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Durable Knockdown and Protection From HIV Transmission in Humanized Mice Treated With Gel-formulated CD4 Aptamer-siRNA Chimeras

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The continued spread of HIV underscores the need to interrupt transmission. One attractive strategy, in the absence of an effective vaccine, is a topical microbicide, but the need for application around the time of sexual intercourse leads to poor patient compliance. Intravaginal (IVAG) application of CD4 aptamer-siRNA chimeras (CD4-AsiCs) targeting the HIV coreceptor *CCR5*, *gag*, and *vif* protected humanized mice from sexual transmission. In non-dividing cells and tissue, RNAi-mediated gene knockdown lasts for several weeks, providing an opportunity for infrequent dosing not temporally linked to sexual intercourse, when compliance is challenging. Here, we investigate the durability of gene knockdown and viral inhibition, protection afforded by *CCR5* or HIV gene knockdown on their own, and effectiveness of CD4-AsiCs formulated in a gel in polarized human cervicovaginal explants and in humanized mice. CD4-AsiC–mediated gene knockdown persisted for several weeks. Cell-specific gene knockdown and protection were comparable in a hydroxyethylcellulose gel formulation. CD4-AsiCs against *CCR5* or *gag*/*vif* performed as well as a cocktail in humanized mice. Transmission was completely blocked by CCR5 CD4-AsiCs applied 2 days before challenge. Significant, but incomplete, protection also occurred when exposure was delayed for 4 or 6 days. CD4-AsiCs targeting *gag*/*vif* provided some protection when administered only after exposure. These data suggest that CD4-AsiCs are a promising approach for developing an HIV microbicide.

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INTRODUCTION

The CAPRISA004 trial demonstrated for the first time that a topical microbicide might protect women against sexually transmitted HIV.[1] Tenofovir gel applied intravaginally before and after sexual intercourse reduced HIV transmission by 39% in a high-risk population. However, these promising results were not replicated in two follow-up studies, probably because of poor compliance with a regimen that required usage just before and after exposure.[2] Compliance might improve for a microbicide that requires less frequent application that is not temporally linked to sexual exposure.[3] Knocking down host genes that are needed for viral transmission, viral genes, or both can interfere with viral transmission and replication,[4–15] including the sexual transmission of viruses such as HIV. Because gene knockdown persists for over a week in most non-dividing cells,[5,16] a microbicide based on RNA interference (RNAi) as its active principle could provide a strategy with a manageable dosing schedule for overcoming problems with compliance. In fact, a previous study of gene knockdown in the genital tract showed protection in mice from intravaginal (IVAG) challenge a week later with a lethal dose of herpes simplex virus type 2.[13,15] siRNAs directed against *CCR5* can effectively silence gene expression *in vitro* for 3 weeks and prevent viral replication in primary macrophages when challenge is delayed for 3 weeks.[5] CCR5 is the coreceptor used almost exclusively during HIV or SHIV sexual transmission.[17,18] Blocking CCR5 by more conventional approaches (antibodies, antagonists, and small molecule inhibitors) inhibits sexual transmission in non-human primates.[19–22]

Accomplishing gene knockdown in HIV-susceptible cells, which are refractory to most transfection techniques that could be used *in vivo*, has been difficult.[5,6] Although targeted delivery has become possible in recent years, even *in vivo*, using fusion proteins, which couple a targeting antibody (Ab) fragment to an RNA binding motif, such as protamine,[12,23,24] we developed an alternative approach using RNA aptamers. These are ideally suited for therapeutic applications like topical microbicides, which require repeated administration, because of economical large-scale synthesis and low immunogenicity.[25,26] Building on the work of other groups targeting prostate cancer[27,28] and virus-infected cells,[29] we recently showed that chimeric RNAs, composed of a known CD4 aptamer[30] covalently linked to an siRNA, could transfect all $CD4^+$ cells susceptible to HIV in the genital tract of female humanized mice, and specifically knockdown

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gene expression in CD4$^+$ cells without any apparent toxicity or immune stimulation.[14] Topical application of a cocktail of CD4-AsiCs targeting *CCR5*, *gag* and *vif* inhibited HIV vaginal transmission with a high dose of HIV$_{\text{JR-CSF}}$ that uniformly infected all control animals.[14] We termed these RNAs, CD4-aptamer-siRNA chimeras (CD4-AsiCs). In that study, CD4-AsiCs were administered according to a complicated schedule—RNAs targeting *CCR5* were administered 1 and 2 days before viral challenge and the CD4-AsiCs targeting viral genes were given 24 hours before and 4 hours after viral challenge. Although the aptamer alone had some antiviral effect, presumably by blocking virion binding to the CD4 receptor, we showed that siRNA-mediated gene knockdown was the principal mechanism underlying the protective effect.[14] RNAs were injected in a liquid solution and mice were kept supine for ~5 minutes to prevent the liquid from immediately leaking out of the genital tract. However, a clinically useful microbicide will need to be formulated in a gel suitable for vaginal retention.

Here, we investigate whether gene knockdown and protection can be achieved using a more practical dosing schedule and RNAs formulated in a gel suitable for clinical use. We used polarized human cervicovaginal explants and immunodeficient NOD/SCID/*IL2Rγ*$^{-/-}$ (NSG) mice, reconstituted with CD34$^+$ cells from human fetal liver and implanted with human fetal thymic grafts (so-called "BLT mice"),[14,31–34] to investigate questions important for developing a clinically useful microbicide based on CD4-AsiCs as the active ingredient. How long does gene knockdown last and how durable is protection? Could CD4-AsiCs be formulated in an inert nontoxic gel that would be retained within the genital tract without interfering with gene knockdown or antiviral efficacy? Could just knocking down *CCR5* or just viral genes provide protection? Are antiviral RNAs protective when administered only after exposure? We found durable protection for ~4 days with gel-formulated CD4-AsiCs targeting just *CCR5* and partial protection from a delayed challenge or when treatment was only given after exposure. Optimization of CD4-AsiCs (and/or repeated application) would likely lead to further improvement.

RESULTS

CD4-AsiCs stably knockdown gene expression for 3 weeks *in vitro*

To assess the durability of gene silencing by CD4-AsiCs, we first incubated resting CD4$^+$ PBMCs from three donors with 1 μmol/l Cy3-labeled CD4-AsiCs against *CCR5*, a concentration that we previously found provided ~50% maximal *in vitro* knockdown[14] (**Figure 1**). AsiC-treated cells were cultured in IL-15 under conditions that minimize cell proliferation but maintain cell viability, and RNA uptake and gene silencing in CD4$^+$ T cells was assessed by flow cytometry over 5 weeks. Uptake and

