

Innate responses to small interfering RNA pools inhibiting herpes simplex virus infection in astrocytoid and epithelial cells

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Abstract

Herpes simplex virus (HSV) is a human pathogen that can cause severe diseases such as encephalitis, keratitis and neonatal herpes. Control of HSV infection may be achieved by using small interfering (si)RNAs. We have designed and enzymatically produced pools of siRNAs targeting HSV. In addition to the target-specific effects, such siRNAs may induce innate immunity responses that may contribute to antiviral effects. HSV has versatile ways of modulating innate immunity, and it remains unclear whether HSV-specific antiviral treatment would benefit from the potential immunostimulatory effects of siRNAs. To address this, cell lines derived from epithelium and nervous system were studied for innate immunity reactions to HSV infection, to siRNA treatment, and to a combination of treatment and infection. In addition, the outcome of HSV infection was quantitated. We show that innate immunity reactions vary drastically between the cell lines. Moreover, our findings indicate only a minimal relation between the antiviral effect and the treatment-induced innate immunity responses. Thus, the antiviral effect is mainly sequence specific and the inhibition of HSV infection is not ascribed to the slight innate immunity induction.

Keywords

Antiviral, herpes simplex virus, interferon, RNA interference, small interfering RNA

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Introduction

Herpes simplex viruses type 1 and 2 (HSV-1 and HSV-2) are ubiquitous human pathogens that cause clinically important diseases such as genital herpes, neonatal herpes, severe ocular infections, meningitis and herpes encephalitis.¹ HSV is capable of modulating innate immunity and escapes adaptive immunity. HSV infection induces the expression of *IFN* genes,^{2–5} and HSV is also able to evade and modulate innate immunity by several mechanisms.^{4,6,7} RNA interference (RNAi) using single-site, small interfering (si)RNAs has already been applied for treatment of HSV infection in cultured cells.^{8–11} A combination of two single-site siRNAs, targeting the *UL29* gene (encoding the essential HSV DNA binding protein ICP8), has enhanced the survival of infected animals in a mouse model of genital herpes.¹² The possible induction of innate immunity by the siRNAs and the consequent effects on the infection control remain unknown.

The antiviral efficacy of the siRNAs could partially arise from the innate responses that such molecules may evoke in the infected cells. As HSV is able to circumvent RNAi,¹³ the immunostimulatory activity of the RNAi treatments may play a role in HSV infection inhibition. Therefore, we studied type I (*IFN- α* , *IFN- β*) and type III *IFN* (*IFN- λ 1* and *IFN- λ 2/3*; *IL-29* and *IL-28A/B*) induction and IFN-stimulated gene (*ISG*) expression during the treatment of HSV-infected and uninfected cells in astrocytic U373MG cells and in keratinocytic HaCaT cells with single-site siRNAs and with enzymatically created siRNA pools (summarized

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Table 1. siRNAs.

Name	Type	Length of siRNA(s) (nt)	Production method	Reference
UL29G	Pool of siRNAs ^a	25–27	Enzymatical, using <i>Giardia intestinalis</i> dicer	Romanovskaya et al. ¹⁶
UL29H	Pool of siRNAs ^a	21	Enzymatical, using human dicer	Romanovskaya et al. ¹⁶
UL29S	Single-site siRNA	21	Synthetic	Palliser et al. ¹²
UL29L	Single-site siRNA	27	Synthetic	Romanovskaya et al. ¹⁶
siGFP	Single-site siRNA	22	Synthetic	Caplen et al. ²⁹
eGFPG	Pool of siRNAs	25–27	Enzymatical, using <i>G. intestinalis</i> dicer	Romanovskaya et al. ¹⁶

^aCovers 653 nts of target sequence.

in Table 1). In addition, we studied the viral shedding and viral gene expression to reveal if the infection was halted at any stage of viral replication.

To create pools of siRNAs, we have applied RNA polymerases of enterobacteria phage T7 and *Pseudomonas* phage phi6 for the enzymatic production of high-quality, double-stranded (ds)RNA for RNAi.^{14–16} The produced target-specific dsRNA molecules were cleaved *in vitro* to pools of canonical 21-nucleotide (nt)-long siRNAs or 27-nt-long dicer-substrate siRNAs using human or *Giardia intestinalis* dicers, respectively. Upon introduction into mammalian cells, the dicer-substrate siRNAs are further processed to 21-nt molecules by the endogenous dicer.¹⁷ Consequently, their effects might differ from the canonical 21-nt siRNAs in RNAi applications.¹⁷ Furthermore, we earlier utilized the enzymatically produced siRNA pools against coxsackie viruses and obtained a broader inhibition of related viral strains than with a single-site siRNA.¹⁵ We have recently studied the inhibition of HSV infection in cell cultures using dicer-substrate and canonical siRNA pools, and found that the siRNA pools do not compromise cell viability in the efficient antiviral concentrations.¹⁶ However, we did observe weak innate responses to the RNA species utilized.¹⁶

Here, we investigated various siRNA preparations for the induction of innate immunity and for their efficacy in inhibiting HSV infection. We describe the innate immunity reactions induced by the siRNAs, and the immunomodulatory capabilities of HSV in cells originating from epithelium and the nervous system. Viral shedding and HSV gene expression profiles in siRNA-treated and untreated cells were characterized.

