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# IL-29/IL-28A suppress HSV-1 infection of human NT2-N neurons

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**Abstract** The newly identified cytokines, IL-28/IL-29 (also termed type III IFNs), are able to inhibit a number of viruses. Here, we examined the antiviral effects of IL-29/IL-28A against herpes simplex virus type 1 (HSV-1) in human NT2-N neurons and CHP212 neuronal cells. Both IL-29 and IL-28A could efficiently inhibit HSV-1 replication in neuronal cells, as evidenced by the reduced expression of HSV-1 DNA and proteins. This inhibitory effect of IL-29 and IL-28A against HSV-1 could be partially blocked by antibody to IL-10Rβ, one of the key receptors for IL-29 and IL-28A. To explore the underlying antiviral mechanisms employed by IL-29/IL-28A, we showed that IL-29/IL-28A could selectively induce the expression of several Toll-like receptors (TLRs) as well as activate TLR-mediated antiviral pathway, including IFN regulatory factor 7, IFN-α, and the key IFN-α stimulated antiviral genes.

**Keywords** IFN · HSV-1 · TLR · Neuron · IRF-7

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## Introduction

Herpes simplex virus 1 (HSV-1) is a highly successful human pathogen that causes various infections within its host, including invasion of the central nervous system (CNS). HSV-1 infection of neurons could lead to encephalitis (HSV-1-induced encephalitis, HSVE; Dennett et al. 1997). Although current antiviral treatment with acyclovir dramatically reduces the mortality rate of HSVE, still above 35% of affected individuals suffer from severe neurological disabilities (Hjalmarsson et al. 2007). Thus, there is a need to develop alternative therapies to treat and prevent HSV-1 infection of CNS.

The interferon (IFN) family of cytokines is now recognized as a key component of the innate antiviral response (Borden et al. 2007). The classification of the IFN family of proteins is mainly based on their sequence, chromosomal location, and receptor specificity (Pestka et al. 2004). There are now three types of human IFNs. Type I IFNs include a number of cytokines, with the predominant members of IFN-α/β, while type II IFN consists of a single cytokine, IFN-γ. Type III IFNs, the newcomers of the IFN family, are composed of IL-29 and IL-28A/B, the latter of which shares 96% amino acid identity (Sheppard et al. 2003). Although IL-29 and IL-28A/B exert biological activities similar to type I IFNs, they appear to have a more specialized role in innate antiviral defense (Ank and Paludan 2009). It is now known that type I IFNs (IFN-α, IFN-β) and type II IFN (IFN-γ) play a crucial role in combating HSV-1 infection. Overexpression of type I IFNs in the CNS can enhance resistance to HSV-1 infection (Carr et al. 1998). HSV-1 proliferation was robust and spread more rapidly from the periphery to the CNS in Mice with null mutation (−/−) in type I IFN receptors (Leib et al. 1999). The protective role of IFNs also extends to the

control of HSV-1 latency. The expression of the IFN-γ transgene significantly delays and reduces the frequency of reactivation of HSV-1 from latency in mice exposed to UV light (Carr et al. 2009). Although the potential anti-HSV-1 activity of IL-29/IL-28A/B has been suggested by the observation that IL-29 priming enhanced HSV-1-induced antiviral cytokine gene expression in human macrophages (Melchjorsen et al. 2006), there is lack of direct evidence to demonstrate the anti-HSV-1 effect of IL-29/IL-28A/B in CNS cells, particularly neurons. The present study was undertaken to determine the effects of IL-29 and IL-28A on HSV-1 infection of human neuronal cells. We also examined the mechanism(s) involved in IL-29/IL-28-mediated anti-HSV-1 activity in neurons.

## Materials and methods

### Reagents

Recombinant human IFN-α and IFN-β were purchased from PBL Biomedical Laboratories (Piscataway, NJ). Recombinant human IL-29 and IL-28A were obtained from PeproTech Inc. (Rocky Hill, NJ). Goat anti-HSV-1 polyclonal antibody was purchased from Chemicon International Inc. (Temecula, CA). Donkey anti-goat IgG was from Molecular Probes (Eugene, OR). Goat anti-human IL-10Rβ polyclonal antibody was purchased from R&D Systems Inc. (Minneapolis, MN).

