBS and differentiated into macrophages after 7-day culture.

Virus and Reagents

HIV Bal, a CCR5-tropic strain, was used for the infection experiments. The virus was obtained from the AIDS Research and Reference Reagent Program of the National Institute of Health (NIH, Bethesda, MD, USA). Rabbit antibodies against interferon regulatory factor 3 (IRF 3), p-IRF3, IRF7, p-IRF7, IFN-stimulated genes (ISGs), and signal transducer and activator of transcription proteins (STATs) were purchased from Cell Signaling Technology (Danvers, MA, USA). All culture plasticwares were obtained from Corning (Corning, NY, USA). Unless otherwise specified, all other culture reagents were purchased from Invitrogen (San Diego, CA, USA).

TLR3 Activation

End1/E6E7 cells were transfected with Poly I:C (TLR3 ligand), using LyoVec™ transfection reagent (InvivoGen, San Diego, CA, USA) according to the manufacturer's manual. LyoVec™-treated cells were used as a vehicle control. The cell culture medium with transfection reagent was replaced with fresh keratinocyte growth medium at 6 h post-transfection. For disruption of TLR3 function, cells were treated with 100 nM of TLR3/dsRNA Complex Inhibitor (abbreviated as TLR3/dsRNA complex inhibitor; EMD Millipore, Inc., Billerica, MA, USA) for 1 h prior to Poly I:C transfection. In order to determine the role of the Janus kinases (JAK)/STAT pathway against HIV, 2 μM JAK inhibitor (JAK Inh) or DMSO was added to the macrophage cultures for 1 h prior to Poly I:C/SN treatment. Culture supernatant (SN) was collected at 48 h post-stimulation for the detection of IFN (IFN-β and IFN-λs) and CC chemokines by enzyme-linked immunosorbent assay (ELISA) or Cytometric Bead Array (CBA).

Preparation of End1/E6E7 SN and HIV Infection

The End1/E6E7 cells were transfected with Poly I:C at different concentrations (0.1, 1, 10 μg/mL), and cell-free SN collected from the Poly I:C-stimulated End1/E6E7 cell cultures was used as End1/

E6E7 SN. TZM-bl cells and primary human macrophages were infected with an equal amount (p24 30 ng/10^6 cells) of cell-free HIV Bal for 3 h at 37 °C in the presence or absence of End1/E6E7 SN.

TZM-bl Assay

The anti-HIV activity was assessed with the TZM-bl, a commonly used cell line for evaluating the efficacy of neutralizing antibodies and small molecular inhibitors in HIV research [21]. TZM-b1 cells contain the reporter gene (firefly luciferase) for the quantitative detection of HIV Tat-dependent luciferase activities after HIV infection. The cells were prepared and maintained as previously described [21].

End1/E6E7 SN Treatment of TZM-bl Cells and Macrophages

TZM-bl cells were seeded in a 96-well microtiter plate (10^4 cells/well) and allowed to achieve confluence overnight at 37 °C. The cells were then treated with or without End1/E6E7 SN for 24 h at a ratio of 10% (volume to volume ratio [v/v]) prior to, simultaneously or post HIV Bal infection. Luciferase activity of TZM-bl cells was measured at 48 h post HIV Bal infection. Macrophages were treated with or without End1/E6E7 SN for 24 h, and then infected with HIV Bal strain. HIV infected-macrophages were cultured in medium conditioned with End1/E6E7 SN. To study the roles of IFN-β or IFN-λs in End1/E6E7 SN-mediated anti-HIV activity, SN was first incubated with the antibodies to IFN-β or IFN-λs receptor for 1 h prior to the addition to macrophage cultures. HIV infectivity was determined at different time points post SN-treatment.

RNA Extraction and Real-Time Polymerase Chain Reaction

Total cellular RNA from End1/E6E7 cells, macrophages, or cell-free SN was extracted using TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA) as described previously [17]. Reverse transcription was performed using the random primer, dNTPs, M-MLV reverse transcriptase, and RNase inhibitor (Promega Co., Madison, WI, USA) according to the manufacturer's instruction. The real-time polymerase chain reaction (RT-PCR) for the quantification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HIV gag, IFN-β, IFN-λs, IRF3, IRF7, ISGs, STATs, and CC chemokines mRNA was performed with IQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, USA) as described previously [17, 22]. The levels of GAPDH mRNA were used as an endogenous reference to normalize the quantities of target mRNA. The primers were synthesized by Invitrogen Inc. The sequences of oligonucleotide primers are shown in online supplementary Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000490586).

Detection of Early Products of HIV RT Strong-Stop DNA

Strong-stop DNA, the first product of HIV reverse transcription, was used for the assessment of viral entry [23, 24]. To study the roles of CC chemokines [regulated on activation, normal T cell expressed and secreted (RANTES), human macrophage inflammatory protein 1 alpha (MIP-1α), MIP-1β] in End1/E6E7 SN-mediated anti-HIV activity, cellular DNA, including genomic and viral DNA products, was isolated at 3-h post-infection with DNaseI-treated HIV Bal using the QIAamp DNA Blood Mini kit (Qiagen, Inc., Valencia, CA, USA). Strong-Stop DNA was then quantified by the RT-PCR with HIV LTR primers (online suppl. Table 1). The concentration of the each DNA sample was normalized by equal DNA loading confirmed with primers for GAPDH.

ELISA and CBA

Cell-free SN from End1/E6E7 cell cultures stimulated with or without Poly I:C for 48 h was collected for analysis of IFN-β, IFN-λs with ELISA kits (IFN-β, eBioscience, San Diego, CA, USA; Human Interleukin-29 (IL-29)/IFN-λ1 and Human IL-28A/IFN-λ2, R&D system Inc., Minneapolis, MH, USA) and RANTES, MIP-1α, MIP-1β with the CBA flex set (BD, Franklin Lakes, NJ, USA). ELISA and CBA were performed according to the manufacturer's instructions.

Western Blotting

Total cell lysates were prepared by 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 were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis precast gels and transferred to an Immunobiolon-P membrane (Millipore, Germany). Nonspecific sites were blocked with 5% nonfat dried milk before being incubated with Antibody. Blots were developed with SuperSignal West Pico Chemiluminescent Substrated (Thermo Fisher Scientific, Waltham, MA, USA). Densitometric analysis was performed by using ImageJ 1.44 software (National Institutes of Health).