Materials and methods

Cell lines and virus

Human glioblastoma-astrocytoma U373MG cells (U-251 MG; ATCC, Manassas, VA, USA) were maintained in high-Glc DMEM (Gibco, Carlsbad,

CA, USA) supplemented with 2 mM L-glutamine and 10% heat inactivated FCS (PromoCell, Heidelberg, Germany). Human HaCaT keratinocyte cells (Department of Dentistry, University of Turku, Finland)¹⁸ were cultured in DMEM supplemented with 7% FCS. The prototype HSV-1 (strain 17+) was propagated in African green monkey kidney cells (Vero; ATCC) maintained in DMEM supplemented with either 2% or 7% FCS for virus propagation and plaque titration, respectively. All cell lines were incubated at 37°C in 5% CO₂. HSV-1 particles were collected from infected Vero cell culture at 72 h post-infection (p.i.) as previously described,¹⁶ and stored in MNT buffer [20 mM MES (Sigma-Aldrich, St. Louis, MO, USA), 100 mM NaCl, and 30 mM Tris, pH 7.4] at –70°C. The viral titer was defined by plaque titration in Vero cells as described previously.¹⁶

dsRNA and siRNA molecules

The HSV- and enhanced GFP (eGFP)-specific dsRNA molecules were produced using a Replicator RNAi kit (Thermo Scientific, Waltham, MA, USA). The DNA template for the HSV-specific dsRNA was a 653-nt-long segment of the *UL29* gene (ICP8) of HSV-1 (strain 17+) in plasmid pET32UL29.¹⁶ The 720-nt-long eGFP-specific sequence was amplified from the plasmid pCR3.1-eGFP.¹⁴ PCR primers used for amplification are presented in Table 2. The siRNA pools were prepared by cleavage of the produced dsRNA molecules with human BLOCK-iT Dicer enzyme (Invitrogen, Carlsbad, CA, USA) or *G. intestinalis* dicer (PowerCut Dicer; Thermo Scientific) and purified by anion-exchange chromatography (Gen-Pak FAX column; Waters, Milford, MA, USA) as described previously.¹⁶ Pools of siRNA, created with *G. intestinalis* dicer, were designated as UL29G and eGFPG, targeting *UL29* or *eGFP*, respectively. The anti-UL29 pool created with human dicer was designated as UL29H. Commercial anti-UL29 single-site siRNAs UL29S (21 nt; UL29.2)¹² and UL29L (27 nt)¹⁶ were purchased from Dharmacon (Lafayette, CO, USA). UL29S was designed using Dharmacon design program to minimize off-target effects,^{12,19} while the others were not.

Table 2. Primers and siRNA sequences.