### Human neuronal cell culture

Human neuroblastoma cell line CHP212 purchased from American Type Tissue Culture (ATCC, Manassas, VA) was cultured in Eagle's MEM-Ham F12 (1:1) medium containing 10% fetal calf serum, 0.1 mM non-essential amino acid, and 1 mM sodium pyruvate. Human NT2-N neurons derived from differentiated Ntera-2clD/1 (NT2) cells (Andrews 1984) were cultured as described previously (Guo et al. 2003; Wan et al. 2008). In brief, NT2 cells were plated at a density of $2.3\times10^6$ per T75 flask and fed twice weekly with DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Iogan, UT) with 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), and 10 μM retinoic acid (RA; Sigma-Aldrich, St. Louis, MO) for up to 6 weeks. The cells were then split (1:4) and grown for an additional 48 h in identical media without RA. Neuronal cells growing atop a monolayer of

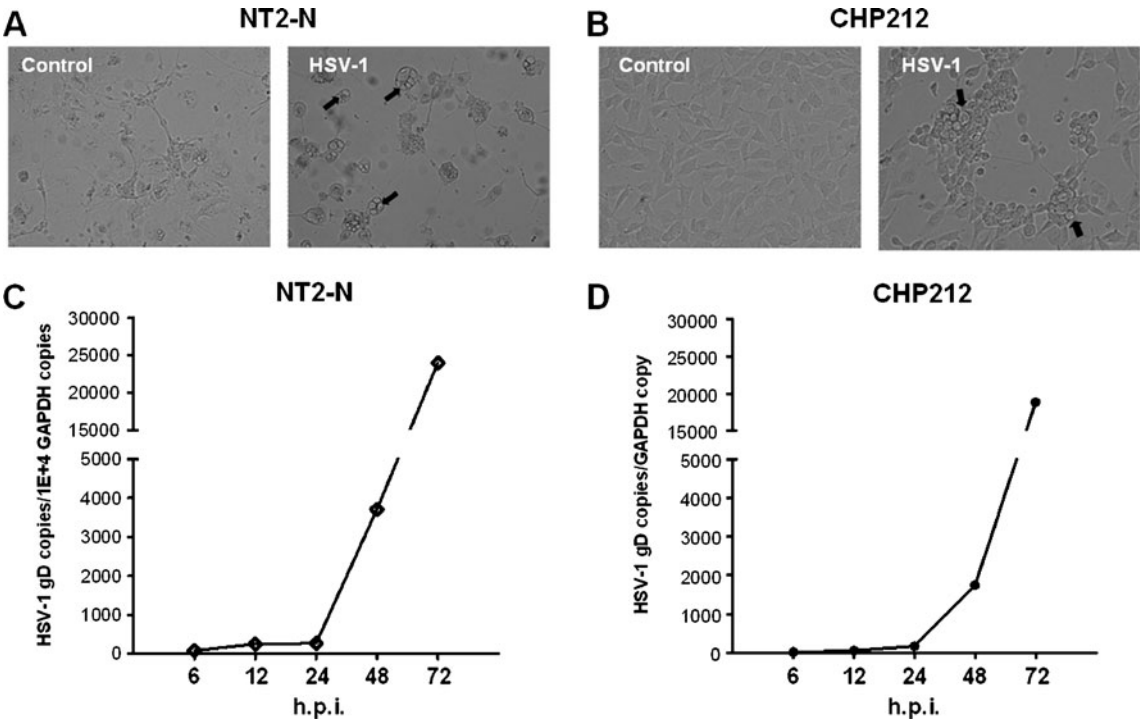


**Fig. 1** HSV-1 replication in human neuronal cells. Morphological view of NT2-N neuron (**a**) or CHP212 cells (**b**) infected with or without HSV-1 and observed at 72 h post-infection. *Arrows* indicate the characteristic cytopathic effect caused by HSV-1 infection in the neurons. NT2-N cells (**c**) and CHP212 cells (**d**) cultured in 24-well plates ($5\times10^5$ cells/well) were then infected with HSV-1 17 syn$^+$ (MOI=0.01) for 90 min. At 6, 12, 24, 48, and 72 h post-infection, genomic DNA was extracted as described in "Materials and methods" and subjected to quantitative real-time PCR to detect the HSV-1 gD level. Since the HSV-1 replication efficiency varies in different neuronal cells, data were expressed as HSV-1 gD DNA copies in 1E+4 GAPDH copies (NT2-N) (**a**) or 1 GAPDH copy (CHP212) (**b**). Representative data of three independent experiments are shown

non-neuronal cells were dislodged with trypsin and plated at a density of $5\times10^5$ cells per well in a 24-well plate for this study. NT2-N neurons have morphologic features similar to primary human neurons and have processes that differentiate into axons and dendrites. NT2-N neurons express cytoskeletal proteins, secretory markers, and surface markers, which are typical characteristics of neurons. They also express functional neuropeptides (Guillemain et al. 2000) and *N*-methyl-D-aspartate (NMDA) as well as non-NMDA glutamate receptors (Younkin et al. 1993).