Data Analysis

When appropriate, data were obtained from at least 3 independent experiments and expressed as mean ± SD. For comparison of the mean of 2 groups, the statistical significance was measured by Student *t* test. To compare the difference between multiple groups, statistical significance was analyzed using a one-way analysis of variance followed by 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

SN from Poly I:C-Stimulated End1/E6E7 Cell Cultures Suppresses HIV Replication

We first determined whether TLR3-activated HCEs can suppress HIV replication. As shown in Figure 1, SN from Poly I:C-stimulated End1/E6E7 cell cultures could inhibit HIV replication in TZM-bl cells. The inhibitory effect on HIV was observed under different SN treatment conditions, either before or simultaneously or after HIV infection. The highest inhibition was observed in the cells pretreated for 24 h with End1/E6E7 SN and then infected with HIV (Fig. 1a). We next examined whether End1/E6E7 SN has the ability to inhibit HIV replication in primary human macrophages. As demonstrated in Figure 2a, HIV Bal infection could induce syncytia in macrophage cultures. However, treatment of HIV-infected macrophages with End1/E6E7 SN sig-

nificantly reduced virus-induced giant syncytia. In addition, HIV replication was also significantly inhibited in macrophages pretreated with End1/E6E7 SN (Fig. 2b–e). The degree of HIV suppression in macrophages was correlated with the doses of Poly I:C used for End1/E6E7 cells stimulation (Fig. 2b, d) and the ratio (volume to volume ratio [v/v]) of SN used for the treatment (Fig. 2c, e).

Activation of TLR3 Induces IFNs

To understand the mechanism(s) of End1/E6E7 SN-mediated HIV inhibition, we determined the effect of Poly I:C on IFNs expression in End1/E6E7 cells. As shown in online supplementary Figure 1a–c, Poly I:C stimulation of End1/E6E7 cells upregulated the expression of IFN-β and IFN-λs at both mRNA and protein levels. And the induction effect of Poly I:C could be compromised by the TLR3/dsRNA complex inhibitor (online suppl. Fig. 1d).

Activation of TLR3 Induces the Phosphorylation of IRF3, IRF7

To determine the mechanism(s) of IFN induction, we examined the effect of TLR3 activation of End1/E6E7 cells on the phosphorylation of IRF3 and IRF7, the key regulators of IFNs [25–28]. As shown in online supplementary Figure 2, Poly I:C stimulation enhanced the phosphorylation of both IRF3 and IRF7.

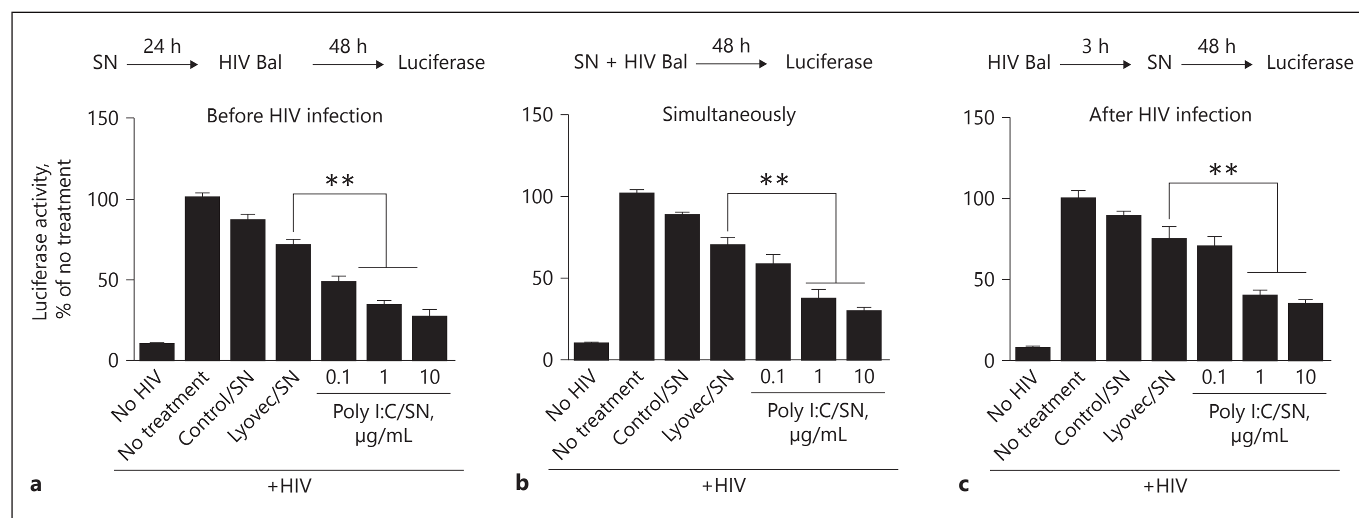


Fig. 1. Effect of supernatant (SN) from Poly I:C-stimulated End1/E6E7 cell cultures on HIV Bal infection of TZM-bl cells. TZM-bl cells were treated with or without SN (10%, volume to volume ratio [v/v]) from End1/E6E7 cell cultures stimulated with Poly I:C at indicated concentrations for 24 h prior to (**a**), simultaneously (**b**) or after HIV infection (**c**). Luciferase activity of TZM-bl cells was measured at 48 h post HIV infection. The results are the mean ± SD of triplicate cultures, representative of 3 experiments (** $p < 0.01$).

Fig. 2. Supernatant (SN) from Poly I:C-stimulated End1/E6E7 cell cultures inhibits HIV replication in macrophages. End1/E6E7 cells were transfected with or without Poly I:C at different concentrations (0.1, 1, and 10 µg/mL) for 6 h and then cultured for 42 h. Cell culture SN collected at 42 h after Poly I:C stimulation was used to treat macrophages. **a** Effect of SN from Poly I:C-stimulated End1/E6E7 cell cultures on HIV-induced syncytium formation in macrophages. The morphology of uninfected and HIV Bal-infected macrophages with or without SN pretreatment was observed and photographed under a light microscope (magnification, ×100). The arrows indicate giant syncytium formation. **b**, **d** Macrophages were pretreated for 24 h with or without SN from Poly I:C-activated End1/E6E7 cell cultures at a ratio of 10% (v/v) and then infected with HIV. Total RNA extracted from macrophages (**b**) or cell-free SN (**d**) was subjected to real-time polymerase chain reaction (RT-PCR) for HIV GAG gene quantification 8 days post HIV infection **c**, **e** Macrophages were pretreated for 24 h with or without SN from Poly I:C-activated End1/E6E7 cell cultures at a ratio of 1, 5, 10, or 20% (v/v) and then infected with HIV. Total RNA extracted from macrophages (**c**) or cell-free SN (**e**) was subjected to RT-PCR for HIV GAG gene quantification 8 days post HIV infection. The data were expressed as HIV gag levels relative (%) to control (without treatment, which is defined as 100%). The results are the mean ± SD of triplicate cultures, representative of 3 experiments (* $p < 0.05$, ** $p < 0.01$).