Name	Type	Sequence ^a	Reference
GAPDH sense	DNA	5'-GAGAAGGCTGGGGCTCAT-3'	Nygardas et al. ¹⁵
GAPDH antisense	DNA	5'-TGCTGATGATCTTGAGGCTG-3'	Nygardas et al. ¹⁵
ISG54 sense	DNA	5'-ACTATCACATGGGCCGACTC-3'	Romanovskaya et al. ¹⁶
ISG54 antisense	DNA	5'-TTTAACCGTGTCCACCCTTC-3'	Romanovskaya et al. ¹⁶
IFN- λ 1 sense	DNA	5'-GGAGCTAGCGAGCTTCAAGA-3'	Romanovskaya et al. ¹⁶
IFN- λ 1 antisense	DNA	5'-GGAAGACAGGAGAGCTGCAA-3'	Romanovskaya et al. ¹⁶
IFN- λ 2/3 sense	DNA	5'-GCCACATAGCCCAGTTCAAG-3'	This study ^a
IFN- λ 2/3 antisense	DNA	5'-TCCTTCAGCAGAAGCGACTC-3'	This study ^a
IFN- α sense	DNA	5'-TGGCTGTGAAGAAATACTTCCG-3'	Peri et al. ²⁵
IFN- α antisense	DNA	5'-TGTTTTTCATGTTGGACCAGATG-3'	Peri et al. ²⁵
IFN- β sense	DNA	5'-TCTCCACGACAGCTCTTTCCA-3'	Peri et al. ²⁵
IFN- β antisense	DNA	5'-ACACTGACAATTGCTGCTTCTTTG-3'	Peri et al. ²⁵
TLR3 sense	DNA	5'-TAGCAGTCATCCAACAGAATCAT-3'	Peri et al. ²⁵
TLR3 antisense	DNA	5'-AATCTTCTGAGTTGATTATGGGTAA-3'	Peri et al. ²⁵
HSV UL54 sense	DNA	5'-GTCCTGCGCTCCATCTCC-3'	This study ^a
HSV UL54 antisense	DNA	5'-GTCGTGCATGACCTGTGC-3'	This study ^a
HSV UL48 sense	DNA	5'-TTTGACCCGCGAGATCCTAT-3'	Broberg et al. ³⁰
HSV UL48 antisense	DNA	5'-GCTCCGTTGACGAACATGAA-3'	Broberg et al. ³⁰
UL29-T7	DNA	5'-TAATACGACTCACTATAGGGATGATGGC CGTAAGGGTGT-3'	Romanovskaya et al. ¹⁶
UL29-Phi6	DNA	5'-GGAAAAAACGCAACTTTTCGCAATCAAT-3'	Romanovskaya et al. ¹⁶
eGFP-T7	DNA	5'-TAATACGACTCACTATAGGGATGGTGAG CAAGGGCGAGGAG-3'	Romanovskaya et al. ¹⁶
eGFP-Phi6	DNA	5'-GGAAAAAACTTGTACAGCTCGTCCAT GCCG-3'	Romanovskaya et al. ¹⁶
UL29S	RNA	5'-CUUUCGCAAUCAAUUCCAUUU-3' 3'-UUGAAAGCGUUAGUUAAGGUU-5'	Palliser et al. ¹²
UL29L	RNA	5'-ACUUCGCAAUCAAUUCCAACCGGUGC-3' 3'-UGAAAGCGUUAGUUAAGGUUGGCCACG-5'	Romanovskaya et al. ¹⁶
siGFP	RNA	5'-GCAAGCUGACCCUGAAGUUCAU-3' 3'-GCCGUUCGACUGGGACUUCAAG-5'	Nygardas et al. ¹⁵ Caplen et al. ²⁹

^aAnnealing temperatures used in qRT-PCR reactions were 60°C for both *IFN- λ 2/3* and *UL54*.

The anti-eGFP single-site siRNA (siGFP) was purchased from Qiagen (Hilden, Germany). The siRNAs used in this study are presented in Table 1. For the sequences of the single-site siRNAs, see Table 2.

Transfection and infection

RNA or water transfections of U373MG and HaCaT cells, propagated in 96-well plates, were carried out using Lipofectamine RNAiMAX (Invitrogen) and the manufacturer's forward transfection protocol. All the experiments were performed 2–5 times ($n = 3–6$ in each experiment).

Four h after transfection, the cells were challenged with a 100- μ l dose of HSV-1(17+) containing 1000 plaque forming units (pfu) per well. In the case of uninfected cells, they were subjected to the same treatment, excluding the virus. Prior to infection, the cells were

washed twice with RPMI 1640 (Gibco) supplemented with 0.1% BSA. Cells were washed three times and covered with 200 μ l of culture medium per well at 60–90 min p.i. At 48 h post-transfection (44 h p.i.) the cells were collected in TRI reagent (MRC, Cincinnati, OH, USA) for RNA extraction. Culture medium was collected prior to this and used to determine the amount of released virus by plaque titration on Vero cells.

Quantitative real-time PCR

Total cellular nucleic acid was isolated from cells using TRI reagent (MRC) according to manufacturer's instructions. After treatment with DNase (Fermentas, Thermo Scientific), the total RNA was reverse transcribed into complementary DNA (cDNA) using RevertAid H Minus Reverse Transcriptase (Fermentas) and random hexamer primers

(Fermentas). For the detection of selected mRNAs (viral or host) the produced cDNA were amplified using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas). Quantitative real-time (qRT)-PCR was performed with a Rotor-Gene Q real-time instrument (Qiagen) as described previously.²⁰ The sequences of the primers are shown in Table 2. All results from the qRT-PCR were normalized to the housekeeping gene, *GAPDH*, copy number in the same sample.

Computational analyses

For statistical analyses, SPSS Statistics 20 (IBM, Armonk, NY, USA) software was used. Statistical significances were calculated with non-parametric Mann–Whitney *U*-test and the threshold was set to $P < 0.05$.