## Virus propagation and infection

A highly neurovirulent HSV-1 17 syn$^+$ strain was obtained as a gift from Dr. Jim Lokeusgard (University of Minnisota Medical School). It was propagated and purified from rabbit skin fibroblasts (CCL68; American Type Culture Collection) by the standard sucrose gradient procedure and used for infection studies at a multiplicity of infection (MOI) of 0.01. After adding the virus, culture plates were incubated at 37°C for 90 min followed by washing with plain medium without FBS. Then, the culture was maintained in fresh complete medium for up to 72 h.

## Real-time PCR

Cellular DNA were extracted from cells lysed in a buffer containing 100 mM KCl, 20 mM Tris, pH 8.4, 500 mg/ml proteinase K (BDH, Poole, UK), and 0.2% (*v*/*v*) NP-40 (BDH). Lysates were incubated at 60°C for 2 h followed by 100°C for 15 min. Total RNA was extracted from cell culture with Tri-Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. RNA samples were treated with deoxyribonuclease I (DNase I; Invitrogen Life Technology) according to the manufacturer's instruction. Real-time RT-PCR was described previously (Guo et al. 2003). The primers for HSV-1 glycoprotein D (gD; Marques et al. 2008), TLRs (Dolganiuc et al. 2006; Fitzner et al. 2008; Liu et al. 2002; Schreiner et al. 2006), IFN-α/β (Tsutsumi et al. 1999), IRFs (Izaguirre et al. 2003; Ye et al. 2009), MxA, OAS-1, protein kinase R (PKR; Dharel et al. 2008), and ISG56 (Hu et al. 2008) were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). PCR was performed with the Brilliant SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). A melting curve analysis was performed to assess primer specificity and product quality by stepwise denaturation of

