LETTERS

An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection

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Herpes simplex virus 2 (HSV-2) infection causes significant morbidity[1] and is an important cofactor for the transmission of HIV infection[2]. A microbicide to prevent sexual transmission of HSV-2 would contribute substantially to controlling the spread of HIV and other infections[3,4]. Because RNA interference (RNAi) provides effective antiviral defence in plants and other organisms, several studies have focused on harnessing RNAi to inhibit viral infection[5]. Here we show that vaginal instillation of small interfering RNAs (siRNAs) targeting HSV-2 protects mice from lethal infection. siRNAs mixed with lipid are efficiently taken up by epithelial and lamina propria cells and silence gene expression in the mouse vagina and ectocervix for at least nine days. Intravaginal application of siRNAs targeting the HSV-2 *UL27* and *UL29* genes (which encode an envelope glycoprotein and a DNA binding protein[6], respectively) was well tolerated, did not induce interferon-responsive genes or cause inflammation, and protected mice when administered before and/or after lethal HSV-2 challenge. These results suggest that siRNAs are attractive candidates for the active component of a microbicide designed to prevent viral infection or transmission.

Most mammalian cells do not take up siRNAs without a transfection reagent. We instilled fluorescein isothiocyanate (FITC)-labelled siRNAs complexed with a transfection lipid into the mouse vagina. The vaginal and ectocervical epithelium, underlying lamina propria and stroma efficiently took up the fluorescent siRNAs (Fig. 1a). When siRNAs targeting enhanced green fluorescent protein (*EGFP*) were administered intravaginally with lipid to transgenic GFP mice that express *EGFP* in every cell from the β-actin promoter[7], GFP expression three days later was down-modulated throughout the vagina and cervix of GFP siRNA-treated mice, but not in control mice (Fig. 1b). Intravaginal siRNAs did not cause systemic silencing in distant organs such as the liver. Silencing persisted without diminution for at least nine days (the total length of the experiments) under conditions in which epithelial turnover was reduced by treatment with medroxyprogesterone acetate (Fig. 1c). Further studies are required to determine how long silencing persists and to assess the effect of menstrual variation on durability. Nonetheless, the extent and persistence of silencing suggests that siRNAs are attractive candidates for the active component of a microbicide. Moreover, their durability suggests that an RNAi-based microbicide might not need to be administered just before sexual intercourse, mitigating one of the main problems with microbicides: compliance.

To determine whether topical siRNA application could protect against sexually transmitted infection, seven siRNAs targeting three essential HSV-2 genes—*UL5* (a component of the helicase–primase complex), *UL27* (envelope glycoprotein B) and *UL29* (a DNA-binding protein)[6]—were designed using the Dharmacon design program[8]. After overnight incubation, siRNA-treated NIH3T3 (Fig. 2a) and Vero (Fig. 2b) cells were infected with HSV-2 strain 186 at a multiplicity of infection (MOI) of 1, and viral replication was assessed by plaque assay 24 h later. UL5.2, UL27.2 and UL29.2 siRNAs significantly reduced viral titre, but GFP siRNA and inverted UL29.2 siRNA did not (Fig. 2b, c).

UL29.2 was the most effective siRNA, suppressing viral replication by 62-fold in NIH3T3 cells and 25-fold in Vero cells. Viral replication by UL29.2 was inhibited at siRNA concentrations of 25 nM, and reached a plateau at 100 nM siRNA (Fig. 2c and data not shown). Gene silencing was specific for the targeted gene. When *UL27* and *UL29* messenger RNAs were quantified by real-time polymerase chain reaction with reverse transcription (RT–PCR) in Vero cells transfected one day earlier with UL27.2, UL29.2 or GFP siRNA and infected with HSV-2, peak *UL27* expression (6 h after infection) was significantly downregulated in response to UL27.2, but not to UL29.2 or GFP siRNA ($P < 0.004$). Conversely, *UL29*, which is expressed earlier than *UL27*, was significantly downregulated both at 4 h and 6 h, and only in response to UL29.2 siRNA ($P < 0.0001$ compared with GFP siRNA) (Fig. 2d). One day later, when infection had amplified by cell-to-cell spread, the expression of all four viral genes examined (siRNA-targeted *UL5*, *UL27* and *UL29* as well as the viral thymidine kinase *TK*) was reduced by siRNAs targeting any of the viral genes (Fig. 2e). These differences were all highly statistically significant. Even the least effective siRNA (UL29.1) reduced viral replication (that is, *TK* expression; $P < 0.002$ compared with GFP siRNA). Control GFP siRNA did not affect viral gene transcription. Viral gene silencing roughly paralleled the inhibition of viral replication, with UL29.2 siRNA proving the most effective, suppressing relative viral gene expression by 4–5-fold ($P < 0.001$ compared with GFP siRNA). UL5.2 and UL27.2 siRNAs each inhibited viral gene expression by ~3-fold ($P < 0.002$ for UL5.2, $P < 0.001$ for UL27.2 compared with GFP).