Results and discussion

In this study, we describe the effects of HSV infection on the cellular innate responses to enzymatically produced antiviral siRNA pools and chemically synthesized single-site siRNAs targeting the essential *UL29* gene of HSV-1. We studied the possible correlation between the antiviral efficacy of siRNAs and their induction of innate immunity. We applied two size classes of siRNAs—21-nt-long and 27-nt-long (Table 1)—and used two cell lines, the astrocytoma-glioblastoma U373MG, representing a host cell of HSV infection in the central nervous system, and the HaCaT keratinocyte cell line, representing an epithelial

type of host cell of HSV-1. Both cell lines produced progeny virus with similar efficiency (Figure 1).

Anti-HSV potency of the siRNAs

The siRNA pools against the *UL29* gene, prepared using human or *Giardia* dicer (UL29H and UL29G, respectively; Table 1), had a significant antiviral activity ($P < 0.01$; Figure 1) when compared with the nonspecific siRNAs targeting the *eGFP* gene (siGFP or eGFP; Table 1) or control treatments in both HaCaT and U373MG cell lines. In accordance with previous studies,¹⁶ both anti-HSV siRNA pools effectively inhibited HSV replication equally (Figure 1), reaching > 99% and 90% decrease in viral shedding in the astrocytic cell line U373MG and HaCaT keratinocytes, respectively, at 44 h p.i. In all cases, the antiviral effectiveness of the 21- and 27-nt single-site siRNAs (UL29S and UL29L, respectively) varied between cell lines but remained significant ($P < 0.05$).

In addition to the effects on viral reproduction, we assessed how the silencing of the early gene *UL29* influences the expression of other viral genes. In lytic HSV infection, HSV genes are expressed in a cascade, starting from α (immediate early) genes, followed by β (early genes) and, finally, γ (late) genes.²¹ The decreased expression levels of the HSV immediate early gene *UL54* and the late *UL48* gene [encoding proteins ICP27 and VP16 (also known as α TIF), respectively] correlated with the inhibition of viral shedding (Table 3, Figure 1). This reflects the general inhibition

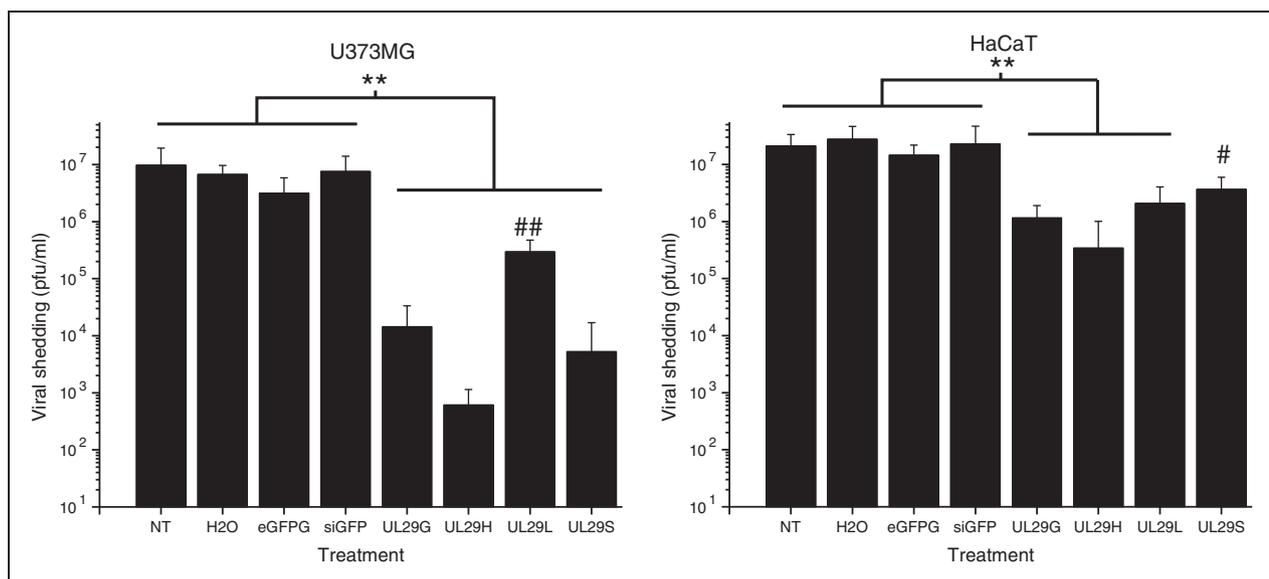


Figure 1. Inhibition of viral shedding with siRNAs. U373MG and HaCaT cells, grown on 96-well plates, were challenged with 1000 pfu of HSV-1 (17+). The amount of infectious virus shed into the culture medium was determined by plaque titration at 44 h p.i. The titers are presented on a logarithmic scale. Mean + SD of six replicates are shown. Significances are shown for the groups of comparison. Non-parametric Mann–Whitney *U*-test was used to determine significances. ** $P < 0.01$; and against all other treatments # $P < 0.05$, ## $P < 0.01$.