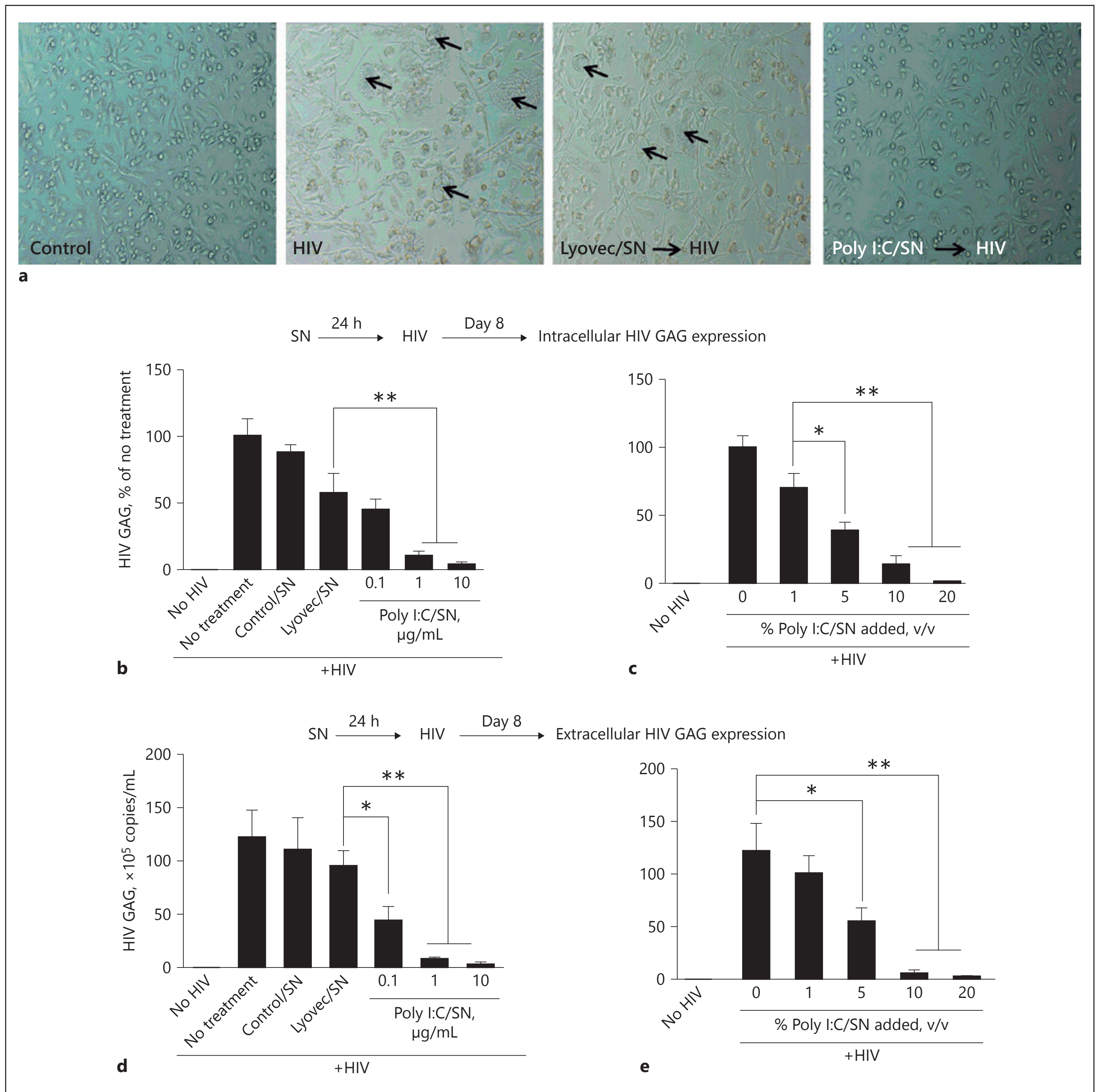
(For figure 2 see next page.)

IFN-β and IFN-λs in End1/E6E7 SN Contribute to HIV Inhibition

To investigate whether the induced IFN-β and IFN-λs are responsible for End1/E6E7 SN-mediated anti-HIV activity, macrophages were cultured in the media containing the anti-IFN-β or anti-IFN-λs receptor subunits (anti-IL-10Rβ and anti-IL-28Rα) antibody prior to the addition of End1/E6E7 SN. As demonstrated in Figure 3a, the antibodies to IFN-β or IFN-λs receptor partially blocked the effect of End1/E6E7 SN on HIV inhibition in macrophages.

End1/E6E7 SN Induces STAT1, STAT2, and STAT3 in Macrophages

Because IFN-β and IFN-λs are the key activators of the JAK/STAT pathways [29–32], we next investigated the