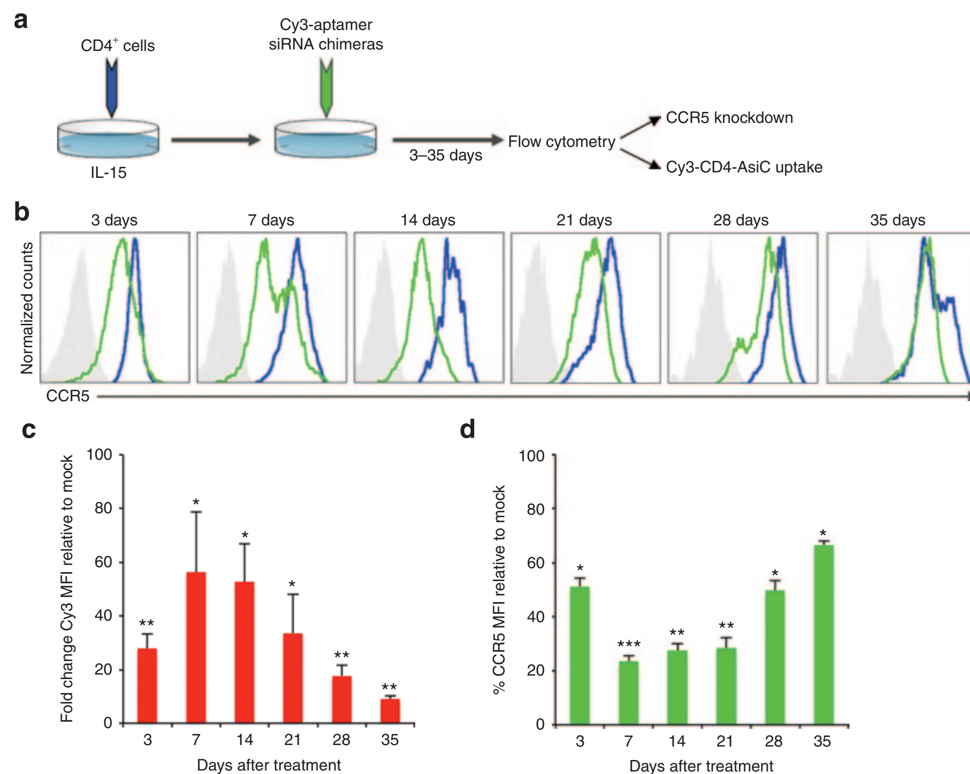

Figure 1 CD4-AsiCs suppress target gene expression for several weeks in resting CD4$^+$ cells *in vitro*. (**a**) *In vitro* silencing experiment design. CD4$^+$ PBMCs, treated with 1 μmol/l Cy3-labeled CD4-AsiCs bearing siRNAs against *CCR5*, were cultured in IL-15 and analyzed over 5 weeks by flow cytometry for uptake of fluorescent siRNA and CCR5. (**b**) Representative flow cytometry histograms of CCR5 levels in gated CD3$^+$ CD4 cells from the blood of one donor after treatment with CD4-AsiCs are shown. Cells treated with *CCR5* CD4-AsiC (green) were compared with mock-treated cells (blue) and unstained (gray) controls. (**c**) Fold increase in Cy3 fluorescence (*$P < 0.05$, **$P < 0.005$, *t*-test) and (**d**) % CCR5 mean fluorescence intensity (MFI) (*$P < 10^{-5}$, **$P < 10^{-6}$, ***$P < 10^{-7}$, *t*-test) in Cy3$^+$ cells, compared with mock-treated cells, are shown. Data represent the mean ± SEM of data from five donors in three independent experiments Maximal target CCR5 knockdown was achieved 7 days after treatment and was maintained for 2 weeks.

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gene silencing increased over the first week. The slow onset of gene silencing by CD4-AsiCs *in vitro* was reproducible (data not shown). *CCR5* expression was stably reduced by ~75% for 3 weeks, and then gradually increased. After 5 weeks of culture, gene expression was still reduced by 25% compared with mock-treated cultured cells. Thus, in slowly dividing CD4 T cells gene knockdown by CD4-AsiCs persists for about 3 weeks.

IVAG application of CD4-AsiCs suppresses target gene expression for 2 weeks in BLT mice

We next evaluated the durability of target gene silencing in humanized mice. A mixture of CD4-AsiCs bearing *CD45* and *CCR5* siRNAs (40 pmol of each per application, the dose used in our previous study)[14] was administered intravaginally twice 24 hours apart to medroxyprogesterone-treated BLT mice. Mice

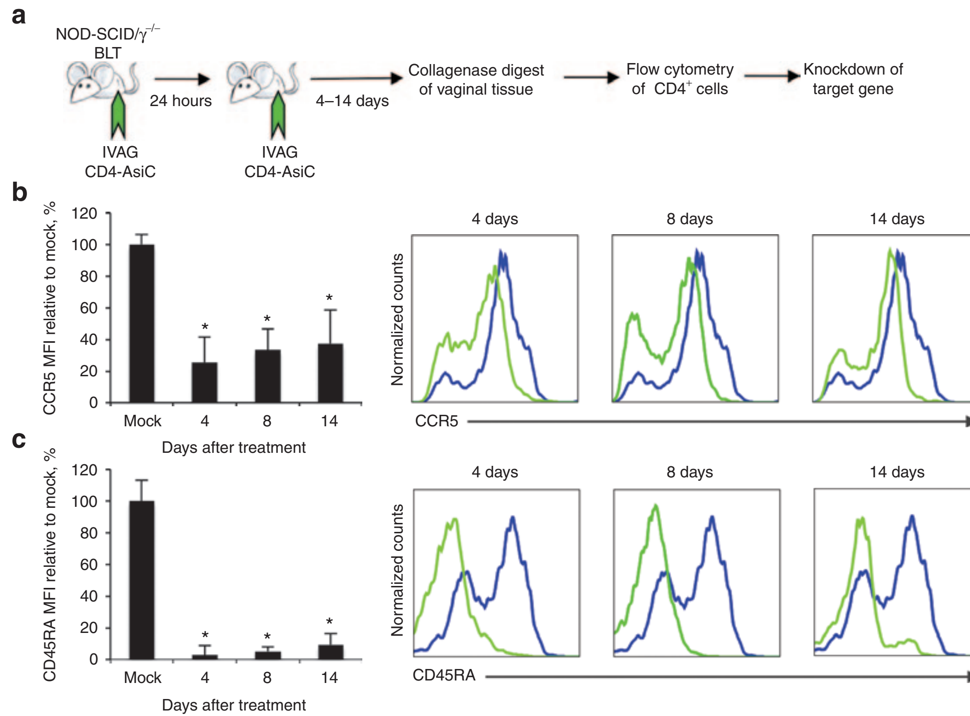

Figure 2 Sustained CD4-AsiC knockdown of *CD45* and *CCR5* in BLT mice. (**a**) Experimental design to assess durability of CD4-AsiC–mediated silencing *in vivo.* BLT mice were treated twice with either phosphate-buffered saline (PBS) ($N = 3$) or a mixture of *CCR5* and *CD45* CD4-AsiCs ($N = 2$ per timepoint) (40 pmol for each target per application). A single cell suspension of the dissected vaginal mucosa was analyzed by flow cytometry by gating on $CD4^+$ cells. (**b**) CCR5 and CD45 knockdown were stable for 2 weeks. Presented are representative histograms (green, treated; blue, mock-treated control mice) and graphs of mean ± SEM mean fluorescence intensity (MFI) of the targeted genes of treated mice relative to mock-treated mice (*$P < 0.01$, *t*-test). These data are representative of two independent experiments.