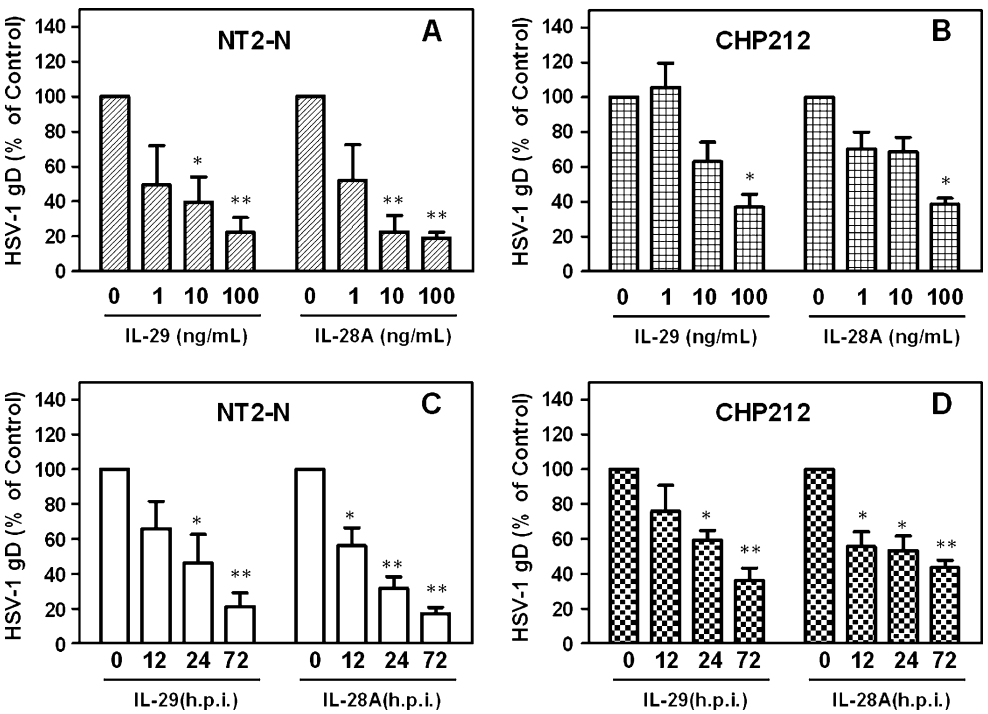


**Fig. 2** Dose- and time-dependent anti-HSV-1 effect of IL-29 and IL-28A. **a**, **b** NT2-N cells and CHP212 cells were cultured in 24-well plates ($5\times10^5$ cells/well) and pretreated with IL-29 or IL-28A at indicated concentrations for 24 h followed with HSV-1 17 syn$^+$ (MOI=0.01) infection for 90 min. At 72 h post-infection, genomic DNA was extracted and subjected to quantitative real-time PCR for HSV-1 gD DNA. **c**, **d** Cells were pretreated with IL-29 (100 ng/ml) or IL-28A (100 ng/ml) for 24 h followed with infection with HSV-1 17 syn$^+$ (MOI=0.01) for 90 min. At indicated time points post-infection, genomic DNA was extracted and subjected to quantitative real-time PCR for HSV-1 gD DNA. The replication of HSV-1 in treated cultures was expressed as percentage of HSV-1 gD DNA levels relative to control (without treatment, which is defined as 100%). Data are expressed as mean ± SD of three different experiments (*$P$<0.05; **$P$<0.01)

the PCR product at a rate of 0.1°C/s to 95°C. All values were normalized using GAPDH mRNA level. For HSV-1, the virus infection level was calculated by copies of HSV-1 in certain GAPDH copies. The relative expression levels of other genes were analyzed using the 2-$\Delta\Delta C_t$ method.

## Neutralizing antibody treatment

NT2-N cells were pretreated with neutralizing antibody to IL-10Rβ (5 μg/ml) or control goat IgG for 1 h followed with IL-29 (100 ng/ml) or IL-28A (100 ng/ml) treatment for 24 h prior to HSV-1 infection (MOI=0.01) for 90 min. The HSV-1 replication was measured by quantitative real-time PCR to detect HSV-1 gD level at 72 h post-infection.

## Immunocytochemical analysis

NT2-N neurons and CHP212 cells were cultured on glass coverslips in a 24-well plate at a density of $2\times10^5$ and $1\times10^5$/well, respectively. Cells were washed with 1× ice-cold PBS (with $Ca^{2+}$ and $Mg^{2+}$) twice, then fixed at 4°C in 4% paraformaldehyde plus 4% sucrose in PBS for 30 min. Subsequently, the cells are permeated in cold methanol (100%) for an additional 10 min followed by 0.2% Triton X-100 for an additional 10 min. Cells were blocked in Block Solution (Pierce, Rockford, IL) for 1 h at room temperature. The cells were then incubated at room temperature with goat polyclonal antibody against HSV-1 gD and IL-10Rβ (1:200) for 1 h. After washing three times with PBS, cells were incubated with FITC-conjugated donkey anti-goat IgG (1:250) or Alexa Fluor 568 donkey anti-goat IgG (1:1,000) for 1 h. For additional Hoechst staining, 1 μg/ml of Hoechst was used to incubate the cells for 1 min. Cells were then viewed under a fluorescence microscope (Zeiss, Germany).

## Data analysis

Where appropriate, the data were presented as means ± SD. The statistical significance was measured by Student's *t*