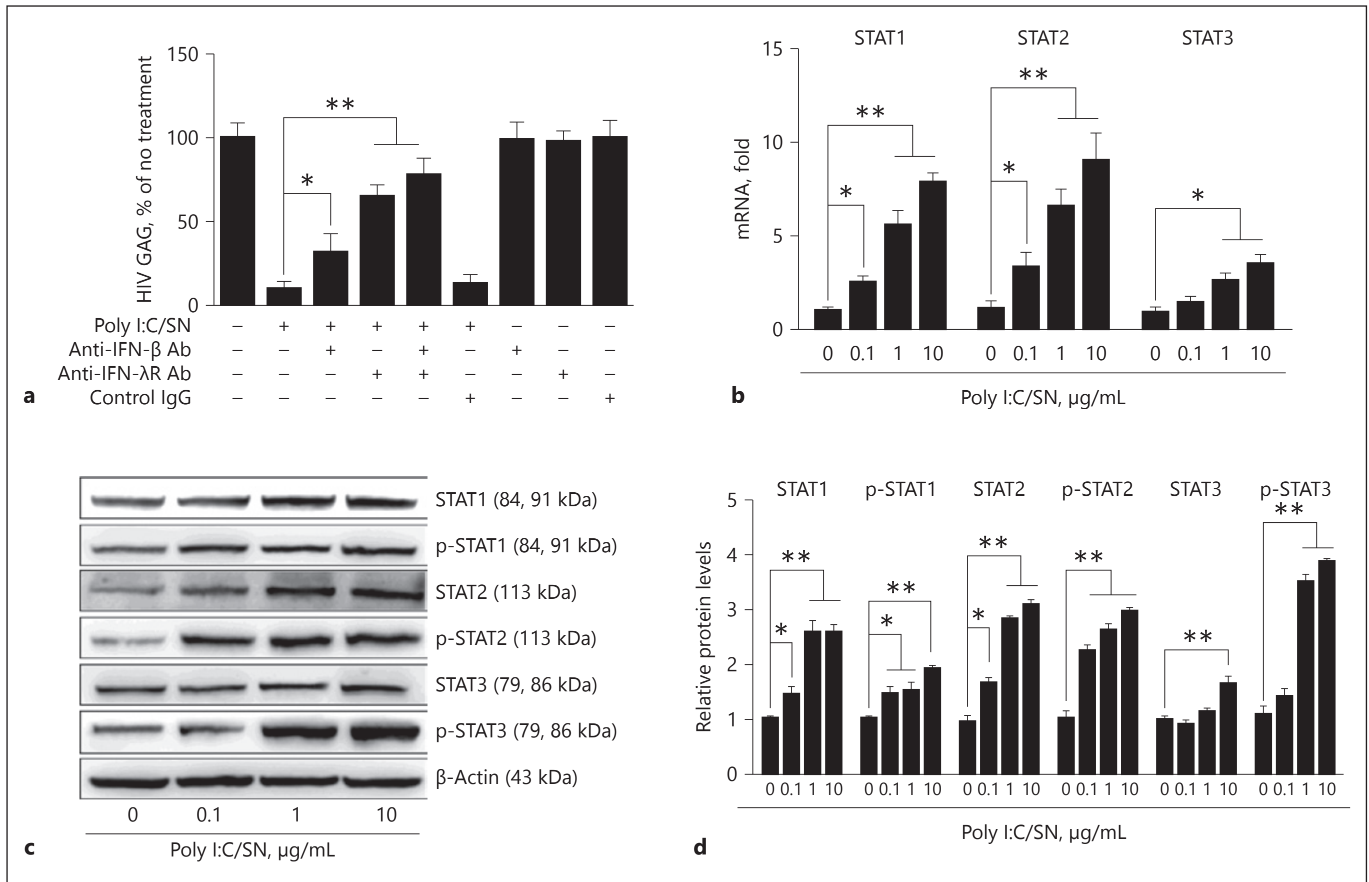


Fig. 3. Antibodies to interferons (IFN)-β or IFN-λs compromise End1/E6E7 cell culture supernatant (SN)-mediated anti-HIV activity. **a** Poly I:C-stimulated End1/E6E7 cell culture SN (10%, v/v) was preincubated with anti-IFN-β (10 μg/mL) for 1 h and then used to treat macrophages 24 h prior to HIV Bal infection. For IFN-λs receptor pretreatment, the anti-IL-10Rβ antibody and anti-IL-28Rα antibody (10 μg/mL) were added to treat macrophages for 1 h prior to the addition of SN (10%, v/v). Total cellular RNA was subjected to real-time polymerase chain reaction (RT-PCR) for HIV GAG gene quantification 8 days post HIV infection. The data were expressed as HIV gag levels relative (%) to control (without treatment, which is defined as 100%). **b** Macrophages were cultured in medium containing SN (10%, v/v) from Poly I:C-stimulated End1/E6E7 cell cultures for 12 h. Total cellular RNA extracted from macrophages was subjected to RT-PCR for STAT1, STAT2, and STAT3 mRNA quantification. **c**, **d** Macrophages were treated with SN (10%, v/v) from End1/E6E7 cell cultures stimulated with indicated concentrations of Poly I:C for 6 h. Cellular proteins were subjected to Western blot assay using antibodies to STAT1, p-STAT1, STAT2, p-STAT2, STAT3, or p-STAT3. β-Actin was used as the loading control. The results are the mean ± SD of triplicate cultures, representative of 3 experiments (* $p < 0.05$, ** $p < 0.01$).

effect of End1/E6E7 SN on the induction of STAT1, STAT2, and STAT3. As shown in Figure 3b–d, End1/E6E7 SN was able to induce the expressions of STAT1, STAT2, and STAT3 in macrophages.

End1/E6E7 SN Induces ISG in Macrophages

The action of IFNs on virus-infected cells elicits an antiviral state, which is characterized by the induction of ISGs. To further determine the mechanism(s) involved in the End1/E6E7 SN-mediated anti-HIV action, we then investigated whether End1/E6E7 SN has the ability to induce the expression of the antiviral ISGs in macrophages. As shown in Figure 4, the expressions of IFN-stimulated gene (ISG15), ISG56, 2′, 5′-oligoadenylate synthetase 1 (OAS 1), OAS 2, myxovirus resistance A (MxA), MxB, and guanylate-binding protein 5 (GBP5). were upregulated in macrophages treated with SN from End1/E6E7 cell cultures.