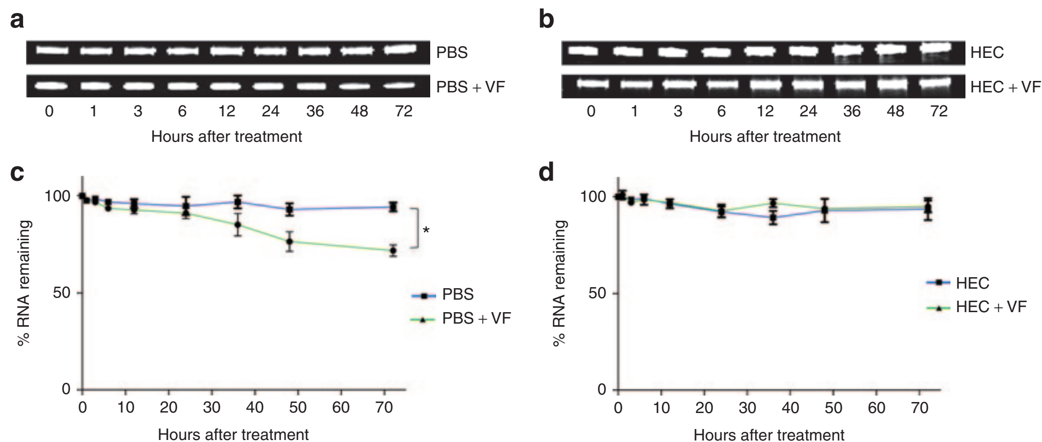

Figure 3 CD4-AsiCs suspended in PBS or hydroxyethyl cellulose (HEC) gel are stable in human vaginal fluid. *CCR5* CD4-AsiCs synthesized using 2′-fluoropyrimidines, mixed in either 100 μl phosphate-buffered saline (PBS) or HEC gel (blue) were added to 100 μl of vaginal fluid (VF, green) obtained from a healthy preovulatory donor. At regular intervals, 10 μl was removed, resuspended in gel loading buffer and frozen at −80 °C before being electrophoresed on a denaturing PAGE gel. Representative ethidium bromide-stained PAGE gels (**a**,**b**) and the average intensity (+SEM) of bands from three independent experiments (**c**,**d**) analyzed by densitometry are shown. HEC-formulated AsiCs are somewhat more stable in vaginal fluid, than unformulated AsiCs. *$P < 0.05$, *t*-test.

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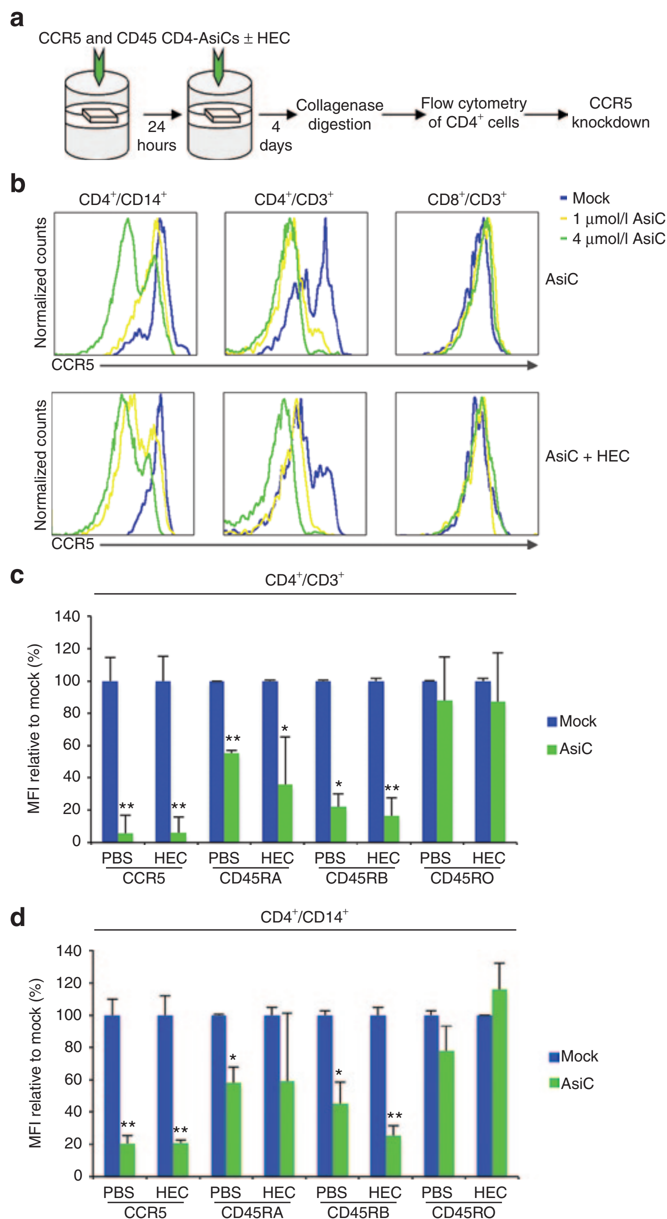

Figure 4 CD4-AsiCs in hydroxyethyl cellulose (HEC) knockdown gene expression in CD4$^+$ cells in polarized human cervicovaginal explants. (**a**) Experimental design. *CCR5* and *CD45* CD4-AsiCs (1 or 4 µmol/l of each) in phosphate-buffered saline (PBS) or HEC gel were applied to agarose-embedded polarized explants twice before tissue was digested with collagenase to a single cell suspension that was stained with fluorescently conjugated mAb, and analyzed by flow cytometry. (**b**) Representative histograms from one of two independent experiments show CCR5 knockdown in CD14$^+$CD4$^+$ monocytes (left), CD3$^+$/CD4$^+$ T cells (middle), but not CD3$^+$/CD8$^+$ T cells (right). Mean ± SEM expression of CCR5 and CD45 isoforms in two donor samples treated with 4 µmol/l CD4-AsiCs is shown in (**c**) CD4$^+$ T cells and (**d**) CD4$^+$/CD14$^+$ monocytes. The *CD45* siRNA is active against *CD45RA* and *CD45RB*, but not *CD45RO*. There was no statistically significant difference in knockdown between AsiCs mixed in saline or HEC gel for either target gene (*$P < 0.05$, **$P < 0.005$, *t*-test CD4-AsiC versus mock treatment).

were sacrificed 4, 8, and 14 days after the last treatment (**Figure 2**). Mock-treated control mice, similarly treated twice with phosphate-buffered saline (PBS), were sacrificed 4 days after the second dose. CD45 and *CCR5* expression in CD4$^+$ cells in the vaginal mucosa were evaluated by flow cytometry of single cell suspensions of collagenase-digested tissue. *CCR5* and *CD45* were stably knocked down for 2 weeks in CD4$^+$ cells in AsiC-treated mice relative to mock-treated controls. The *CD45* CD4-AsiC was more effective at gene knockdown (mean fluorescence intensity reduced to 5–10% of control over 2 weeks) than the *CCR5* construct (mean fluorescence intensity reduced to 20–35% of baseline). A similar experiment conducted in mice prepared from a different donor showed similarly stable gene knockdown (data not shown).

CD4-AsiCs maintain activity when formulated in hydroxyethylcellulose

Because a liquid microbicide is not practical for human use, we next evaluated whether uptake and silencing is maintained when CD4-AsiCs are formulated in a hydroxyethyl cellulose (HEC) gel. HEC is an FDA-approved nonionic, nontoxic, chemically stable, water-soluble polymer that has been used as the universal placebo in all HIV microbicide trials since 2005.[35] CD4-AsiCs were highly stable in HEC gel or PBS over 72 hours (**Figure 3**). In the presence of vaginal fluid, the AsiCs in HEC did not decay at all, whereas the AsiCs in PBS decayed slightly (only by ~20% over 72 hours). To examine the effect of the gel on gene knockdown, *CCR5* or *CD45* CD4-AsiCs, premixed in PBS or HEC, were added twice to polarized human cervicovaginal explants, and single cell suspensions, prepared by collagenase digestion, were analyzed for *CCR5* and CD45 expression by flow cytometry 4 days after the second treatment (**Figure 4**). *CD45* mRNA is alternatively spliced to produce three main isoforms; the *CD45* siRNA is specific for the CD45RA and RB isoforms, but is inactive against the RO isoform. Formulation in HEC did not significantly affect gene silencing. Gene knockdown of *CCR5* and the siRNA-sensitive isoforms of CD45 (but not CD45RO) occurred in CD4$^+$ T cells and tissue macrophages, but not in CD8$^+$ T cells, suggesting that cell-specific targeting was maintained.