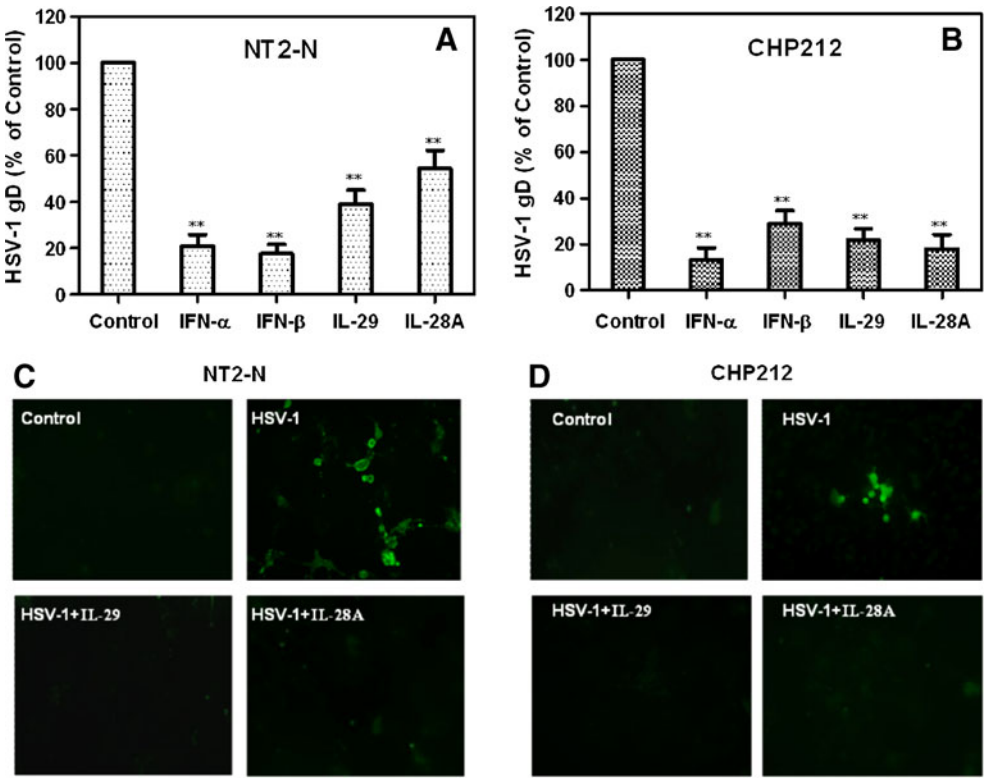


**Fig. 3** IL-29 and IL-28A inhibit HSV-1 protein expression in human neuronal cells. **a**, **b** Effect of IFNs on HSV-1 replication. NT2-N cells (**a**) and CHP212 cells (**b**) were cultured in 24-well plates at $5\times10^5$ cells per well and pretreated with IFN-α (100 U/ml), IFN-β (100 U/ml), IL-29 (100 ng/ml), or IL-28A (100 ng/ml) for 24 h. Cultures were then infected with HSV-1 17 $syn^+$ (M.O.I=0.01) and further cultured for 72 h. Genomic DNA was extracted from infected cells and subjected to quantitative real-time PCR for HSV-1 gD DNA. The data are expressed as HSV-1 gD levels relative to control (without treatment, which is defined as 100%). The results are mean ± SD of triplicate cultures, representative of three experiments (*$P<0.05$; **$P<0.01$). **c**, **d** Immunofluorescence assay showing IL-29 and IL-28A inhibit HSV-1 protein expression. NT2-N cells (**c**) and CHP212 cells (**d**) pretreated with or without IL-29 (100 ng/ml) or IL-28A (100 ng/ml) for 24 h were infected with HSV-1 (MOI=0.01) for 90 min and washed. Uninfected cells are considered as control. At 72 h post-infection, cells were fixed and the expression of HSV-1 proteins was determined by immunofluorescence assay using a polyclonal anti-HSV-1 gD antibody. A representative experiment was shown (magnification ×200)

test. Calculations were performed with Stata Statistical Software (StataCorp, College Station, TX). Values of $P < 0.05$ were considered statistically significant.

## Results

### NT2-N neurons and CHP212 cells were susceptible to HSV-1 infection

In vivo study demonstrated that HSV infection of neurons followed a lytic or latent course, causing the destruction or apoptosis of infected neurons (Esaki et al. 2010). In order to provide direct and experimental evidence that HSV-1 can infect human neurons, we infected human neuronal cells with HSV-1 17 $syn^+$ strain. Following HSV-1 infection, NT2-N cells showed significant changes in morphology, including loss of axon terminal and dendrite and expansion of cell body (Fig. 1a), while CHP212 cells became rounded and detached (Fig. 1b), showing HSV-1 cytopathic effects. HSV-1 infection-induced morphological changes were confirmed by the increased expression of HSV-1 gD gene during the course of HSV-1 infection (Fig. 1c, d).