Inhibition of JAK/STAT Pathway Compromises the Induction of ISGs and Anti-HIV Activity by SN from Poly I:C-Stimulated End1/E6E7 Cells

To investigate whether the induction of ISGs and anti-HIV activity by SN from Poly I:C-stimulated End1/E6E7 cell cultures are associated with JAK/STAT signaling, we

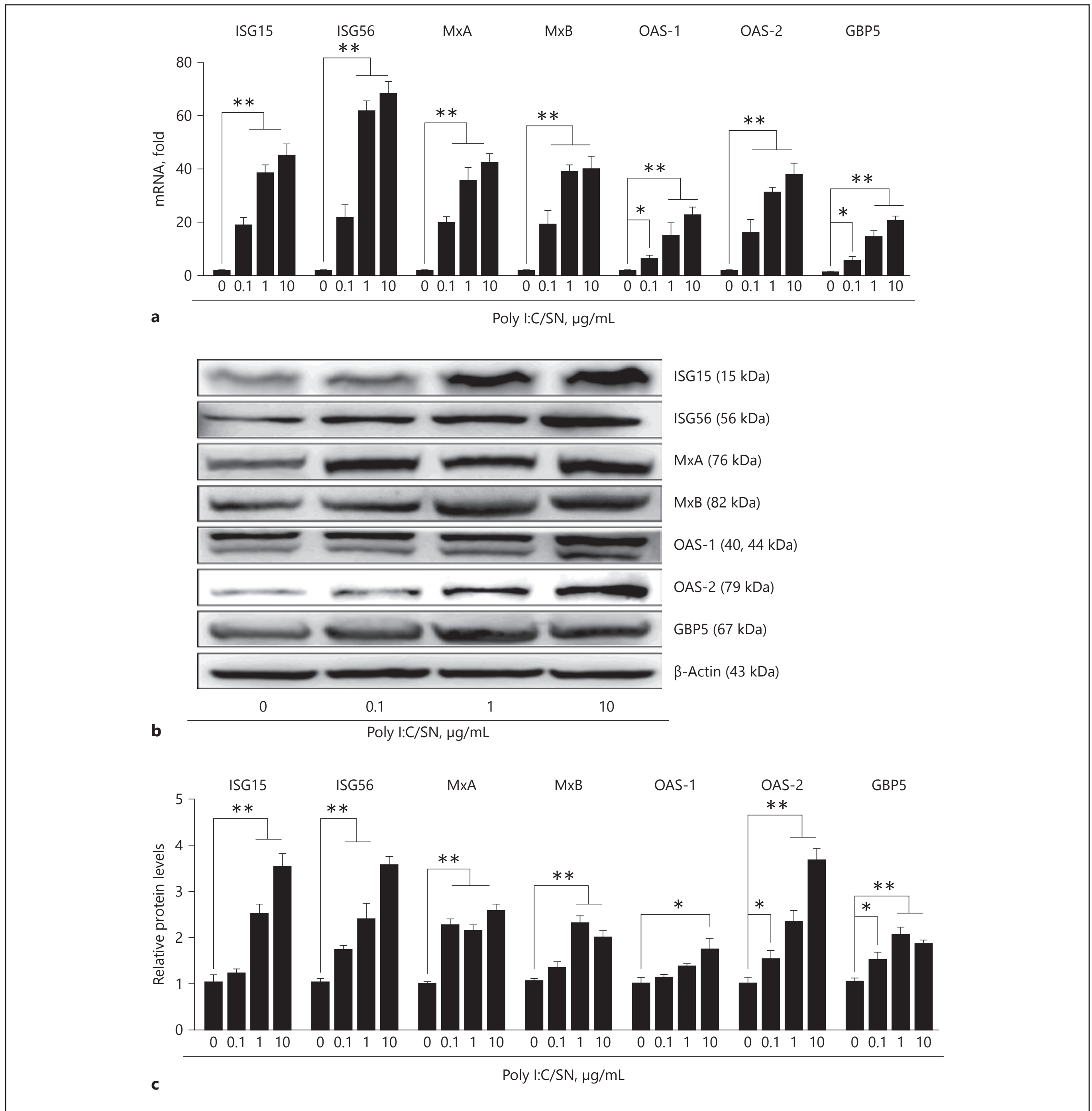


Fig. 4. Effect of supernatant (SN) from Poly I:C-stimulated End1/E6E7 cell cultures on the expression of IFN-stimulated genes (ISGs) in macrophages. End1/E6E7 cells were transfected with or without 1 μg/mL Poly I:C for 6 h and then cultured for 42 h after removal of Poly I:C. **a** Macrophages were cultured with SN (10%, v/v) from End1/E6E7 cell cultures stimulated with Poly I:C at indicated concentrations for 12 h. Total cellular RNA extracted from macrophages was subjected to real-time polymerase chain reaction for ISG15, ISG56, MxA, MxB, OAS-1, OAS-2, GBP5 mRNA quantification. The data were expressed as RNA levels relative (fold) to control (without SN treatment, which is defined as 1). **b**, **c** Macrophages were treated with SN (10%, v/v) from End1/E6E7 cell cultures stimulated with indicated concentrations of Poly I:C for 24 h. Cellular proteins were subjected to Western blot assay using antibodies to ISG15, ISG56, MxA, MxB, OAS-1, OAS-2, GBP5. β-Actin was used as a loading protein control. The results are the mean ± SD of triplicate cultures, representative of 3 experiments (* $p < 0.05$, ** $p < 0.01$).