We next assessed whether gel formulation affects gene knockdown in the genital tract of humanized female mice. CD4-AsiCs against *CCR5* and *CD45* (40 pmol each) were administered with or without gel formulation twice (spaced by 24 hours) to the genital tract of humanized mice. Four days later, vaginal tissue was harvested, digested with collagenase, and single cell suspensions were analyzed for target gene expression by flow cytometry (**Figure 5**). Groups of mice treated with PBS or naked siRNAs (±HEC), served as negative controls. Knockdown of *CCR5* and targeted *CD45* isoforms was similar in mice treated with CD4-AsiCs in PBS and in HEC gel and was specific to CD4$^+$, compared with CD8$^+$, T cells. Naked siRNAs did not induce gene knockdown in T cells and may have been immunostimulatory, because they upregulated CD45RO in both CD4$^+$ and CD8$^+$ lymphocyte populations. CD4-AsiCs in PBS increased CD45RO expression to a lesser extent, but only in CD8$^+$ cells. Although this upregulation was not observed in other experiments, we cannot rule out a low level of immune stimulation by the *CCR5* or *CD45* CD4-AsiC.

It should also be noted that the degree of knockdown in CD4$^+$ T cells in this *in vivo* experiment was less than we observed in our published study[14] or in **Figure 2** above or in data not shown. Some batches of humanized mice, depending on the human donor, are especially prone to develop graft versus host disease (GvHD) or

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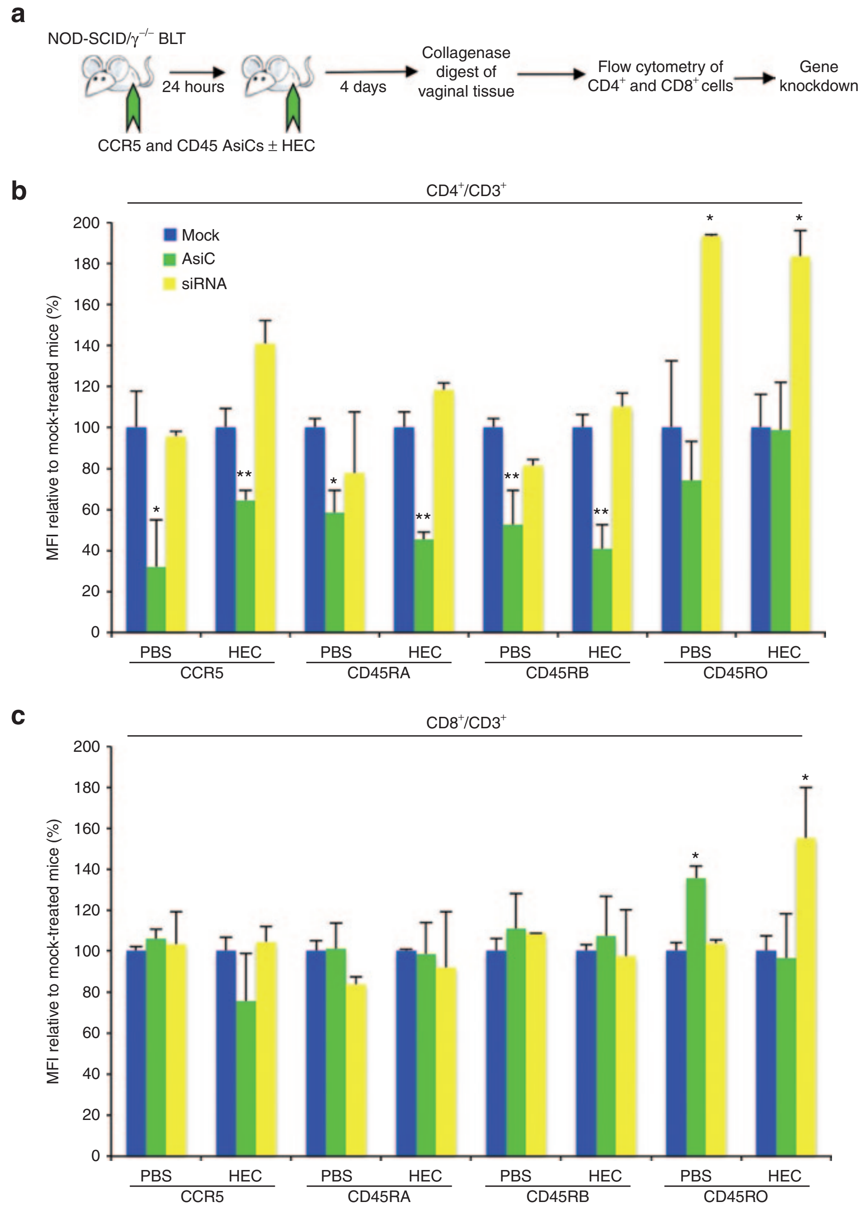

Figure 5 CD4-AsiCs in hydroxyethyl cellulose (HEC) knockdown gene expression in CD4$^+$ cells in the vaginal mucosa of BLT mice. (**a**) Experimental design to compare knockdown by CD4-AsiCs (40 pmol of each RNA per application) *in vivo* applied in phosphate-buffered saline (PBS) or HEC gel. (**b**,**c**) Groups of BLT mice were treated IVAG with vehicle alone (mock, *N* = 3), CD4-AsiCs (*N* = 3), or naked siRNAs (*N* = 2). A single cell suspension of the extracted vaginal mucosa was analyzed by flow cytometry to assess target gene silencing in (**b**) CD4$^+$ T cells and (**c**) CD8$^+$ T cells. Knockdown was restricted to CD4$^+$ cells. Data are representative of three independent experiments. Graphs show mean ± SEM MFI, normalized to mock-treated mice (*$P < 0.05$, **$P < 0.005$, *t*-test relative to mock).

a wasting syndrome that has been attributed to their conditioning regimen. Either of these conditions may cause more immune activation and increased immune cell trafficking into and out of mucosal tissues, which might affect knockdown. This batch of mice had a high incidence of wasting, which might be responsible for the atypically poor gene knockdown in this experiment.

Targeting either HIV-encoded genes or host factors using CD4-AsiCs blocks vaginal transmission of HIV to humanized mice

We previously showed that IVAG application of a cocktail of CD4-AsiCs against *CCR5*, *gag*, and *vif* inhibits HIV transmission to BLT mice.[14] We next tested the relative contribution of targeting host or viral genes to HIV inhibition. Using the same dosing regimen, we administered *CCR5* CD4-AsiCs 48 hours and 24 hours before IVAG challenge or *gag/vif* CD4-AsiCs 24 hours before and 4 hours after IVAG challenge. Mice treated with the cocktail of host and HIV-specific AsiCs given at the same times were used as a positive control, and mice treated with PBS served as a negative control (**Figure 6**). All control mice became infected and had detectable blood HIV p24 antigen and plasma RNA within 4 weeks of infection, whereas all mice treated with the cocktail, the *CCR5* CD4-AsiC or the *gag/vif* CD4-AsiCs showed no evidence of infection. Their CD4