### IL-29 or IL-28A inhibits HSV-1 infection

We next examined whether IL-29 or IL-28A has the ability to inhibit HSV-1 replication in neuronal cells. Both IL-29 and IL-28A significantly inhibited HSV-1 gD DNA synthesis in NT2-N neurons and CHP212 cells in a dose- and time-dependent fashion (Fig. 2). Since IFN-α or IFN-β was also demonstrated to inhibit HSV-1 infection of neurons, we compared the inhibitory effects of these three IFNs. The degree of IL-29 or IL-28A-mediated HSV-1 inhibition was similar to that of IFN-α or IFN-β (Fig. 3a, b). The anti-HSV-1 effects of IL-29 or IL-28A were also supported by immunofluorescence observations that either IL-29- or IL-28A-treated and infected neuronal cells expressed the reduced levels of HSV-1 proteins compared with untreated and infected cells (Fig. 3c, d).

### Involvement of IL-10Rβ in anti-HSV-1 effect of IL-29 or IL-28A

We have previously demonstrated that human neurons could express the receptors (IL-28Rα and IL-10Rβ) for both IL-29 and IL-28A (Zhou et al. 2009a). As shown in Fig. 4a, b, both NT2-N and CHP212 cells express IL-10Rβ at both mRNA and protein levels. To examine whether these receptors are involved in the anti-HSV-1 effects of IL-29 or IL-28A in NT2-N and CHP212 cells, we incubated NT2-N neurons with antibody against the extracellular domain of IL-10Rβ prior to IL-29 or IL-28A treatment and HSV-1 infection. As indicated in Fig. 4c, antibody to IL-10Rβ partially blocked the ability of either IL-29 or IL-28A to inhibit HSV-1 replication, while control IgG had little effect.

### IL-29 or IL-28A activates TLR-mediated antiviral pathway

TLR-mediated immune system plays an essential role in the innate immunity of host cells against viral infections. We thus examined whether IL-29 or IL-28A could activate the TLRs and TLR pathways. Among the ten known human TLRs, the expression of TLR3 and TLR9 in neuronal cells was upregulated by both IL-29 and IL-28A treatment (Fig. 5a). We also investigated whether IL-29 or IL-28A has the ability to induce the expression of IRFs, the key regulators of type I IFNs. We found that NT2-N neurons treated with IL-29 or IL-28A expressed higher levels of IRF7 than untreated cells, while other IRFs were not affected (Fig. 5b). Our subsequent experiments demonstrated that NT2-N neurons treated with IL-29 or IL-28A expressed significantly higher levels of IFN-α than untreated cells (Fig. 5c). In contrast, both IL-29 and IL-28A had little effect on IFN-β expression in NT2-N neurons (Fig. 5c). Furthermore, both IL-29- and IL-28A-treated NT2-N neurons expressed higher levels of IFN-α stimulated antiviral genes (ISGs), including MxA, OAS-1, PKR, and ISG56 than untreated control cells (Fig. 5d).

## Discussion

In the present study, we demonstrated that IL-29 or IL-28A could inhibit HSV-1 infection of human neuronal cells. The inhibitory effects of IL-29/IL-28A on HSV-1 in neuronal cells are comparable to those of IFN-α and IFN-β (Fig. 3), suggesting that these new members of the IFN family possess the crucial function of classical type I IFNs in human neuronal cells. HSV-1 travels to the neuronal body of the sensory ganglia within the first 24 h following infection, which assures escape from the adaptive immune response (Shimeld et al. 2001). Thus, the host's innate immune response plays a key role in blocking HSV-1 infection and spread to the CNS (Conrady et al. 2009). To determine the underlying mechanism(s) of anti-HSV-1 effect by IL-29 or IL-28A, we investigated the impact of these antiviral cytokines on TLR-mediated antiviral pathway, the crucial element in host innate immune system (Noppert et al. 2007).

It has been documented that CNS cells express all the known TLRs (1–10), including both glial cells and neurons (Bsibsi et al. 2006; Bsibsi et al. 2002; Lafon et al. 2006). We previously reported that human neuronal cells express

