Intravaginal ring eluting tenofovir disoproxil fumarate completely protects macaques from multiple vaginal simian-HIV challenges

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Topical preexposure prophylaxis interrupts HIV transmission at the site of mucosal exposure. Intermittently dosed vaginal gels containing the HIV-1 reverse transcriptase inhibitor tenofovir protected pigtailed macaques depending on the timing of viral challenge relative to gel application. However, modest or no protection was observed in clinical trials. Intravaginal rings (IVRs) may improve efficacy by providing long-term sustained drug delivery leading to constant mucosal antiretroviral concentrations and enhancing adherence. Although a few IVRs have entered the clinical pipeline, 100% efficacy in a repeated macaque vaginal challenge model has not been achieved. Here we describe a reservoir IVR technology that delivers the tenofovir prodrug tenofovir disoproxil fumarate (TDF) continuously over 28 d. With four monthly ring changes in this repeated challenge model, TDF IVRs generated reproducible and protective drug levels. All TDF IVR-treated macaques ($n = 6$) remained seronegative and simian-HIV RNA negative after 16 weekly vaginal exposures to 50 tissue culture infectious dose SHIV162p3. In contrast, 11/12 control macaques became infected, with a median of four exposures assuming an eclipse of 7 d from infection to virus RNA detection. Protection was associated with tenofovir levels in vaginal fluid [mean 1.8×10^5 ng/mL (range 1.1×10^4 to 6.6×10^5 ng/mL)] and ex vivo antiviral activity of cervicovaginal lavage samples. These observations support further advancement of TDF IVRs as well as the concept that extended duration drug delivery devices delivering topical antiretrovirals could be effective tools in preventing the sexual transmission of HIV in humans.

controlled drug delivery | PrEP | nonhuman primate | pharmacokinetics

The prodrug tenofovir disoproxil fumarate (TDF) and its hydrolysis product tenofovir (TFV) have been the major focus of HIV prevention research. TDF is one component of the two-drug mixture Truvada, the first US Food and Drug Administration–approved oral preexposure prophylaxis (PrEP) agent against HIV acquisition in discordant couples and other high-risk populations (1), and TFV is the active pharmaceutical ingredient in the first vaginal gel to show partial HIV protection (2). Both molecules have a long safety record and have been widely used in humans with minimal toxicity. The 1% (wt/wt) TFV vaginal gel formulation administered 30 min before virus exposure provided complete protection (6/6) against multiple simian-HIV (SHIV) challenges (3, 4) and retained partial activity protecting four of six macaques that were treated once weekly with gel and exposed to virus twice weekly; the second exposure being 3 d after gel application (4). Unfortunately, the TFV gel provided only partial protection in clinical trials when women were asked to use the gel before and after coitus and no protection when daily dosing was evaluated (2, 5). There are many factors, both social and biological, that may have contributed to the TFV gel clinical trial outcomes. However, as far as we know the most important factor in all gel trials to date is the difficulty of sustaining high adherence to frequent dosing (6). Simply put, if the course of protective antiretroviral levels does not match the kinetics of viral exposure, PrEP will not be highly effective. This motivates the development of long duration, drug delivery systems such as intravaginal rings (IVRs) that may overcome these limitations by facilitating adherence and providing less variation in mucosal drug levels (7, 8).

Matrix IVRs delivering the nonnucleoside reverse transcriptase inhibitor (NNRTI) dapivirine and the entry inhibitor maraviroc are being advanced through the clinical pipeline. Neither of these rings, which are currently in phase 3 and phase 1 trials, have been evaluated for efficacy in macaques. Among all antiretroviral IVR efficacy studies conducted to date, only one formulation conferred protection against a single, high-dose viral challenge in nonhuman primates, whereas the remainder yielded partial protection (9–11). None has been evaluated in repeated vaginal challenge models designed to mimic the repeated viral exposure during human sexual transmission (12). Reasons for failure of these device–drug combinations to provide complete protection in macaque models are complex and may reflect differences in the model system [species, use of depo-medroxyprogesterone (DMPA), viral dose, and strain], the pharmacologic properties of the drugs tested,

Significance

Topical prevention of HIV is designed to pharmacologically interrupt sexual transmission at the genital mucosa. Attempts at preventing transmission in women using vaginal gels have yielded disappointing results in part because of poor rates of adherence. Controlled topical drug delivery using intravaginal ring technology should improve efficacy and adherence by providing sustained mucosal delivery of antiretrovirals. In this paper, we describe a reservoir intravaginal ring that delivers tenofovir disoproxil fumarate (TDF) for 1 month. The ring protected pigtailed macaques from weekly vaginal simian–human immunodeficiency virus challenges for 4 mo. The sterilizing performance of this drug delivery system supports the concept that an intravaginal ring delivering TDF could be an effective tool for prevention of HIV sexual transmission in women.