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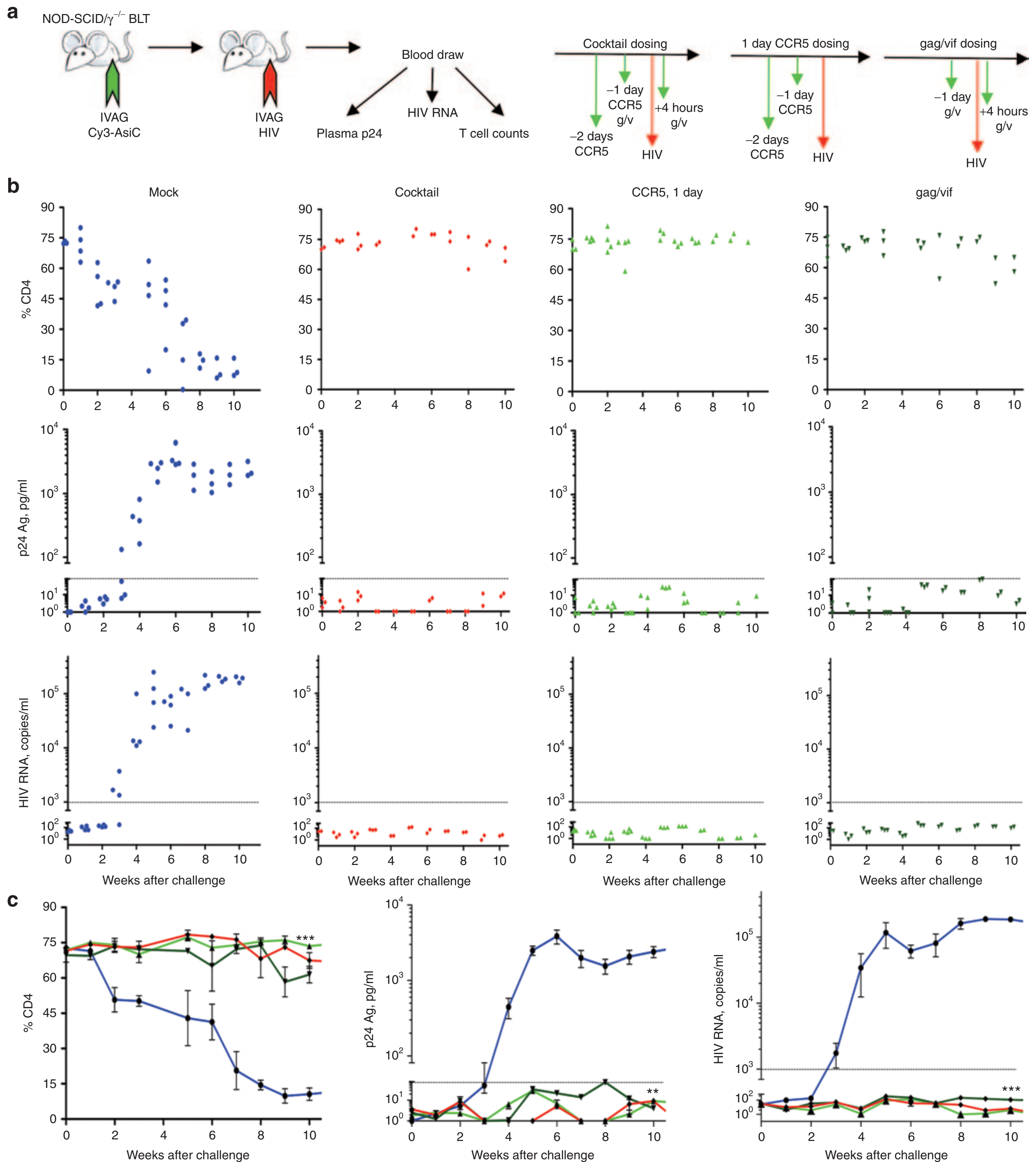

Figure 6 CD4-AsiCs targeting *CCR5* or viral genes inhibit HIV transmission to BLT mice. (**a**) Experimental design for HIV_{JR-CSF} challenge. The dosing schedule is the same as in ref. 14. Four mice were assigned to each group. (**b**) Four treatment groups were compared: phosphate-buffered saline (PBS) controls (blue), CCR5 and viral gene cocktail (red), CCR5 (bright green), or gag/vif (dark green). Shown is the effect of treatment on circulating $CD4^+/CD8^+$ T cell ratio (top), plasma HIV p24 Ag (middle), and plasma HIV RNA assessed by qRT-PCR (bottom). (**c**) Mean ± SEM for each group (**P < 0.01; ***P < 0.001, analysis of variance). Dashed lines indicate the detection threshold.

counts remained normal and none had detectable HIV p24 antigen or RNA in their plasma above background during the 10 weeks of observation. At weeks 5 and 8, the p24 Ag level of mice treated with AsiCs targeting just *gag* and *vif* had p24 Ag levels just at the threshold of detection. However, the qRT-PCR assay for HIV *gag* mRNA, which is a more sensitive test for plasma virus, remained below the threshold of detection over the 10 weeks. We therefore interpret these data as showing that

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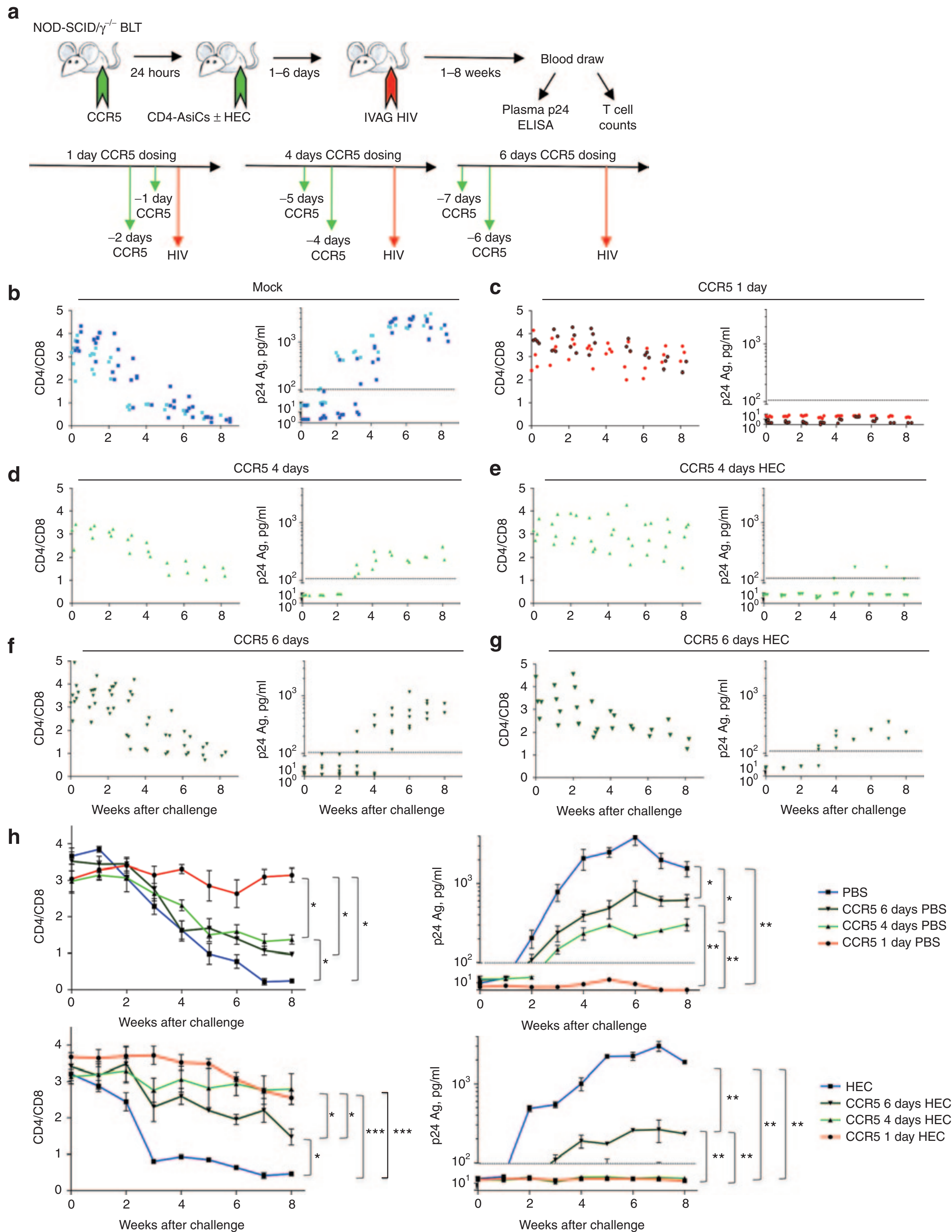