of HSV replication and gene expression at 44 h p.i. in cells treated with virus-specific siRNA. Nevertheless, there is a change in the balance of immediate early and late genes depending on the treatment given (Table 3). When *UL29* (early gene, β) is inhibited, relatively fewer copies of γ genes (expressed after β genes) are present than α gene RNAs (expressed before β genes) in the infected cells (Table 3). This change in the ratio of α to γ genes is evident in U373MG cells. In HaCaT cells, the effect is more subtle, as is the antiviral effect (Figure 1). Altogether, the efficacy of the antiviral effect and the relative decrease in γ gene expression correlate. These results favor the explanation that the siRNAs work in a sequence specific manner.

Innate responses in siRNA-treated and HSV-infected U373MG and HaCaT cells

We focused on the cellular innate response, in particular on the changes in type I and III IFN gene expression, in cells treated with the siRNA preparations and infected with HSV-1. There is evidence that HSV can modulate or evade host innate responses.^{4,6,7} The effects of HSV infection on the studied innate responses in U373MG cells were limited (non- or water-transfected, infected and uninfected cells; Figure 2). This provided a platform to investigate the role that siRNA-induced IFN response play in antiviral efficacy. In contrast, HaCaT cells infected with HSV evoked robust innate responses (non- or water-transfected, infected and uninfected cells; Figure 3). This represented a combination of viral innate immunity

modulation mixed with treatment-induced effects. In uninfected cells, siRNA treatments themselves caused some innate responses (Figures 2 and 3), in accordance with previous studies.¹⁶ Nevertheless, these responses had minimal correlation with antiviral effectivity of a given siRNA preparation (Figure 1).

Innate cellular responses in U373MG cells. Astrocytic cells, including U373MG, express TLR3 and produce IFN type I and type III molecules.^{16,22,23} However, U373MG cells were unable to mount a distinct IFN response to the HSV infection (infected cells with non- and water transfection; Figure 2). The gene expression levels indicated a significant response to some of the siRNA treatments (Figure 2).

Transfection of U373MG cells with siRNAs caused only minimal alterations in *IFN- α* gene expression (Figure 2a). However, the UL29H siRNA pool and single-site UL29L siRNAs increased *IFN- β* and *- λ* expression (Figure 2b–d) and levels of *TLR3*- and *ISG54*-specific mRNAs (Figure 2e, f). Compared with the controls, UL29H- and UL29L-treated U373MG cells had significant *IFN* and *IFN*-stimulated gene expression responses irrespective of HSV infection (Figure 2b–f). Significant *IFN* responses to UL29L (Figure 2b–d) did not correlate with its anti-HSV activity (Figure 1).

Previous studies using astrocytes indicate that transfected and complexed dsRNA is recognized in these cell types via a melanoma differentiation-associated gene 5 cytosolic RNA sensor. However, the TLR3-dependent pathway is employed only when the dsRNA is naked in the culture media.²⁴ We observed a significant increase in *TLR3* mRNA levels for all siRNA preparations (Figure 2e), except for short siRNAs. Although TLR3 is involved in the sensing of HSV infection,^{25,26} especially in astrocytes,^{22,23} we did not detect alterations in *TLR3* gene expression after HSV infection (non- or water-transfected, infected and uninfected cells; Figure 2e). There was a significant upregulation of *IFN- β* , *- λ* and *TLR3* in UL29G-treated and infected cells compared with their uninfected counterparts. The HSV infection modified the *IFN- λ 2/3*, *TLR3* and *ISG54* gene expression in UL29H siRNA-treated cells (Figure 2d–f). The observed induction profile for IFN-stimulated gene 54 (*ISG54*) was similar to that of *TLR3*, which is also an ISG (Figure 2e, f) in different treatment groups.

The antiviral effect of the siRNA preparations (Figure 1) was not directly related to the type I or type III *IFN* gene induction. The UL29H siRNA pool induced the highest expression of *IFN- β* and *IFN- λ* , but the equally effective UL29G pool or single-site UL29S siRNA induced only low or undetectable expression of *IFN- β* or *IFN- λ* expression in U373MG cells (Figure 2b–d). Moreover, the longer siRNAs (27 nt), UL29G and UL29L (Table 1), had very similar effects on the

Table 3. Relative HSV late (γ) to immediate early (α) gene expression.