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and/or inadequate drug release profile of the IVRs. We hypothesize that IVRs that maintain a consistent level of mucosal TDF may provide greater protection. This notion is supported by the higher cell permeability and at least 100 times greater potency of TDF compared with TFV against HIV in vitro (13). The bioactive form, TFV diphosphate (TFV-DP), has the further advantage of a long intracellular half-life (60–150 h) (14, 15) that could mitigate lapses in adherence and may prove advantageous compared with antiretrovirals (e.g., dapivirine) that can readily diffuse from the intracellular to the extracellular compartments in response to drug concentration gradients in time and space (16).

Developing IVRs to elute the thermally and hydrolytically unstable TDF at therapeutically relevant rates is a challenge. Although TDF is more hydrophobic than TFV, its polarity is too high for adequate polymer solubility to deliver protective doses of the drug from common elastomers used in IVR technology such as silicone and poly(ethylene-covinyl acetate) (13). Furthermore, TDF is susceptible to hydrolysis, making formulation of this drug in any aqueous topical dosage form impossible (17, 18) and precluding its use in gels. We engineered a class of IVRs capable of achieving high fluxes of hydrophilic antiretrovirals such as TFV and TDF using hollow hydrophilic polyether urethane (HPEU) elastomeric tubes (Fig. 1 *A* and *B*) (19). The hydrophilic–hydrophobic balance of HPEU can be tailored to solubilize and deliver drugs with a range of physical properties (20) and at fluxes that achieve clinically relevant concentrations (21). We assessed the ability of TDF IVR designs to achieve monthly sustainable protective levels of TDF/TFV in vaginal fluid and tissue by performing pharmacokinetic (PK) studies in pigtailed macaques. Pigtailed macaques were chosen for the study because of their similarities in hormonal cycling, vaginal architecture, and microflora to women, and previous studies in this model provide data on intracellular TFV-DP levels that correlate with protection (4). The IVR design that generated high and consistent drug levels in vaginal fluids and tissues was then evaluated in a more extensive terminal PK study to determine the levels of TFV-DP in the female reproductive tract and lymph nodes. Finally, we tested the ability of the IVR to provide continuous protection against infection by repeated weekly vaginal 50 tissue culture infectious dose ($TCID_{50}$) SHIV162p3 challenges over multiple monthly IVR changes coupled to ex vivo viral inhibition in cervicovaginal lavage (CVL) samples.

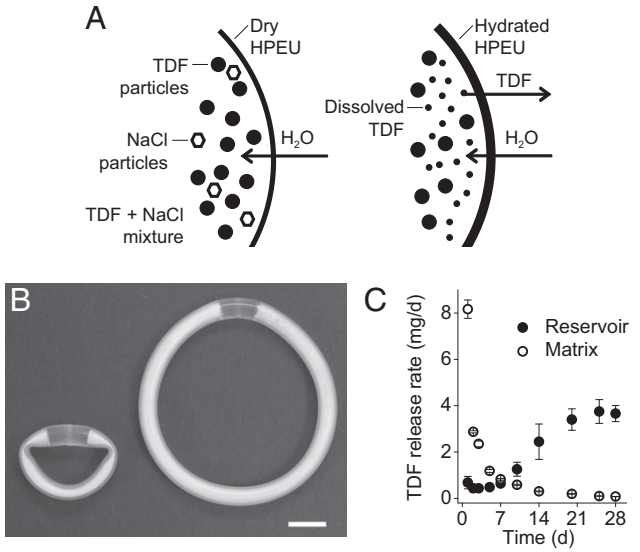

Fig. 1. TDF IVR design and in vitro release. (*A*) Schema depicting mechanism of drug release from TDF reservoir IVR. Vaginal fluid hydrates the swellable HPEU tubing (*Left*) and water is driven into the osmotically active drug–NaCl core along a gradient, resulting in TDF dissolution and elution from the IVR (*Right*). NaCl aids in establishing soluble drug in the core and achieving equilibrium drug release (Fig. 2*B*). (*B*) Photograph of macaque (left) and human (right) TDF IVR. (Scale bar: 1 cm.) (*C*) Comparison of 28-d in vitro TDF release rates from matrix ($n = 3$) and reservoir ($n = 6$) IVRs under simulated vaginal conditions (2). Data represented as mean ± SD. The SDs of some of the collected data are too small to be visualized.