To investigate whether siRNAs could protect mice from HSV-2 infection, groups of 5–10 medroxyprogesterone-pretreated mice were given lipid-complexed UL29.2 intravaginally 2 h before and 4 h after vaginal challenge with $2\,LD_{50}$ (2×10^4 plaque-forming units, p.f.u.) of HSV-2 wild-type virus. Mice treated with <250 pmol UL29.2 siRNA were not protected, mice treated with 250 pmol siRNA were partially protected, and 500 pmol siRNA gave optimal protection (data not shown). We therefore administered 500 pmol siRNA in subsequent experiments.

UL29.2 siRNA provided highly significant protection, as assessed daily by a clinical disease scoring system or by survival (Fig. 3a, b). Although 75% of infected mice treated with GFP siRNA (15/20) or no siRNA (13/17) died, only 25% of mice treated with UL29.2 (5/20) died (time to death comparison by log-rank test: $P < 0.001$ versus no treatment, $P < 0.003$ versus GFP siRNA). Although 55% of UL29.2-treated mice developed some signs of infection, surviving

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mice were free of clinical disease by day 11. A longitudinal regression analysis of disease severity over time and between groups showed robust protection in UL29.2-treated mice ($P < 0.001$ versus no treatment, $P < 0.006$ versus GFP siRNA when analysed with respect to time course; $P < 0.001$ versus either control when analysed between groups).

Mice treated with UL27.2, which was less effective *in vitro*, were less effectively protected. Sixty per cent (6/10) of mice survived the lethal vaginal challenge ($P < 0.009$ compared with untreated, $P = 0.10$ compared with GFP siRNA). UL27.2 protection from disease severity was significant by longitudinal regression analysis ($P < 0.001$ compared with untreated, $P < 0.005$ compared with GFP siRNA with respect to time; $P < 0.01$ and $P = 0.05$ when analysed between the respective groups). The clinical advantage was also evident by quantifying shed virus six days after infection (Fig. 3c). Although all infected mice not given siRNAs shed virus on day six, no virus was detected in 70% of UL29.2- and 50% of UL27.2-treated mice. No virus was isolated from three out of nine GFP siRNA-treated mice, but this was not significantly different from mice not treated with siRNAs. Comparison of virus recovered from UL29.2 siRNA-treated mice with GFP siRNA-treated mice also was not significant ($P = 0.09$ by Wilcoxon rank sum test). However, the geometric mean viral titre was reduced from 1,226 p.f.u. ml^{-1} in untreated mice to 7.9 p.f.u. ml^{-1} in mice that received UL29.2 ($P < 0.01$). Viral shedding at day six predicted survival, as 18 out of 19 mice from which virus was cultured died, whereas none out of 15 mice with undetectable virus died.

One concern about using RNAi against viruses is escape from RNAi by mutation of the targeted sequence. Escape mutation has been shown for polio, HIV and hepatitis C[9–11]. We cloned and