Figure 7 Protection from delayed HIV challenge in BLT mice–treated IVAG with *CCR5* CD4-AsiCs. (**a**) Experimental design. *CCR5* CD4-AsiCs of 80 pmol were administered for each treatment in either saline or HEC gel. Mice treated with phosphate-buffered saline (PBS) (dark blue) or HEC gel (light blue) served as negative controls, and mice treated with *CCR5* CD4-AsiCs suspended in PBS (red symbol without outline) or in HEC gel (red symbol with black outline) 2 and 1 days before infection (red) were positive treatment controls. Mice were observed for 8 weeks after HIV challenge. Results from (**b–g**) individual mice and the (**h–k**) mean ± SEM for each treatment group, pooled from two independent experiments, show CD4/CD8 ratio measured by flow cytometry (left) and plasma p24 Ag measured by ELISA (right). (**P* < 0.01; ***P* < 0.001, ****P* < 0.0001, *t*-test relative to mock). Dashed lines indicate the detection threshold.

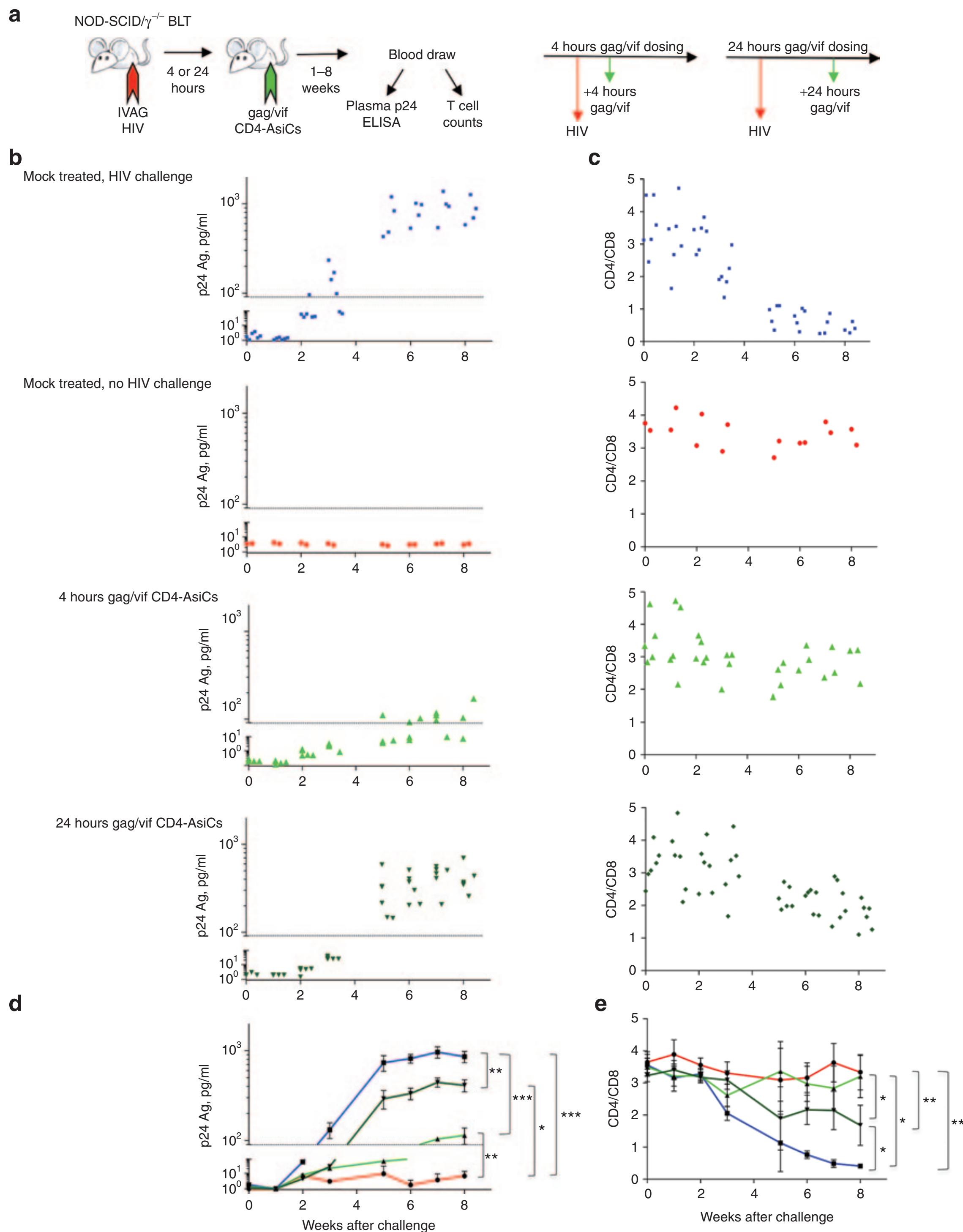

Figure 8 Test of *gag/vif* CD4-AsiCs for post-exposure prophylaxis of BLT mice. (**a**) Experimental design. CD4-AsiCs targeting *gag/vif* (80 pmol of each) were administered once, either 4 or 24 hours after HIV_{JR-CSF} challenge. Controls were phosphate-buffered saline (PBS)-treated mice either infected (blue) or uninfected (red). Mice were observed for 8 weeks after HIV challenge. Plasma p24 Ag and circulating CD4/CD8 ratios for (**b**,**c**) individual mice and (**d**,**e**) mean ± SEM for each treatment group, pooled from two independent experiments, are shown. Bright green, 4 hours treatment; dark green, 24 hours treatment (*$P < 0.05$; **$P < 0.005$, ***$P < 0.0005$, analysis of variance). Dashed lines indicate the detection threshold.

knockdown of either viral genes or *CCR5* provides sterilizing protection from transmission. In this experiment, there was no statistically significant difference in any parameter between mice treated with the cocktail or the AsiCs targeting *CCR5* or viral genes.

Mice pretreated with *CCR5* CD4-AsiCs, formulated or not in HEC, are partially protected from delayed HIV challenge

We next wanted to see if the sustained gene knockdown we observed in mice-treated IVAG with unformulated or

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gel-formulated *CCR5* CD4-AsiCs translates into protection from delayed HIV challenge. Thus, in two independent experiments, we pretreated groups of humanized BLT mice IVAG twice with 80 pmol of *CCR5* CD4-AsiCs either 7 and 6 days, 5 and 4 days, or 2 and 1 days before vaginal challenge with HIV (**Figure 7**). For the delayed challenge groups, the CD4-AsiCs were either dissolved in PBS or in HEC gel. The CD4-AsiC–treated mice were compared with mock control animals, which were treated IVAG with either PBS or HEC gel 2 and 1 days before IVAG challenge with HIV. Mice were observed for 8 weeks following challenge. All mock-treated mice became infected and had detectable p24 antigenemia and greatly reduced $CD4^+$ T cell counts within 4 weeks of exposure. The gel on its own did not reduce viral loads or ameliorate the decline in CD4 T cells. As before (**Figure 6**), mice treated with *CCR5* CD4-AsiCs 2 and 1 days before viral challenge were completely protected. However, when HIV exposure was delayed for 4 or 6 days after the last CD4-AsiC application, protection was incomplete in all groups. The most protected mice were those treated with HEC-formulated CD4-AsiCs and challenged 4 days later. In this group, there was no significant difference in mean p24 Ag level or CD4/CD8 ratio throughout the course of the experiment, compared with the completely protected animals who were challenged without delay, and only one mouse showed signs of infection with a slight increase in p24 antigenemia and a modest decline in CD4/CD8 ratio. All mice in the other delayed challenge groups became infected, but had significantly reduced viral burden, as measured by HIV p24 Ag that was 1–1.5 logs less than in PBS-treated controls. Moreover, the rate of $CD4^+$ T cell decline was significantly less than in mock-treated controls. There was no significant difference in transmission sequelae (p24 Ag or CD4/CD8 ratio) depending on whether the challenge was performed 4 or 6 days after treatment with unformulated *CCR5* CD4-AsiCs. Mice treated with HEC-formulated CD4-AsiCs and challenged 6 days later had significantly less plasma viremia and a less steep drop in CD4/CD8 ratio than mice treated with unformulated CD4-AsiCs. Thus, protection from transmission was only partial for delayed challenge, and formulation of CD4-AsiCs in HEC improved protection in both the 4 and 6 days delayed challenge groups.