Treatment	U373MG	HaCaT
	$\gamma:\alpha^a$	$\gamma:\alpha^a$
NT	36 ± 9	47 ± 6
H ₂ O	44 ± 6	36 ± 10
eGFPG	26 ± 6	38 ± 4
siGFP	40 ± 11	40 ± 10
UL29G	7 ± 2	27 ± 8
UL29H	8 ± 4	26 ± 5
UL29L	15 ± 4	32 ± 7
UL29S	8 ± 3	39 ± 22

NT: no treatment; eGFPG: non-specific pool of siRNAs; siGFP: non-specific single-site siRNA.

^aUL27 (γ) and UL54 (α) mRNA expression measured by gene-specific quantitative RT-PCR from HSV-infected HaCaT and U373MG cells 2 d p.i. ($n \geq 6$) and normalized to *GAPDH* expression. The averages (\pm) SD are shown.

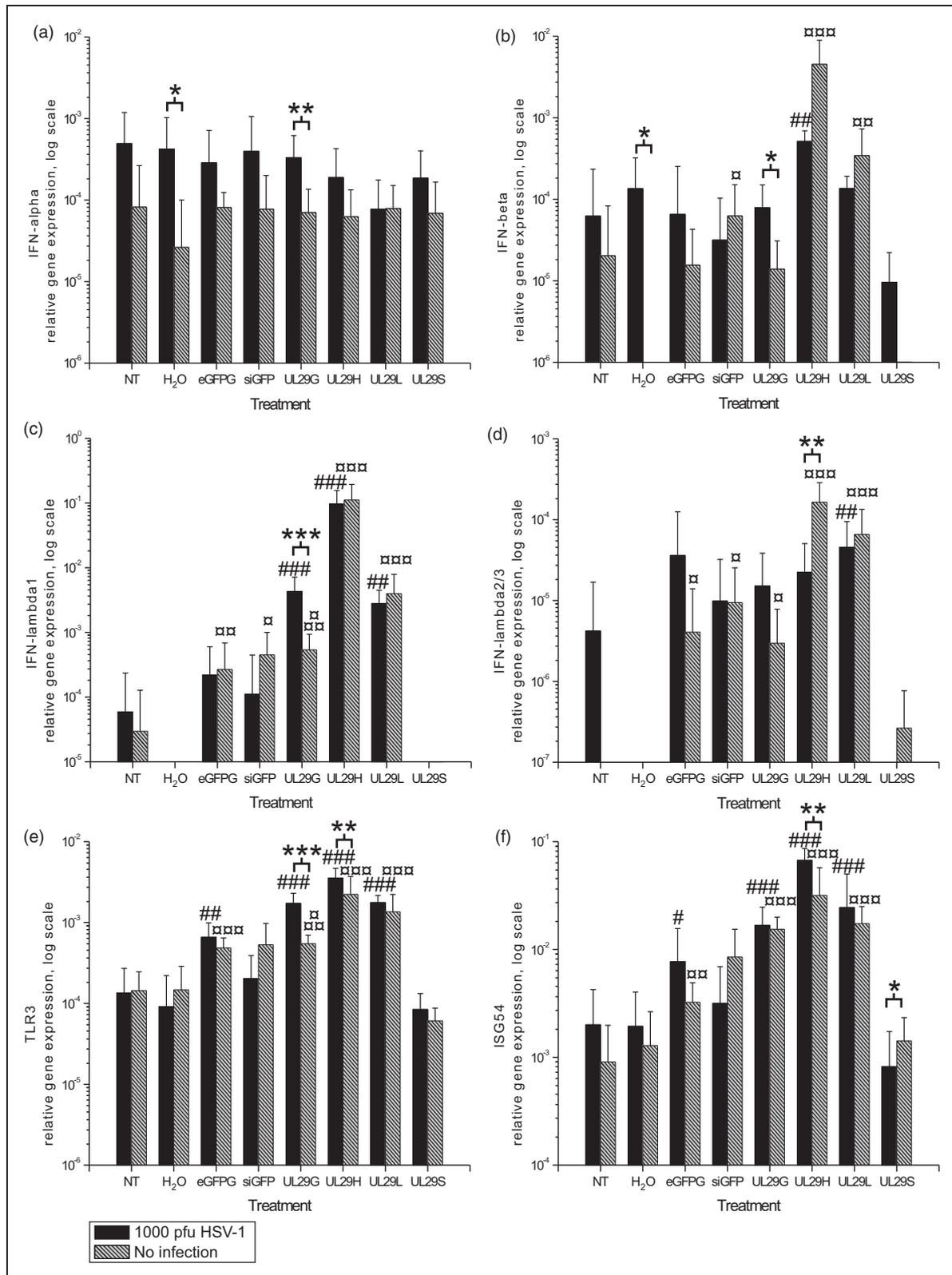


Figure 2. Effects of HSV-I infection on the siRNA-induced innate responses in astrocytic cells. (a) *IFN-alpha*, (b) *IFN-beta*, (c) *IFN-lambda1* (IL-29), (d) *IFN-lambda2/3* (IL-28 A/B), (e) *TLR3* and (f) *ISG54* gene expression levels were measured by quantitative RT-PCR from uninfected (gray bars) and HSV-infected (black bars) U373MG cells at 48 h post-transfection (44 h p.i.). The relative expression, normalized to *GAPDH* gene expression, is shown on a logarithmic scale for each siRNA or control treatment. SDs of at least eight replicates are shown. A non-parametric Mann–Whitney *U*-test was used to determine significances. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (HSV-infected versus uninfected); # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ as compared with infected controls (NT and H₂O); □ $P < 0.05$, □□ $P < 0.01$, □□□ $P < 0.001$ as compared with non-infected controls (NT and H₂O).