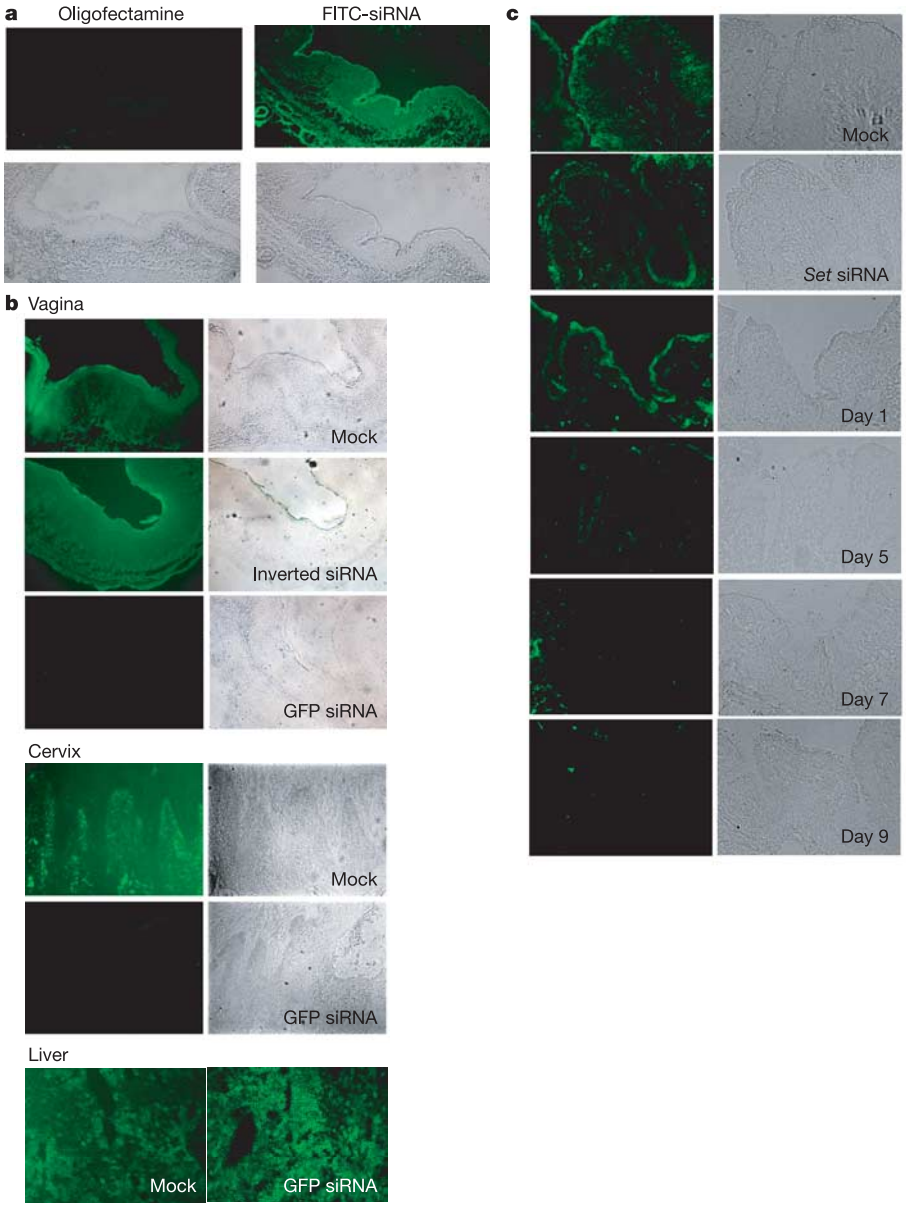

Figure 1 | siRNAs administered intravaginally are efficiently taken up by vaginal tissue and durably silence endogenous *EGFP* expression. **a**, FITC-siRNA mixed with Oligofectamine is efficiently taken up throughout the mucosa and submucosa. Sections were obtained 24 h after administration. **b**, siRNA targeting *EGFP*, but not an inverted control sequence, silences *EGFP* expression throughout the mouse vagina and cervix in GFP transgenic mice three days after administration. Liver *EGFP* expression is unaffected. **c**, Silencing persists for at least nine days in the vagina of GFP transgenic mice treated with GFP siRNAs. Data are representative of at least two experiments. An siRNA targeting an irrelevant gene (*Set*) was administered to control mice.

sequenced HSV-2 DNA from the day 6 vaginal swab from one UL29.2-treated mouse that died and from one control mouse. No mutations were found in 150-nucleotide stretches of *UL29*, which included the targeted sequence, in 24 sequences analysed from each mouse. Escape mutation is not anticipated to be as problematic for DNA viruses (such as HSV-2) as for RNA viruses.

The cervicovaginal mucosa of siRNA-treated, HSV-2-infected mice at day 6 was also spared (Fig. 3d). In control infected mice that were pretreated with no siRNA or GFP siRNA, the mucosal epithelium was partially denuded, and dying cells and inflammatory infiltrates were prominent. Multinucleated cells with intranuclear inclusion bodies—a hallmark of HSV-2 infection—were also evident. In contrast, in UL27.2 or UL29.2 siRNA-treated mice, the epithelium was intact and there were few apoptotic bodies and scarcely any inflammatory cells.