Inhibition of HIV transmission in mice treated with *gag/vif* CD4-AsiCs after exposure

A potential clinical application of a topical microbicide is post-exposure prophylaxis. We previously showed that siRNAs targeting an *HSV-2* gene–administered IVAG after exposure provided protection from a lethal challenge dose of virus.[15,17] To determine whether interfering with HIV gene expression after viral exposure could protect humanized mice from transmission, we challenged groups of BLT mice IVAG with an HIV viral dose that uniformly results in infection and then 4 or 24 hours later administered 80 pmol of *gag/vif* CD4-siCs IVAG (**Figure 8**). Control mice were treated with PBS 4 hours after HIV or mock viral challenge. All mice were evaluated for plasma p24 antigen and CD4 and CD8 T cell counts. All mock-treated mice became infected and had reduced $CD4^+$ T cell counts. Mice treated after exposure with *gag/vif* CD4-AsiCs were partially protected, but protection was better in mice treated at 4 hours versus 24 hours. Although two mice became infected in the 4 hours group, the infected mice had low levels of p24 Ag that were close to background and did not show evidence of reduced CD4 T cell counts. All the mice treated 24 hours after exposure became infected, but their viral burden was reduced by about 0.5 logs. The rate of CD4/CD8 ratio decline was also significantly less rapid than the control mice. Thus, viral-targeted CD4-AsiCs reduced viral burden, more substantially if administered sooner after exposure, and even protected some mice completely when administered 4 hours after exposure.

DISCUSSION

The success of any microbicide requires adequate patient compliance.[1,3,13,35,36] Establishing durable resistance to infection should eliminate the need for microbicide administration just before a sexual encounter, thereby facilitating adherence. RNAi, which knocks down target genes for as long as three weeks *in vitro* and *in vivo*,[5,16] should provide a means for long-lasting protection. Here, we used the BLT mouse model to show that CD4-AsiCs knockdown gene expression in HIV-susceptible cells in the female genital tract for at least two weeks *in vivo*. Knockdown was comparable when CD4-AsiCs were formulated in HEC, a water-soluble FDA-approved polymer already used in HIV microbicide trials that promotes retention in the female genital tract.[35] Gel formulation actually improved protection from delayed challenge. Thus, HEC formulation should be used to evaluate siRNA-based microbicides in future animal studies, as they are currently being used to assess small molecule *CCR5* inhibitors.[37] CD4-AsiCs can be formulated in a clinically applicable microbicide gel without loss of cell-type specificity, knockdown activity or effectiveness. Sterilizing protection from a high dose challenge in BLT mice was uniformly achieved when mice were treated to knockdown *CCR5* and HIV *gag* and *vif* together or the coreceptor or viral genes individually. Protection by targeting just *CCR5* should mean that the same microbicide could be used to protect against all viral clades with minimal risk of viral escape or drug resistance.[5,6] When challenge was performed around the time of treatment, 13 of 13 evaluable mice resisted becoming infected. These results are encouraging in a model that results in 100% transmission, especially considering that HIV sexual transmission in humans is very inefficient, requiring hundreds of sexual exposures on average to become infected.[18] Directing knockdown to all $CD4^+$ cells capable of being infected could complement gp160-targeted AsiCs, which are effective only against cells already infected with the virus.[38] Because the human *CCR5* sequence we targeted is well conserved in *Rhesus macaques,* and preliminary data suggest that the human CD4 aptamer binds to macaque CD4, we are planning to test the *CCR5* CD4-AsiCs in non-human primates challenged with SHIV virus, which should help us evaluate the promise of CD4-AsiCs for human microbicide use.[19,21,22]

When challenge was delayed for 4 days after AsiC application in HEC gel, all mice, but one, were completely protected. No sterilizing protection occurred with 4 days delayed challenge when RNAs were administered in saline or when challenge was delayed until 6 days after the treatment was completed. However, both viral load and the decline in CD4 counts were significantly reduced in AsiC-treated mice under all these conditions. Why gel formulation improved protection is not clear. Contributing factors might be improved stability, longer

interface time, more uniform coverage of the luminal surface or better toleration without inflammation of the epithelial tissue when RNAs are incorporated into a gel. It will be worthwhile to evaluate whether CD4-AsiCs can be incorporated into slow-releasing vaginal rings without compromising activity and function to provide even longer term protection.

The decline in protection after 1 week was unexpected, given the sustained target gene knockdown. Although we cannot conclusively rule out[39] resistance, a more likely explanation might be recruitment of new CD4$^+$ cells to the genital tract that occurred after treatment. This may be a special problem in the BLT model. These mice have a high rate of GvHD and wasting, which has been attributed to the conditioning regimen. These effects can vary from batch to batch of BLT mice[31,40–42] and were more severe in the mice used in the delayed challenge experiments in **Figure 6**. GvHD leads to increased T cell activation and tissue trafficking. As HIV preferentially infects activated T cells,[43] transmission may be enhanced in mice with GvHD. In addition, the durability of gene knockdown is reduced in activated cells because siRNAs are diluted with each cell division. siRNAs may also be more rapidly degraded in activated, than in resting, T cells. All of these considerations suggest that HIV may be more difficult to inhibit in the setting of GvHD. These factors may also apply to women with ongoing vaginal or systemic infection or other conditions associated with inflammation, who are more susceptible to transmission,[44–46] and for whom it may be more challenging to achieve durable knockdown.

Post-exposure prophylaxis is possible using antiretroviral drugs.[47,48] Our first attempt here to show protection from viral transmission after exposure met with mixed success. However, two of four mice that were treated within 4 hours of exposure did not become infected and all the treated mice had less severe signs of infection as measured by T cell counts and plasma viremia. These data suggest that interfering with viral gene expression after infection may be effective *in vivo*.

Improvements in the design of CD4-AsiCs or altered dosing likely can enhance protection from delayed challenge and post-exposure prophylaxis. In these experiments, we used the same doses as in our previous study, 40–80 pmol/mouse/injection, which translates to <0.2 mg/kg/mouse. Although these doses are practical, improved design might reduce the dose, and hence, the cost of the microbicide, which is an important consideration for global use. The CD4-AsiCs, which are our first design, could be optimized for more efficient uptake into cells or escape from endosomes (the bottlenecks in intracellular delivery), stability within cells (the $t_{1/2}$ in serum or genital fluid is already >3 days[14,28,30] and doesn't need improvement), or efficiency of incorporation into the RNA-induced silencing complex or target gene interference. This might be accomplished by optimizing the aptamer sequence, the linker between the aptamer and the siRNA[49] or choice of siRNA sequence, altering the chemical modifications of the RNA bases (here only the long strand was modified with 2′-fluoropyrimidines),[30] or attaching small molecules to enhance cellular uptake or endosomal escape. Drug optimization will likely not only enhance protection, but might also reduce the drug concentration and cost needed for effective prevention.