Results and Discussion

TDF IVR Design. IVRs are divided into two well-known controlled release device categories: matrix and reservoir. Matrix devices exhibit drug release rates that decrease with time, whereas reservoir devices have a rate controlling membrane that allows for more consistent drug flux (Fig. 1*A*) (8). Because matrix devices are simple and more easily fabricated, we originally explored the same matrix design strategy as the dapivirine ring (22, 23). Polyether urethane (PEU) matrix TDF devices containing 10 wt% TDF showed a 20-fold reduction in drug release rates in vitro from day 1 to day 28 (Fig. 1*C*). A similar effect was seen in vivo in rhesus macaques ($n = 3$) in which more than a 2-log reduction was noted in mean TDF vaginal fluid concentrations from 1.5×10^5 ng/mL (range 2.3×10^4 to 3.3×10^5) on day 3 to 6.3×10^2 ng/mL (range 0 to 2.2×10^3) on day 21 (Fig. 2*A*) and no detectable drug on day 28 (13). The chemical stability of TDF in this device was also unacceptable. Although high levels of TFV were still detected in vaginal fluid at day 28, TDF levels were undetectable.

Given the poor performance of the TDF matrix device, we investigated reservoir IVR designs composed of HPEU tubing with a drug-bearing core sealed by induction-melt welding into a torus (19). The tubing wall acts as rate-controlling membrane for drug release while providing the necessary mechanical support for ring retention in the vaginal canal. Simply filling the device with TDF alone resulted in a long drug-release lag time of more than 20 d (Fig. 2*B*). Therefore, we included osmotic agents to attract vaginal fluid into the core to solubilize TDF and rapidly establish a concentration gradient of soluble and diffusible drug to drive release (Fig. 1*A*). A reservoir IVR designed to deliver a TDF formulation with NaCl (14 wt%) as an osmotic excipient was selected by scanning a series of osmoattractants in vitro (Fig. S1*A*). Fig. 1*B* shows human and macaque reservoir IVRs with outer diameters of 55 and 25 mm, respectively. This IVR design delivers in vitro 0.4–4 mg/day TDF over 28 d, with an average release rate of 2.3 ± 0.3 mg/d [macaque reservoir IVR (mean ± SD; $n = 3$); Fig. 1*C*]. This HPEU-reservoir IVR design eliminated the need for exposing TDF to elevated temperatures required for creation of thermoplastic matrix IVRs. Functionally, we found that the maximum rate of NaCl release preceded the large increase in TDF release (Fig. 2*C*), implying that core hydration was rate-limiting in achieving sufficient drug release. Finally, we found that we could load the wall of IVRs with TDF after ring fabrication by exposing the loaded device to elevated temperature, which decreased the lag time of drug elution (Fig. 2*D*), potentially providing a loading dose of TDF. We were able to obtain release rates of milligram per day quantities of TDF in vitro from the reservoir device and thereby achieve drug levels in vaginal fluid at all time points, exceeding the macaque (4) and clinical correlate of protection (24) of ~1,000 ng/mL of TFV in vaginal fluid. This was not the case with the inadequate release observed over time from the matrix TDF IVR (Fig. 2*A*) (13). Although the in vitro release rate from the reservoir design was not constant over 28 d, the IVRs generated reproducible TFV levels (Fig. 3*A*) over the first 15 d when the in vitro release rate changed the most, potentially implying early saturation of the system with drug or differences between the in vitro and in vivo TDF release rates.