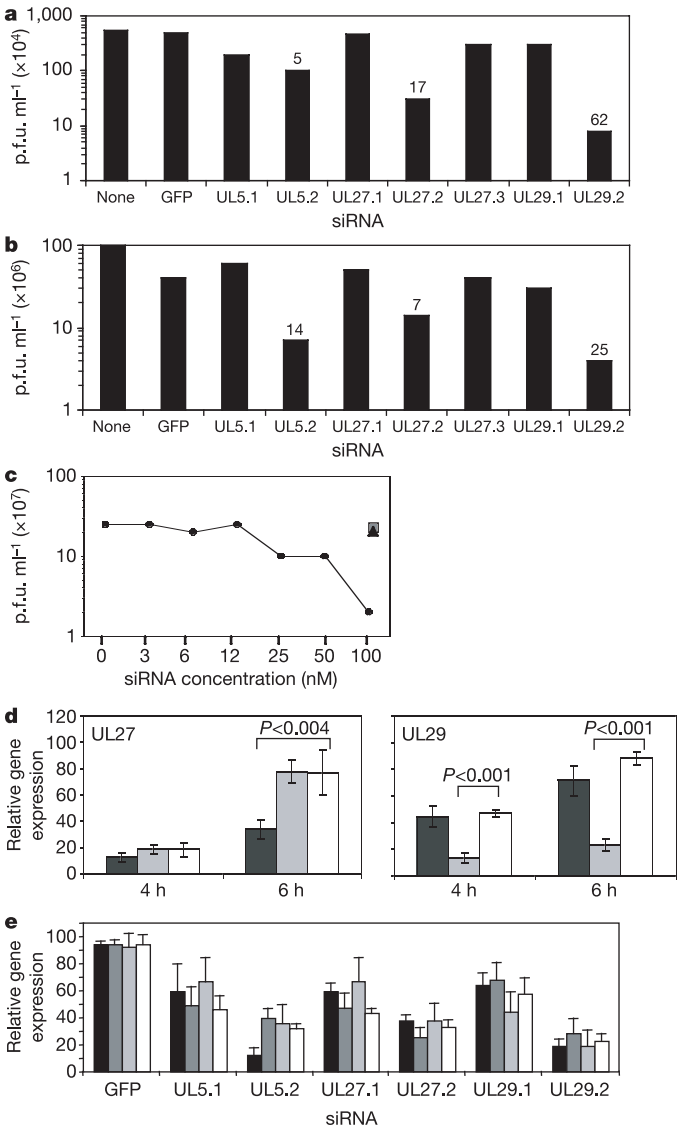

Figure 2 | siRNAs targeting HSV-2 reduce viral replication. NIH3T3 (**a**) or Vero (**b**–**e**) cells were transfected overnight with siRNA, then infected with HSV-2 and harvested 20 h later. Values above bars show fold reduction in viral plaques. Data are representative of five independent experiments. **c**, Dose-response curve, showing effect of treatment with UL29.2 (filled circles), inverted UL29.2 (filled square) or GFP (filled triangle) siRNA. **d**, **e**, Gene silencing by real-time RT–PCR was specific at 4 h or 6 h, that is, before cell-to-cell spread (UL27.2, dark grey; UL29.2, light grey; GFP, white) (**d**), but expression of all viral genes (*UL5*, black; *UL27*, dark grey; *UL29*, light grey; *TK*, white) was suppressed at 24 h (**e**). Data show mean ± s.d. from one of two experiments.

To investigate the effects of delaying siRNA treatment until after HSV-2 exposure, 500 pmol of UL27.2 or UL29.2, or a mixture of both (250 pmol each), was administered intravaginally 3 and 6 h after infection. Mice receiving UL27.2 or UL29.2 alone had no survival advantage compared with mice given GFP siRNA (2/6 survived) or no siRNA (1/6 survived) (Fig. 3e). However, 5/6 mice given both UL27.2 and UL29.2 siRNA survived ($P = 0.11$ compared with GFP siRNA; $P < 0.04$ compared with no siRNA). Therefore, post-exposure treatment might be effective. Targeting multiple genes will probably work better than targeting a single gene.

Under certain circumstances, siRNAs can induce the interferon (IFN) pathway and trigger inflammation[12–15]. We therefore analysed vaginal tissue for inflammatory infiltrates (Fig. 4a) and induction of interferon and interferon-responsive genes 24 and 48 h after siRNA treatment (Fig. 4b). siRNA treatment did not cause an inflammatory infiltrate. Moreover, *Ifnb* and the principal interferon-responsive genes, *Oas1* and *Stat1*, were not significantly induced when analysed by quantitative RT–PCR. As expected, HSV-2 infection in the absence of siRNAs (used as a positive control) activated interferon-responsive genes.