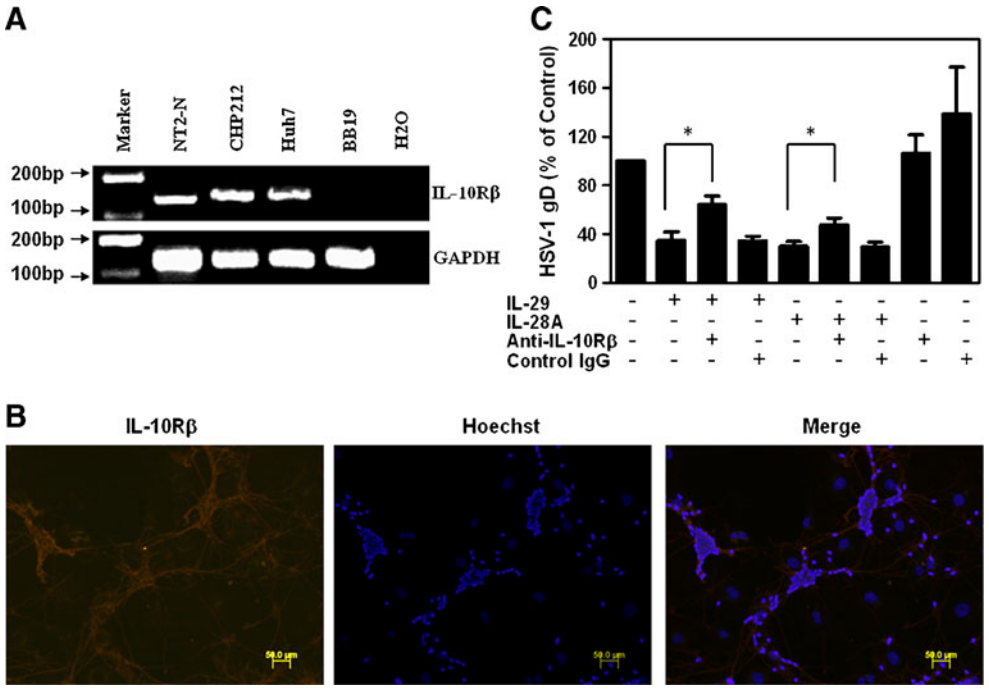


**Fig. 4** Neutralization effect of antibody against IL-10Rβ on IL-29/IL-28A-mediated anti-HSV-1 activity. **a** Expression of IL-10Rβ mRNA in neurons. RNA isolated from NT2-N, CHP212, Huh7, and BB19 cells was subjected to the real-time RT-PCR with the specific primers for IL-10Rβ and GAPDH. Huh7 cells were used as a positive control and BB19 cells were used as a negative control for IL-10Rβ expression. The RT-PCR-amplified products were analyzed by 2% agarose electrophoresis. **b** Expression of IL-10Rβprotein in NT2-N neurons by immunochemistry staining. NT2-N cells were incubated with polyclonal anti-IL-10Rβ antibody and subsequently observed under an immunofluorescence microscope. *Scale bar*, 50 μm. **c** NT2-N cells were incubated with or without IL-29 (100 ng/ml) or IL-28A (100 ng/ml) and/or antibody to IL-10Rβ (5 μg/ml) for 24 h prior to HSV-1 infection (MOI=0.01) for 90 min. Goat IgG antibody was used as control. The HSV-1 replication was measured by quantitative real-time PCR to detect HSV-1 gD level at 72 h post-infection. Values are the mean ± SD of three different cultures (*$P$<0.05)