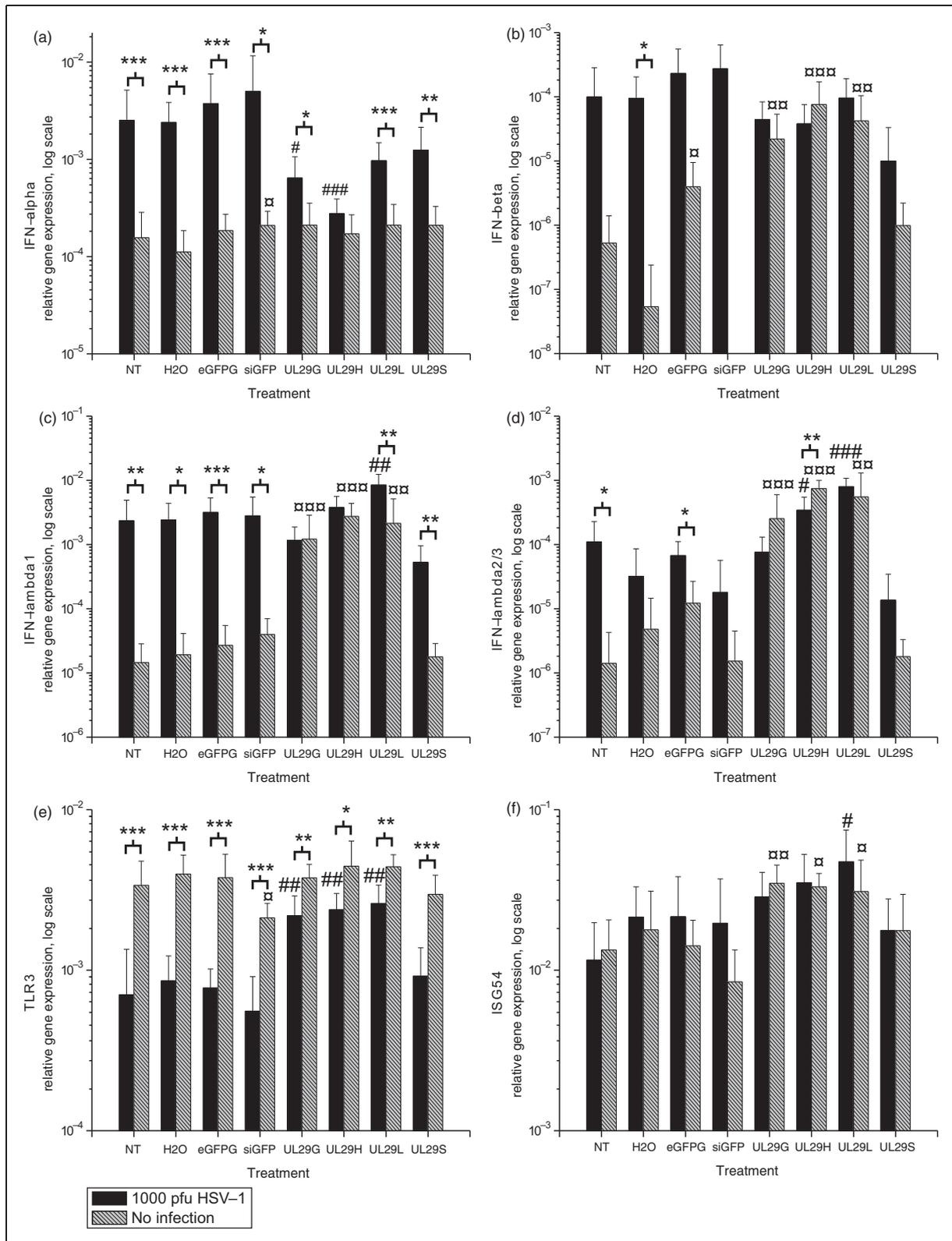


Figure 3. Effects of HSV-I infection on the siRNA-induced innate responses in HaCaT cells. (a) *IFN-alpha*, (b) *IFN-beta*, (c) *IFN-lambda1* (IL-29), (d) *IFN-lambda2/3* (IL-28 A/B), (e) *TLR3* and (f) *ISG54* gene expression was measured by quantitative RT-PCR from uninfected (gray bars) or HSV-infected (black bars) HaCaT cells at 48 h post-transfection (44 h p.i.). The relative gene expression, normalized to *GAPDH* gene expression, is shown on a logarithmic scale for each siRNA or control treatment. SDs of at least eight replicates are shown. Non-parametric Mann-Whitney *U*-test was used to determine significances. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (HSV-infected versus uninfected); #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 as compared with infected controls (NT and H₂O); □*P* < 0.05, □□*P* < 0.01, □□□*P* < 0.001 as compared with non-infected controls (NT and H₂O).

gene expression of *IFN* type I and III, and on *TLR3* and *ISG54* gene expression in HSV infection, but their anti-HSV potency was significantly different (Figure 2 and Figure 1, respectively) in the U373MG cells. This suggests that the antiviral effects of the siRNAs are sequence-specific. The difference in the antiviral efficiency of the target-specific and nonspecific siRNAs, both single-site siRNAs and pools, support such a conclusion (Figure 1).

Innate cellular responses in HaCaT cells. In HSV-infected HaCaT cells, we observed type I and type III IFN responses (Figure 3a–d), consisting of distinct IFN- α and IFN- λ 1 responses to HSV, but the IFN- β and IFN- λ 2/3 responses were modest. The virus-induced effects overruled those caused by the siRNA pools or single-site siRNAs. The HSV infection-induced IFN- α gene expression was markedly suppressed when the cells were treated with anti-HSV siRNAs in comparison with the nontreated but infected controls (Figure 3a). This may reflect the significant reduction in HSV replication by these siRNA treatments (Figure 1), which, in turn, suppressed virus-induced IFN- α gene expression.