Vaginal instillation of siRNAs targeting essential viral genes protects mice from vaginal challenge with a lethal dose of HSV-2. The treatment was well-tolerated without causing inflammation or inducing interferon-responsive genes. This efficient and lasting silencing deep in the vaginal tissue was unexpected, and augurs well for using siRNAs to prevent or treat sexually transmitted viral and parasitic infections. Our results, together with impressive results in lung models of viral infection[16–20], suggest that siRNA uptake at mucosal surfaces may be particularly efficient and involve mechanisms not present in internal organs.

Much work needs to be done to develop siRNAs as the basis for a microbicide. These experiments were done without optimizing the siRNAs for silencing efficiency or chemical modifications that enhance resistance to endogenous RNases[21]. siRNAs would also need to be formulated in a vehicle acceptable for vaginal retention. The effect of menstrual variation on protection, especially on the durability of silencing, needs to be evaluated. Viral sequence variability also needs to be addressed. However, by targeting relatively well-conserved viral sequences in essential viral genes or by combining siRNAs that target multiple viral genes, the related problems of viral sequence diversity and potential escape mutation might be mitigated. Although we did not find evidence of escape mutation, this might take longer than six days to develop. Any extension of our results to the designing of an HIV microbicide would also require demonstrating silencing in resident tissue macrophages, dendritic cells and T cells, which are rare in normal, uninflamed vaginal tissue.

Finally, cost is an important consideration for a microbicide designed for global use. Only 500 pmol siRNA was required to protect mice in this study. The manufacturing cost of a single application for humans, crudely estimated on the basis of scaling up by weight and current costs, is $8. If silencing is durable and treatments can be spaced, this is a realistic cost. Given the devastating global epidemic and the unlikelihood of there being an effective HIV-l vaccine soon, we feel that investigating whether RNAi can be harnessed for use in microbicides is a sensible approach.

Note added in proof: In the advance online publication of this Letter, in the second sentence of the fourth paragraph '25 μM, and reached a plateau at 100 μM' should read '25 nM, and reached a plateau at 100 nM'. In addition, the *x* axis of Fig. 2c should read 'siRNA concentration (nM)'. These errors have been corrected for print.

METHODS

Mice. BALB/c mice (5–8 weeks old) were obtained from Taconic Farms; FVB.Cg-Tg(GFPU)5Nagy mice were from Jackson Laboratories[7]. Mice were