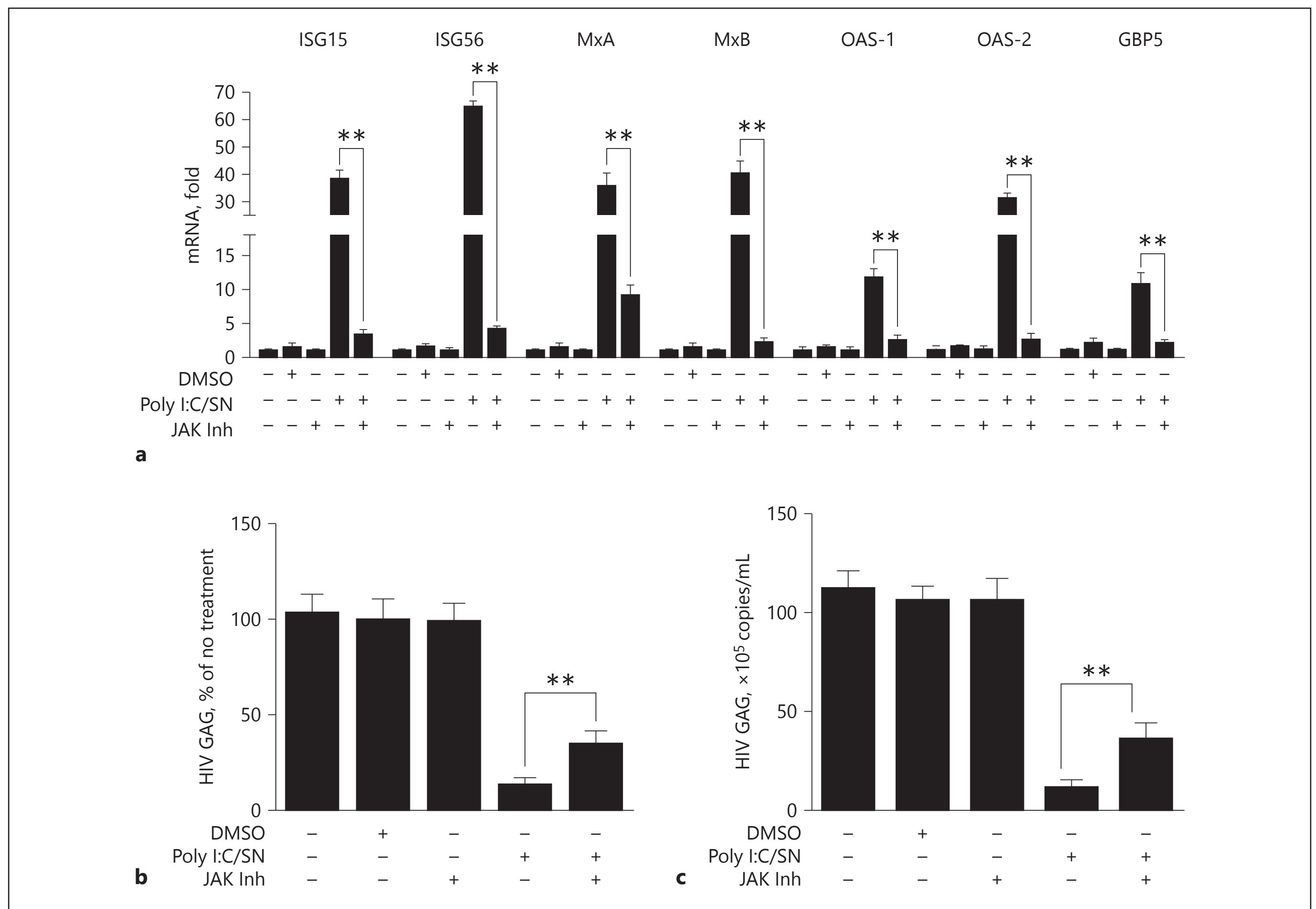


Fig. 5. JAK Inhibitor compromises the induction of interferons (IFN)-stimulated genes (ISGs) and anti-HIV activity by SN from Poly I:C-stimulated End1/E6E7 cell cultures. **a** Macrophages were treated with 2 μM JAK inhibitor (JAK Inh) or DMSO for 1 h and then incubated with Poly I:C/SN for additional 12 h. Cellular RNA was subjected to real-time polymerase chain reaction (RT-PCR) for indicated genes and GAPDH RNA. Data are expressed as indicated gene RNA levels relative (fold) to untreated control, which is defined as 1. **b**, **c** Macrophages were treated with or without JAK inhibitor (JAK Inh) for 1 h and incubated with Poly I:C/SN for additional 24 h prior to HIV infection. After washing away unbound virus, fresh medium containing Poly I:C/SN or JAK Inhibitor was added to the cultures. Total RNA extracted from macrophages (**b**) or cell-free supernatant (**c**) was subjected to RT-PCR for HIV GAG gene quantification 8 days post HIV infection. Data are shown as mean ± SD for 3 independent experiments (* $p < 0.05$, ** $p < 0.01$).

examined the effect of the JAK/STAT pathway inhibitor (JAK Inh). In the presence of JAK Inh (2 μM), Poly I:C/SN-induced expression of ISGs was inhibited (Fig. 5a). In addition, JAK Inh compromised Poly I:C/SN-mediated anti-HIV effects (Fig. 5b, c).

Role of CC Chemokines in Poly I:C-Mediated Anti-HIV Activity

We next examined the effect of Poly I:C stimulation on the expression of CC chemokines in End1/E6E7 cells. As shown in Figure 6a, b, Poly I:C induced the expression of CC chemokines (RANTES, MIP-1α, MIP-1β) at both mRNA (Fig. 6a) and protein (Fig. 6b) levels in a Poly I:C concentration-dependent manner. The strong-stop DNA is the first product of HIV reverse transcription [33, 34]. So we could measure the HIV entrance by detection of the 199-nucleotide minus strong-stop DNA. As shown in Figure 6c, pretreatment of macrophages with SN from Poly I:C-stimulated End1/E6E7 cell cultures blocked HIV entry into macrophages, while neutralization of RANTES, MIP-1α, and MIP-1β partially blocked the effect of End1/E6E7 SN on HIV inhibition in macrophages (Fig. 6d).