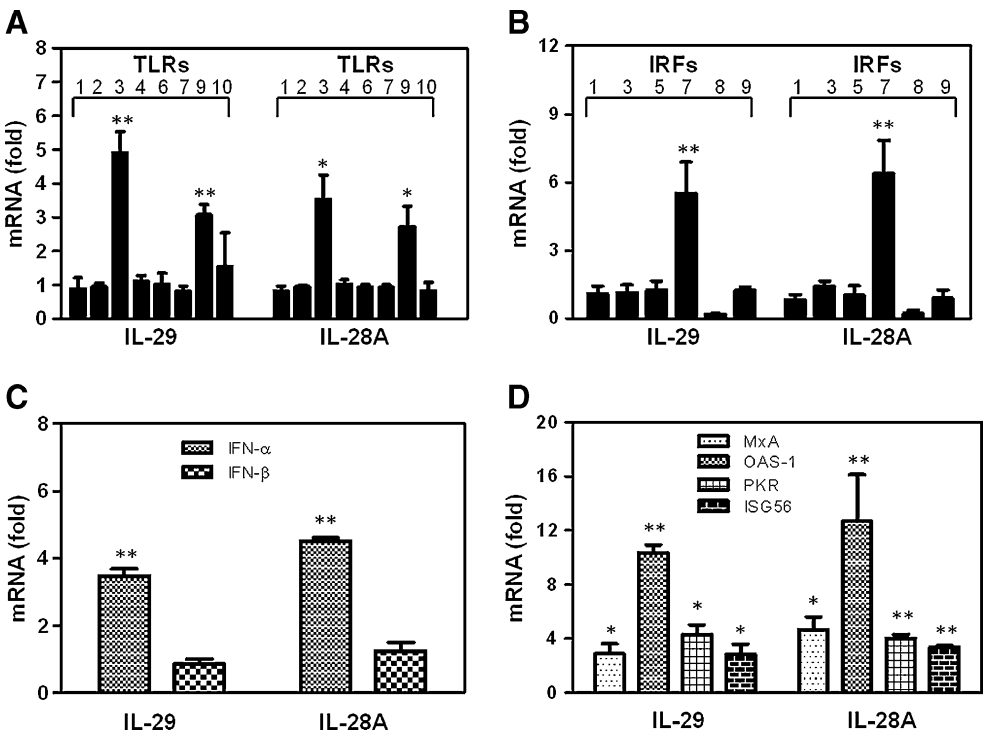


**Fig. 5** Effect of IL-29 and IL-28A on TLR-mediated antiviral pathway. NT2-N cells were treated with IL-29 or IL-28A for 12 h, and total cellular RNA was extracted from cell cultures for quantitative real-time PCR assay using specific primers for TLRs (**a**), IRFs (**b**), type I IFN (**c**), and ISGs (**d**). Data are expressed as the type III IFN-mediated increase in induction (*n*-fold) relative to untreated control, which is regarded as 1. All the specimens are normalized based on GAPDH mRNA levels. Values are the mean ± SD of three different cultures (*$P$<0.05; **$P$<0.01)

TLRs (Zhou et al. 2009b). Our findings that IL-29 or IL-28A induced TLR3 and TLR9 expression in neuronal cells provide the first experimental evidence to support the notion that the anti-HSV-1 action of IL-29 or IL-28A involves the TLR-mediated innate immunity in neuronal cells. Both TLR3 and TLR9 are involved in host innate immunity against HSV-1. TLR3 stimulation by polyI:C before infection reinforces an innate immune response of neuroprotection against HSV-1 (Boivin et al. 2008). Similarly, TLR9 participates in an early and rapid defense in response to HSV-1 infection (Krug et al. 2004; Rasmussen et al. 2007). Although it remains to be determined how IL-29 or IL-28A activates TLR3 and TLR9, there is a growing body of evidence showing that TLRs can be activated by endogenous ligands other than those pathogen-associated molecular patterns (Marsh et al. 2009). It has been shown that IFN-α enhances TLR responsiveness by upregulating TLR3, 4, and 7 (Siren et al. 2005).

In addition to their effect on TLR expression, IL-29 or IL-28A also selectively upregulated the expression of IRF7, a crucial positive regulator of IFN-α (Honda et al. 2005). Since IFN-α exerts its functions through multiple antiviral cellular factors, we examined whether there is elevated expression of ISGs in human neuronal cells treated with IL-29 or IL-28A. Our findings that both IL-29 and IL-28A treatment induced the expression of MxA, OAS-1, PKR, and ISG56 in neuronal cells are consistent with the report that IL-29 induced ISG expression in other cell systems (Dumoutier et al. 2004). Several studies also documented that IL-29 or IL-28A-mediated antiviral activity is linked to its ability to activate ISG3 and several antiviral genes (Doyle et al. 2006; Kotenko et al. 2003). These data support the notion that the anti-HSV-1 ability of IL-29 or IL-28A in neuronal cells is likely through participating in the establishment of an antiviral state as type I IFNs. Nevertheless, future studies are necessary in order to determine whether IL-29 or IL-28A and type I IFNs share similar intracellular signaling pathways that lead to antiviral potencies (Marcello et al. 2006).

As a potential new therapeutic agent for the treatment of patients with hepatitis C infection, IL-29 may have fewer adverse effects than IFN-α as IL-29 receptors are more cell-specific than the widely distributed IFN-α receptors (Ank and Paludan 2009). Our data that both IL-29 and IL-28A were able to inhibit HSV-1 infection of human neuronal cell provide support for using IL-29 or IL-28A for the clinical treatment of HSV-1 infection of the CNS. However, more extensive studies are required to incorporate human subjects and/or animal models to determine the in vivo impact of IL-29 or IL-28A/B on HSV-1 infection.

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