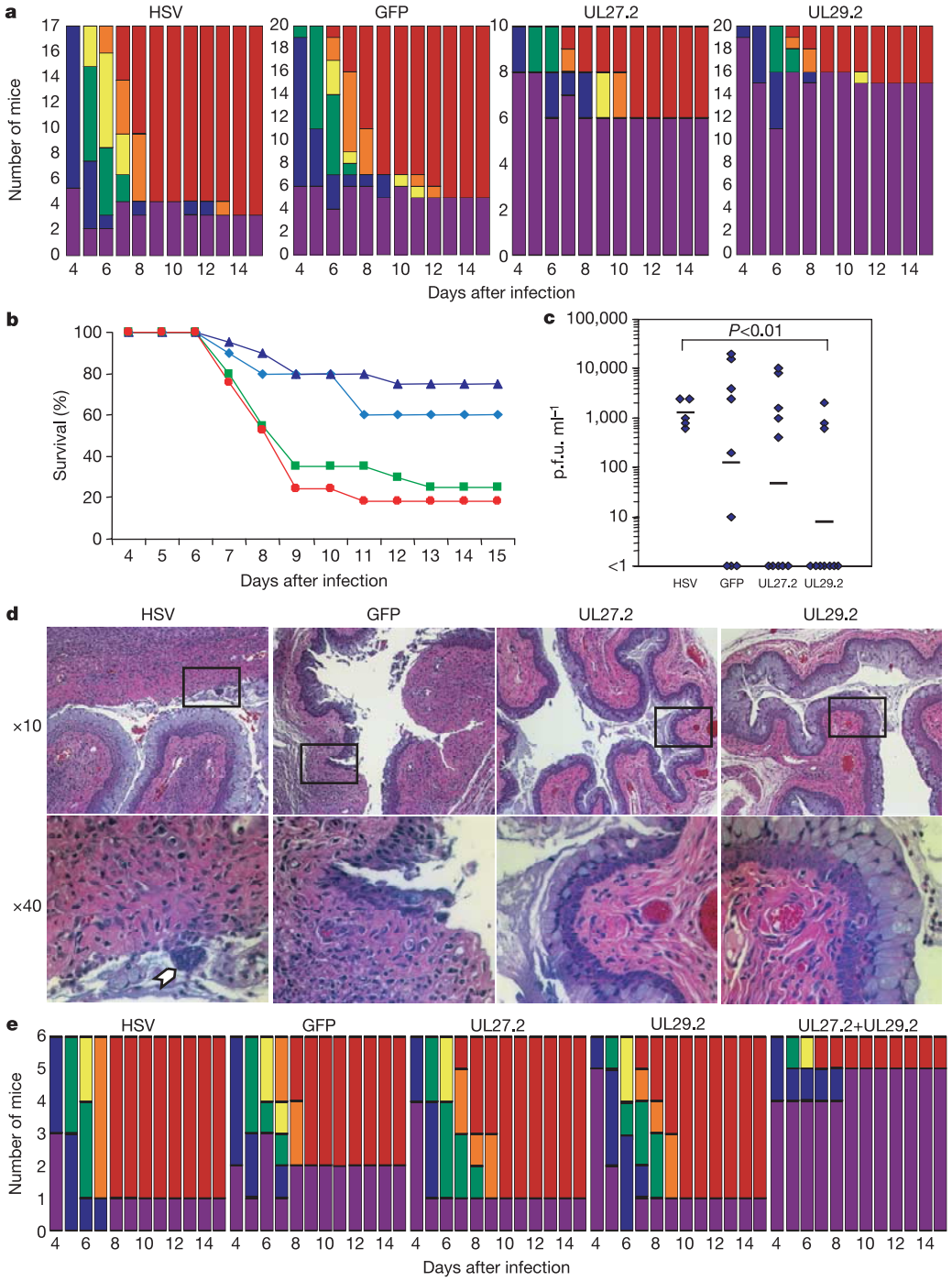

Figure 3 | siRNAs protect mice from lethal HSV-2 infection. a–d, Mice given lipid-complexed siRNA intravaginally 2 h before and 4 h after infection with ~2 LD_{50} HSV-2 were analysed for disease severity (**a**) (see colour code provided in Methods), survival (**b**) (HSV only, red; GFP, green; UL27.2, light blue; UL29.2, dark blue), viral shedding on day 6 (**c**) and cervicovaginal histopathology on day 6 (**d**). **a**, **b** show data from three experiments. Transfection of lipid alone did not affect HSV-2 disease (not shown). **c**, Vaginal viral shedding. Bars represent geometric mean titre. **d**, The epithelium is preserved after UL27.2 or UL29.2 siRNA treatment, with decreased inflammatory infiltrates and fewer dying cells. Boxes indicate areas magnified in lower panels. White arrow points to a multinucleated cell with viral inclusion—a hallmark of HSV-2 infection. **e**, A combination of UL27.2 plus UL29.2 siRNA, but neither siRNA alone, protects from HSV-2 disease after exposure. Data are representative of two experiments.

subcutaneously injected with 2 mg medroxyprogesterone acetate (Sicor), and then 1 week later were infected vaginally with 2×10^4 p.f.u. (~2 LD_{50}) HSV-2 strain 186 (ref. 22). siRNA (500 pmol) was complexed with Oligofectamine (Invitrogen) according to the manufacturer's protocol, and was then administered intravaginally (in a maximum volume of 12 μl) either 2 h before and 4 h after HSV-2 infection or 3 h and 6 h after HSV-2 infection. Clinical signs of infection were graded according to a five-point scale: 0, no signs of infection (purple); 1, slight genital erythema and oedema (blue); 2, moderate genital inflammation (green); 3, purulent genital lesions (yellow); 4, hind limb paralysis (orange); 5, death (red)[22]. Viral shedding was determined by swabbing the vaginal cavity (using a Micropur swab, PurFybr Inc.) on day 6 after infection, and titrating the virus on Vero cells. In some cases, the vagina was dissected at the indicated times and either fixed in 10% formalin (Sigma) for paraffin embedding and sectioning, or stored in RNAlater (Qiagen) for RNA isolation.