TLR3 is expressed in HaCaT cells (Figure 3e),²⁷ which are also capable of processing dicer-substrate RNAs to trigger RNAi.²⁸ In these cells, the HSV infection inhibited *TLR3* gene expression both in untreated and siRNA-treated cells (Figure 3e). The downregulation of *TLR3* gene expression correlated with the extent of HSV infection (Figure 1, Figure 3e). The highest expression occurred in cells treated with the effective anti-HSV siRNAs. This is in accordance with our earlier observation regarding the modulation of *TLR3* expression by HSV infection in monocytic cells.²⁵ Only minor differences in *ISG54* expression, in comparison with nontreated cells, were observed in HSV-infected or uninfected siRNA-treated cells (Figure 3f) at 48 h post-transfection. In general, the type I and III *IFN* gene expression, induced by the siRNA pools, was low or equal to the HSV-induced *IFN* expression in the keratinocyte cell line, supporting the concept that the inhibition of HSV infection by the siRNA pools was sequence-specific, and not a result of *IFN* induction by the siRNA pools.

As different cell lines give varying responses to HSV and the siRNA treatments, it remains to be studied what happens in an *in vivo* application. A slightly elevated immune response might enhance adaptive antiviral immunity, whereas too high a response to the treatment might cause adverse effects in a multicellular organism. Thus, the UL29G pool, causing only slight immune responses and having very effective antiviral properties, could be a prime candidate for an *in vivo* approach for the treatment of, for example, herpes keratitis.

Conclusions

The effects of HSV on the innate immunity responses depended on the studied cell line. Immunostimulation by the siRNA preparations had minimal effect against HSV. The antiviral effect of the siRNAs was mainly sequence specific. The inhibition efficacy varied significantly between the different anti-HSV siRNA molecules with similar innate responses. This was most evident in the U373MG cells, as the cells did not respond to HSV infection. In contrast, HaCaT cells reacted strongly to the HSV infection by mounting innate immunity responses. In addition, HSV was able to downregulate innate response in these epithelial cells as HSV-infected cells had diminished *TLR3* gene expression compared with the baseline. The HSV replication inhibition correlated only weakly with the innate responses caused by the siRNA pools and single-site siRNAs of different length. In conclusion, HSV was capable of resisting a slight increase in intracellular innate immunity in HaCaT and U373MG cells, whereas the virus remained vulnerable to sequence-specific RNA interference.

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