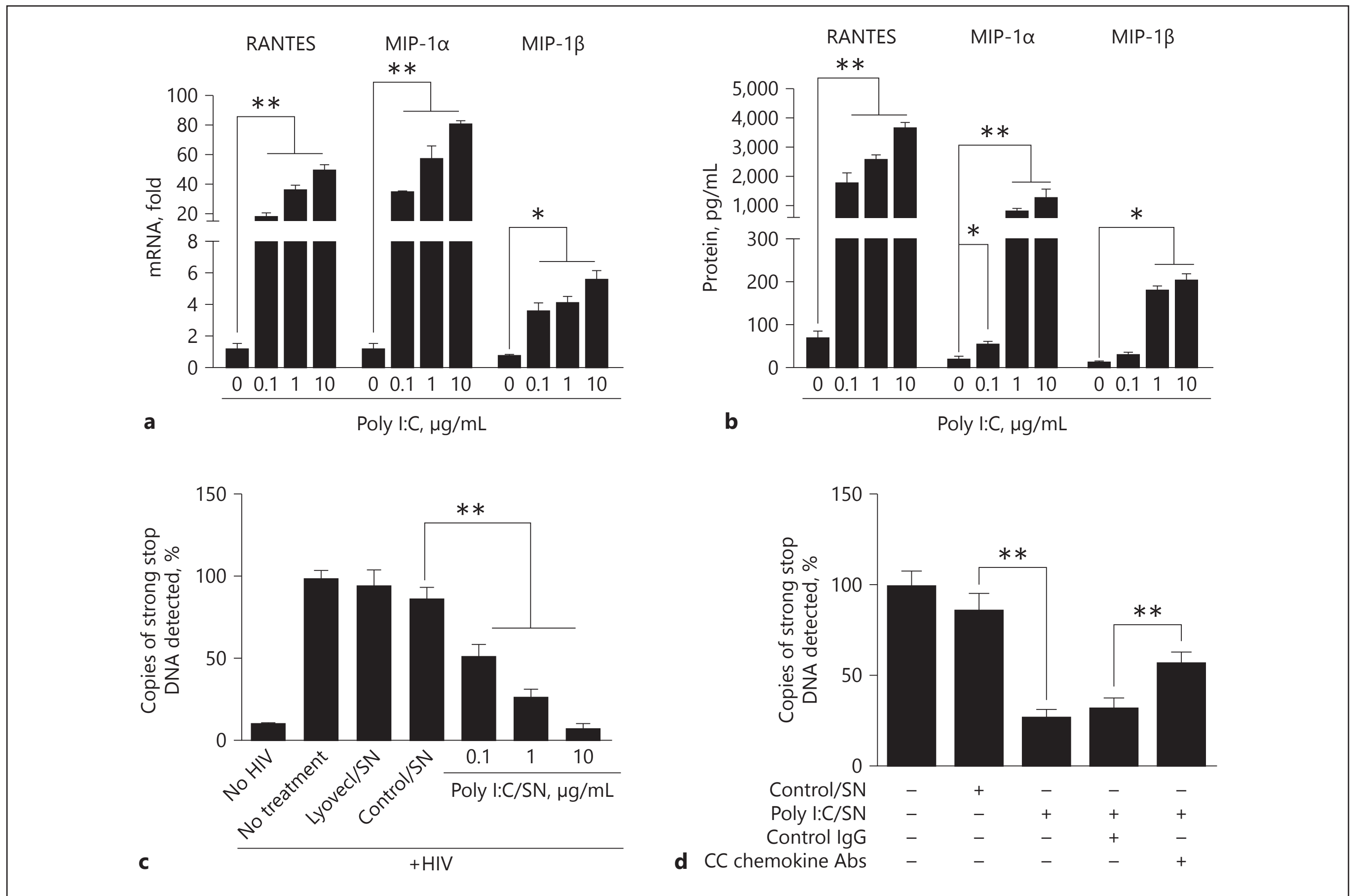


Fig. 6. Toll-like receptor (TLR)3 signaling of human cervical epithelial cells induces CC chemokines. End1/E6E7 cells were transfected with or without Poly I:C at indicated concentrations for the mRNA (**a**) and protein (**b**) detection of CC chemokines (RANTES, MIP-1α, MIP-1β). Data are expressed as RNA relative (fold) to untreated control, which is defined as 1. **c** HIV strong-stop DNA was detected in macrophages pretreated with SN (10%, v/v) from End1/E6E7 cell cultures activated by Poly I:C at different concentrations (0.1, 1, and 10 μg/mL). **d** Poly I:C/SN was incubated with 20 μg/mL control IgG or a mixture of neutralization antibodies to RANTES, MIP-1α, and MIP-1β for 1 h and then added to the macrophages for an additional 1 h prior to infection with DNase I-treated HIV (Bal). HIV strong-stop DNA was quantified at 3 h post-infection. The number of copies was normalized to the control (without treatment, which is defined as 100%). Data are shown as mean ± SD for 3 independent experiments (* $p < 0.05$, ** $p < 0.01$).

Discussion

During HIV sexual transmission, HCEs are directly in contact with the virus or virus-infected cells in FRT. Therefore, it is of great interest to determine whether HCEs are involved in the interactions between the FRT innate immunity and HIV infection. In this study, we demonstrated for the first time that HCEs express the functional TLR3/IFN signaling system, and produce a number of HIV restriction factors. We used HCEs cell line (End1/E6E7 cells) for this study, as these cells are derived from normal cervical epithelium [20], which has been extensively used and accepted as an in vitro cell model [18–20]. In our early work, we examined the difference in the TLR3 activation efficiency between the direct addition and transfection of Poly I:C in End1/E6E7 cells [17], showing that the levels of induced IFNs were significantly higher in End1/E6E7 cells transfected with Poly I:C as compared to that by direct treatment. Therefore, we used the transfection technique for Poly I:C stimulation of HCEs in this in vitro study. However, it may be unnecessary to activate TLR3 of HCEs in vivo by transfection of Poly I:C, given differences between in vitro and in vivo microenvironments in terms of the response to Poly I:C treatment.

We demonstrated that immunologically activated End1/E6E7 cells could release IFNs (IFN-β and IFN-λs)