Viruses and transfection assays. For *in vitro* studies, 186ΔKpn, a replication-competent, *TK*-negative mutant of strain 186syn + (ref. 23) was grown in Vero cells as described[24]. Vero or NIH3T3 cells (ATCC) (4×10^5 cells per well in 6-well plates in 1 ml of complete medium, plated one day earlier), were treated

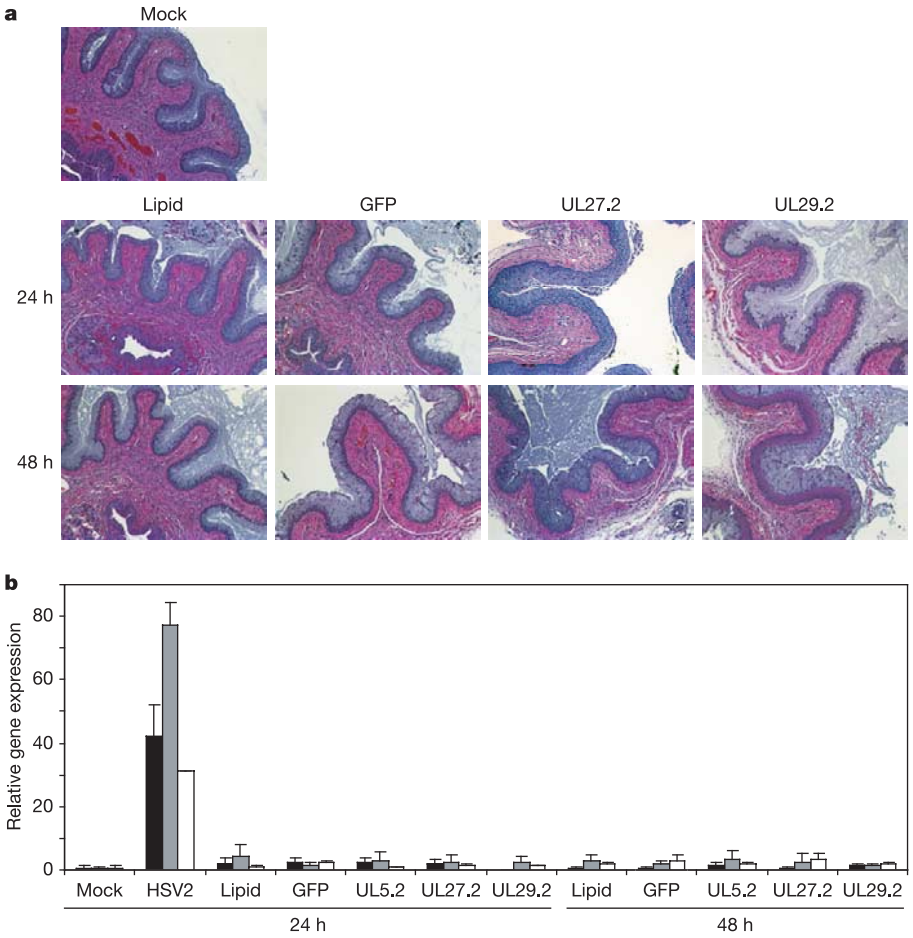

Figure 4 | Topical lipid-complexed siRNAs do not activate inflammation or interferon-responsive genes. a, **b**, Vaginal tissue, dissected 24 h or 48 h after administering 500 pmol of lipid-complexed siRNA, was assessed by haematoxylin-eosin staining for inflammation (**a**, ×10 magnification) and by quantitative RT–PCR for expression of *Ifnb* (black) and the interferon-responsive genes *Stat1* (grey) and *Oas1* (white) relative to the control gene *Gapdh* (**b**). HSV-2 infection was used as a positive control for interferon induction. In siRNA-treated mice, no HSV-2 was administered. None of the siRNAs induced a significant change in interferon-responsive gene expression compared to mock-treated mice given only PBS intravaginally. Data show mean ± s.d.

with 100 pmol or the indicated concentration of siRNA. The siRNA had been complexed with TransIT-TKO (Mirus) to transfect Vero cells or with TransIT-siQuest (Mirus) for NIH3T3 cells, according to the manufacturer's instructions. The medium was replaced after overnight incubation at 37 °C, and 2 h later HSV-2 186ΔKpn was added at an MOI of 1. After 1 h at 37 °C, the medium was again replaced. Cells were harvested 24 h later and viral titre determined by plaque assay on Vero cells. For mouse experiments, wild-type HSV-2 strain 186syn + virus was used[25]. An aliquot of virus used for each mouse experiment was also assayed by plaque assay to confirm viral titre.