MATERIALS AND METHODS

CD4-AsiC synthesis. CD4-AsiCs were synthesized by *in vitro* transcription and purified as previously described.[14,28,30]

Flow cytometry. Direct immunostaining of CD3, CD4, CD8, CD14, CD45, and CCR5 was performed using 1/20 dilutions (1/5 dilution for CCR5) of fluorescently conjugated murine mAb for 30–60 minutes at 4 °C (BioLegend, San Diego, CA). Cells were stained in PBS containing 0.5% FCS, 1 mmol/l EDTA, and 25 mmol/l HEPES. Samples were washed twice in the same buffer. Data for samples in one and two-color experiments were acquired using the FACSCalibur (BD Biosciences, Franklin Lakes, NJ), whereas in multi-color experiments, they were acquired using the FACS-Aria II (BD Biosciences). All data analysis was performed using FlowJo (Treestar, Ashland, OR).

Viruses. HIV_{BaL} virus, which was used to infect cervicovaginal explants, was obtained from the NIH AIDS Research and Reference Reagent Program (NIH-ARRRP). Viral stocks were generated by infecting pooled PBMCs that had been stimulated with phytohemagglutinin (4 µg/ml) in RPMI supplemented with 10% fetal bovine serum (H10) + 60 IU/ml rIL-2 (Chiron, Charlotte, NC). $HIV\text{-}1_{JR\text{-}CSF}$ viral stocks, which were used to infect BLT mice, were produced through transfection of HEK293 cells as described.[14] Supernatant virus was concentrated 1:50 using the PEG-it Virus Precipitation Solution (System Biosciences, Mountain View, CA) as per the manufacturer's protocol. p24 Ag levels in culture supernatants were measured by HIV-1 p24 Ag enzyme-linked immunosorbent assay (ELISA) kit (Perkin Elmer, Waltham, MA).

BLT mice. All *in vivo* experiments were performed using NOD/SCID/$IL2R\gamma^{-/-}$ (NSG) mice, bearing humanized bone marrow following reconstitution with CD34$^+$ cells from human fetal liver, and surgical human thymic graft ("BLT mice"), prepared as previously described.[14,31] Uniformity of HIV infection in HIV challenge experiments was obtained by requiring high levels of human immune reconstitution. Criteria for BLT reconstitution were >25% of peripheral blood cells within a lymphocyte gate on forward-versus-side scatter plots; >50% of cells in the lymphocyte gate are human (hCD45$^+$/mCD45$^-$); and >40% of human cells in the lymphocyte gate are T cells (hCD3$^+$). Approximately 1 week before use, mice were treated with 2 mg medroxyprogesterone subcutaneously (Besse, New York, NY). Equal numbers of mice generated from a single fetal donor were assigned to each experimental group. In some experiments, all mice were from 1 donor; in others, mice from two or three donors were used. In some experiments, some of the experimental mice died from a transplantation-related wasting syndrome during the course of the experiment. These deaths occurred equally in HIV-infected and -uninfected groups and were not related to viral load or CD4 counts or treatment, and thus were thought to be unrelated to either HIV infection or treatment. Animal work was approved by the Animal Care and Use Committees of Massachusetts General Hospital, University of Massachusetts Medical School, and Harvard Medical School.

HIV-1 infection. For HIV infection of BLT mice, $HIV_{JR\text{-}CSF}$ (10^5 $TCID_{50}$) diluted in PBS to a 10 µl final volume was applied atraumatically to the vaginal mucosa using 10 µl XL pipette tips (Neptune Scientific, San Diego, CA). Using this challenge dose, all control mice became infected.

Vaginal stability assay. *CCR5* CD4-AsiC (2 nmol) synthesized using 2′-fluoro-pyrimidines suspended in either 100 µl PBS or HEC gel were added to 100 µl of vaginal fluid obtained from a healthy preovulatory donor. At regular intervals, 20 µl was removed, resuspended in gel loading buffer, and frozen at −80°C before resolution by PAGE analysis. RNA content was analyzed by ethidium bromide staining and densitometry, and amounts were calculated relative to RNA content at time 0.

Human cervical polarized tissue explants. Human cervical tissue was obtained from healthy donors undergoing hysterectomy for benign conditions. Tissue was immediately sectioned into ~3 mm^3 specimens and then

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oriented on the membrane of a 12-transwell system (Corning, Tewksbury, MA) with the apical epithelial surface facing up. Explants were then embedded in 3% agarose as described[14,28–30] and the integrity of the agarose seal was tested using Trypan blue. The polarized explants were cultured in 200 µl H10 medium. Cy3-labeled AsiCs, in 50 µl Optimem (Invitrogen), were applied to the apical surface, and the explants were then incubated at 37 °C for 4–6 hours before adding 150 µl H10 to each well. The treatment was repeated 24 hours later and then the tissue was incubated for an additional 48 hours at 37 °C before collagenase digestion and analysis. Tissues were obtained in accordance with institutional regulations concerning human samples.

Collagenase digestion of vaginal tissue. For experiments to assess gene silencing in explants, specimens were removed from the transwell 48 hours after the second application of RNA. Ten polarized explants per treatment condition were pooled and digested in 10 ml RPMI containing 1 mg/ml collagenase II (Sigma) for 30 minutes at 37°C with shaking. Samples were disrupted in a gentleMACS dissociator (Miltenyi, Auburn, CA) using the C.01 program for 30 minutes at 37 °C both before and after collagenase digestion. Cell suspensions were passed through a 70 µm cell strainer (BD Falcon, Franklin Lakes, NJ), washed with 30 ml RPMI and then stained for flow cytometry analysis. The same procedure was followed for vaginal tissue dissected from individual BLT mice.

In vivo *treatment and HIV protection*. Xylazine/Ketamine anesthetized mice were treated with CD4-AsiCs by atraumatic application to the vaginal mucosa of PBS or HEC solution in 15 µl according to the indicated schedule. Mice were kept supine for 5 minutes after each application. The HEC gel formulation was prepared by mixing the HEC gel (NIH-ARRRP) in a 1:1 ratio with sterile PBS. For AsiC preparations, RNAs were added to the diluted mixture, vortexed, and applied IVAG according to the experimental protocol. Mice were sacrificed at times indicated for each experiment. HIV protection was assessed in NSG-BLT mice, treated IVAG with the specified concentrations of CD4-AsiCs or PBS (mock controls) in a 15 µl volume, that was divided into two 7.5 µl applications administered atraumatically using 10 µl XL pipette tips (Neptune).

Analysis of HIV infection. Blood was obtained by venipuncture of the facial vein at weekly intervals for 8–10 weeks following HIV challenge. Cells were pelleted by centrifugation and plasma was stored at −80 °C until analysis. Cell pellets were twice treated with RBC lysis buffer (Sigma), washed with flow cytometry buffer described above and stained using a 1/20 dilution of CD3, CD4, and CD8 mAb. Viral RNA was extracted from 75 µl of plasma using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was reverse transcribed using SuperscriptIII (Invitrogen) and HIV-gag-specific primers[37] according to the manufacturer's protocol. qRT-PCR was performed as above. The remaining serum was aliquoted for p24 Ag ELISA (Perkin Elmer) performed as per the manufacturer's instructions.

Statistical analysis. Data for most experiments were analyzed by Student's t-test. All P values are for two-tailed significance tests. For analysis of data based on independent experiments using samples from multiple donors, one-way analysis of variance with Dunnett multiple comparison test was performed using GraphPad Prizm (GraphPad Software, San Diego, CA). P values below 0.05 were considered significant. The limit of detection was calculated using the method of ref. 50 and is shown as the average of the calculated limit of detection for each individual assay.

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