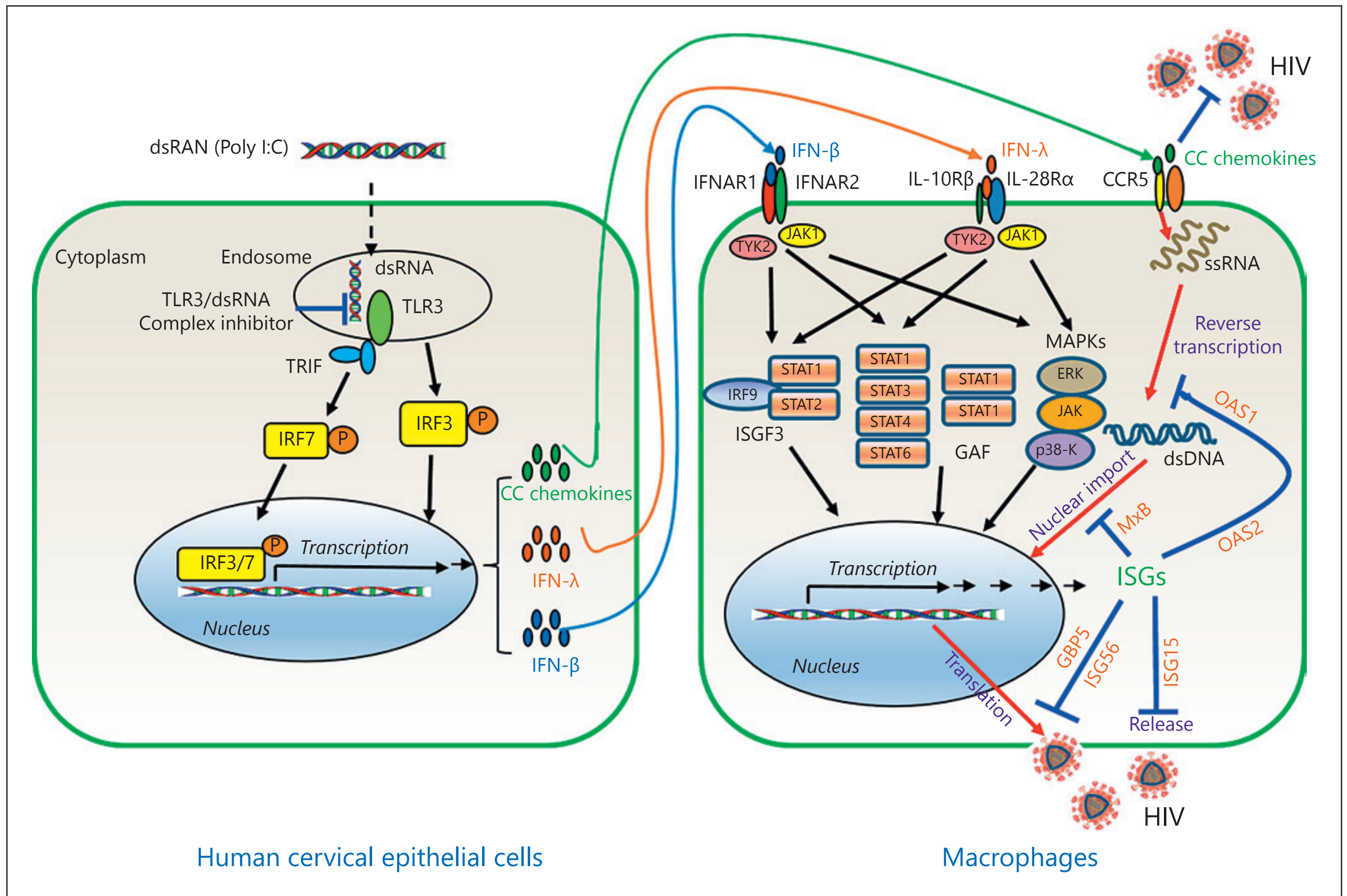


Fig. 7. Schematic diagram of mechanisms for human cervical epithelial cells (HCEs)-mediated HIV suppression in macrophage. Through the activation of toll-like receptors (TLRs) signaling pathway regulated by interferons (IFN) regulatory factors (IRFs), particularly IRF3 and IRF7, Poly I:C induces IFN-β and IFN-λs in HCEs, resulting in the activation of JAK/STAT pathway and the induction of IFN-stimulated genes (IFN-stimulated gene [ISG]15, ISG56, MxA, MxB, OAS-1, OAS-2, and GBP5). These cellular factors have the ability to restrict HIV replication at multiple steps. In addition, Poly I:C stimulation of HCEs also induces CC chemokines (RANTES, MIP-1α, MIP-1β), the ligands for HIV entry co-receptor CCR5.

and the multiple ISGs that potently inhibited HIV infection of macrophages. It is well known that IFN-β and IFN-λs have the ability to inhibit a number of viruses, including HIV [35, 36]. The vital role of IFN-β and IFN-λs in HCEs SN-mediated HIV inhibition was demonstrated by the several observations: (A) the pre-incubation of End1/E6E7 SN with the antibodies to IFN-β or IFN-λs receptor largely blocked End1/E6E7 SN-mediated anti-HIV activity in macrophages (Fig. 3a); (B) End1/E6E7 SN treatment of macrophages induced the expressions of STAT1, STAT2, and STAT3 (Fig. 3b–d), as well as the ISGs (ISG15, ISG56, OAS-1, OAS-2, MxA, MxB, and GBP5; Fig. 4); and (C) TLR3 activation had little effect on the expressions of IL-10, IL-22 and IL-26, all of which share IL-10Rβ, a subunit receptor for IFN-λs [37] (data not shown). We also found that the JAK/STAT signaling pathway plays a role in End1/E6E7 SN-mediated anti-HIV activity, as JAK Inh, the inhibitor of JAK/STAT signaling pathway, could effectively block the induction of ISGs (Fig. 5a), and HIV inhibition by End1/E6E7 SN (Fig. 5b, c).

In addition to the IFNs and the ISGs, we also examined other HIV-related cellular factors released by TLR3-activated End1/E6E7 cells. We were particularly interested in CC chemokines (RANTES, MIP-1α, MIP-1β), the ligands for HIV entry co-receptor CCR5 on macrophages [38], as they can compete with HIV for the co-receptor and block entry of the virus into the target cells [38, 39]. We found that Poly I:C stimulation could induce expressions of RANTES, MIP-1α, MIP-1β in End1/

E6E7 cells at both mRNA (Fig. 6a) and protein levels (Fig. 6b). The CC chemokines contributed to End1/E6E7 SN-mediated HIV inhibition in macrophages as evidenced by reduced expression of HIV strong-stop DNA (Fig. 6c), the first product of HIV reverse transcription [33, 34]. In addition, the antibody to CC chemokines (RANTES, MIP-1α, MIP-1β) partially blocked the effect of End1/E6E7 SN on HIV inhibition in macrophages (Fig. 6d).

In conclusion, we have provided the experimental evidence that immunological activation of HCEs by Poly I:C can mount an effective and intracellular immune response, producing IFNs and a number of HIV restriction factors. While the accurate mechanism(s) for HCEs-mediated HIV inhibition remains to be determined, it is likely that IFNs (IFN-β and IFN-λs) released from End1/E6E7 cells are responsible for the anti-HIV action, as they can bind to the IFN receptors on macrophages, resulting in the production of the ISGs that inhibit HIV at several steps of the viral replication cycle (Fig. 7). In addition to the induction of the ISGs, TLR3-activated End1/E6E7 cells also produced the CC chemokines. These observations support the assumption that HCEs are likely to be involved in the FRT mucosal innate immune defense against viral infections [17]. However, due to the limitation of our in vitro study, future investigations with suitable animal models are necessary in order to confirm that TLR3 signaling of HCEs is indeed effective in producing antiviral factors and inhibiting HIV infection. These future studies will be crucial for developing TLR3 activation-based interventions to control HIV mucosal transmission in FRT.

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Disclosure Statement

The authors declare that they have no competing financial interests to disclose.

Author Contribution

L.Z. and W.-Z.H. conceived and designed the experiments; X.-Q.X., L.G., and J.-B.L. performed the experiments; X.-Q.X. and X.W. analyzed the data; J.G., Y.L., H.L., and R.-H.Z contributed to reagents, materials, and analysis tools; P.X. proofread the paper; L.Z. and W.-Z.H. wrote the paper.

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