siRNAs. siRNAs (Dharmacon) were prepared according to the manufacturer's instructions. FITC-labelled siRNA was a previously described sequence targeting CD4 (ref. 26). The sequence for silencing *EGFP* has been described[26]. The sequences for HSV-2 (GenBank accession number NC 001798) siRNAs were: UL5.1 (nt 12838–12856) sense 5′-CUACGGCAUCAGCUCCAAA-3′, antisense 5′-UUUGGAGCUGAUGCCGUAG-3′; UL5.2 (nt 12604–12622) sense 5′-UGUGGUCAUUGUCUAUUAA-3′, antisense 5′-UUAAUAGACAAUGACCACA-3′; UL27.1 (nt 54588–54606) sense 5′-GUUUACGUAUAACCACAUA-3′, antisense 5′-UAUGUGGUUAUACGUAAAC-3′; UL27.2 (nt 54370–54388) sense 5′-ACGUGAUCGUGCAGAACUC-3′, antisense 5′-GAGUUCUGCACGAUCACGU-3′; UL27.3 (nt 54097–54115) sense 5′-UCGACCUGAACAUCACCAU-3′, antisense 5′-AUGGUGAUGUUCAGGUCGA-3′; UL29.1 (nt 59715–59733) sense 5′-CCACUCGACGUACUUCAUA-3′, antisense 5′-UAUGAAGUACGUCGAGUGG-3′; UL29.2 (nt 60324–60342) sense 5′-CUUUCGCAAUCAAUUCCAA-3′, antisense 5′-UUGGAAUUGAUUGCGAAAG-3′; inverted UL29.2 sense 5′-AACCUUAACUAACGCUUUC-3′, antisense 5′-GAAAGCGUUAGUUAAGGUU-3′.

Quantitative RT–PCR. Total RNA (1 μg) was isolated using the RNeasy RNA isolation kit (Qiagen) and reverse transcribed using Superscript III (Invitrogen) and random hexamers, according to the manufacturer's protocol. Real-time PCR was performed on 0.2 μl of complementary DNA, or a comparable amount of RNA with no reverse transcriptase, using Platinum Taq Polymerase (Invitrogen) and a Biorad iCycler. SYBR green (Molecular Probes) was used to detect PCR products. Reactions were performed in 25 μl in triplicate. Primers were: *Gapdh* forward 5′-TTCACCACCATGGAGAAGGC-3′, *Gapdh* reverse 5′-GGCATGGACTGTGGTCATGA-3′, *TK* forward 5′-CGATCTACTCGCCAACACGGTG-3′, *TK* reverse 5′-GAACGCGGAACAGGGCAAACAG-3′, *UL5* forward 5′-TCGCTGGAGTCCACCTTCGAAC-3′, *UL5* reverse 5′–CGAACTCGTGCTCCACACATCG-3′, *UL27* forward 5-CAAAGACGTGACCGTGTCGCAG-3′, *UL27* reverse 5′-GCGGTGGTCTCCATGTTGTTCC-3′, *UL29* forward 5′-GCCAGGAGATGGACGTGTTTCG-3′, *UL29* reverse 5′-CGCGCTGTTCATCGTTCCGAAG-3′, *Stat1* forward 5′-TTTGCCCAGACTCGAGCTCCTG-3′, *Stat1* reverse 5′-GGGTGCAGGTTCGGGATTCAAC-3′, *Oas1* forward 5′-GGAGGTTGCAGTGCCAACGAAG-3′, *Oas1* reverse 5′-TGGAAGGGAGGCAGGGCATAAC-3′, *Ifnb* forward 5′-CTGGAGCAGCTGAATGGAAAG-3′, *Ifnb* reverse 5′-CTTGAAGTCCGCCCTGTAGGT-3′.

PCR parameters consisted of 5 min Taq activation at 95 °C, followed by 40 cycles of 95 °C × 20 s, 60 °C × 30 s, and 69 °C × 20 s. Standard curves were generated and the relative amount of mRNA was normalized to *Gapdh* mRNA. Specificity was verified by melt curve analysis and agarose gel electrophoresis.

Tissue sections and microscopy. For fluorescence microscopy, dissected tissue was placed in optimal cutting temperature compound (TissueTek) and snap-frozen in LN_2. For haematoxylin-eosin stained sections, tissues were fixed in 10% formalin and paraffin-embedded. Microscopy was performed and scored (by an operator blind to the treatment condition) on a Zeiss Axiovert 200M microscope using Slidebook acquisition and analysis software (Intelligent Imaging).

Statistical analysis. *In vitro* data were analysed by Student's *t*-test. Survival distribution was calculated using the Kaplan and Meier method[27], and the univariate comparison of survival for control versus treated groups was tested using a log-rank test, comparing two groups at a time[28]. The approach of generalized estimating equations was used to model disease scores collected over time and to compare disease severity of control versus treated groups[29]. All *P*-values are for two-tailed significance tests.

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