Drug PK Study in Pigtailed Macaques. We investigated the TDF reservoir IVRs in pigtailed macaques ($n = 6$) in a "28 + 2" day study (28 d of IVR exposure and 2 d after removal) for drug

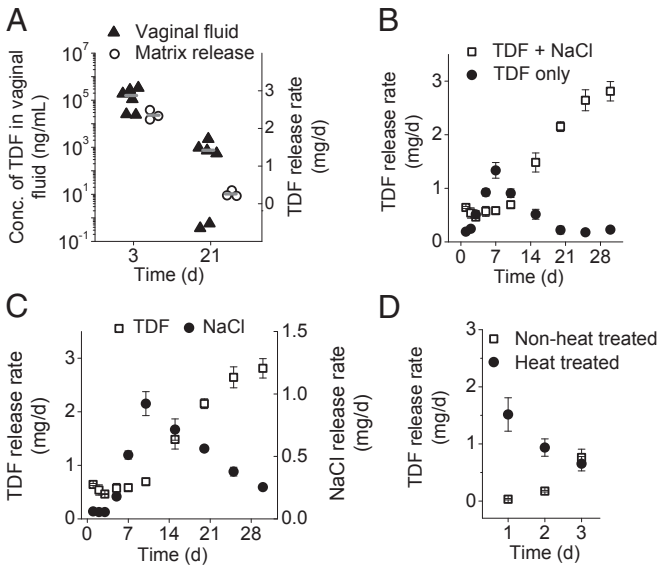

Fig. 2. Matrix release and design evaluation of reservoir TDF IVR. (*A*) Comparison of TDF released from matrix PEU IVR in vitro on days 3 and 21 to drug concentrations in vaginal fluid of Chinese rhesus macaques ($n = 3$) in a 28 + 3-d study. Each data point represents a single sample and the bar corresponds to the mean for that dataset. (*B*) In vitro TDF release rate from HPEU reservoir IVR filled with TDF and TDF-NaCl formulation under simulated vaginal conditions ($n = 3$). Data represented as mean ± SD. (*C*) In vitro TDF and NaCl release rates from HPEU reservoir IVR under simulated vaginal conditions ($n = 3$). Data represented as mean ± SD. (*D*) Comparison of initial in vitro TDF release (up to day 3) from heat-treated and control-unheated TDF IVR ($n = 3$). Heat treatment of the TDF reservoir IVR at 65 °C for 5 d increased TDF release in the first 2 d. Data represented as mean ± SD.

distribution and concentration in vaginal fluid and tissue (Fig. 3 *A* and *B* and Fig. S2). The TDF IVR provided high TFV (Fig. 3*A*, *Upper*) and TDF (Fig. 3*A*, *Lower*) mean vaginal fluid concentrations of 7.2×10^4 ng/mL (range 7.1×10^3 to 3.5×10^5) and 1.0×10^2 ng/mL (range 5 to 6.1×10^5), respectively. The former consistently exceeded the TFV concentration of 1,000 ng/mL recovered in cervicovaginal aspirates that correlated with protection in women receiving 1% TFV gel (24). In addition, we detected comparable but more variable concentrations of the more potent TDF. Levels of both drugs appear stable in vaginal fluid from days 3 to 28 and levels seemed similar proximally and distally (Fig. 3*A*). TFV levels also appeared stable over time in proximal and distal tissues (Fig. 3*B*), whereas TDF levels were more variable (Fig. S2). This suggests that despite the variable drug release in vitro, the IVRs exhibited a minimal lag time to reach high antiretroviral concentrations in the macaque vaginal vault.

Because TDF hydrolytically converts into TFV, we found variable levels of TDF in swabs and there was no quantifiable TDF detected in vaginal swabs 2 d after ring removal. However, mean TFV levels in swabs were 4.4×10^3 ng/mL (range 2.2×10^2 to 6.9×10^4) and in tissue were 2.9×10^3 ng/g (range 5.4×10^2 to 2.5×10^4) 2 d after ring removal (Fig. 3 *A* and *B*), exceeding the TFV levels detected after TFV gel application in clinical studies (2, 25). In tissues, the TFV concentrations exceeded the in vitro IC_{50} by ~80 times. These high and sustained vaginal fluid and tissue levels of TFV likely reflect the diffusion of luminal prodrug through the vaginal tissue and its hydrolysis to TFV (8). We do not know the instantaneous in vivo TDF release rate in the macaque vagina. However, as determined by the amount of recovered TDF from the IVRs after use in this PK study, the time-averaged TDF release rate was similar under both in vitro and vivo conditions (Fig. S1*B*). It has been difficult for others in practice to obtain and sustain these levels of drug in vivo; our IVR delivered ~50-fold more TDF in vivo compared with the one other TDF IVR in the literature (26). These data and the prolonged half-life of intracellular TFV-DP strongly suggest that this drug–device combination would pharmacologically tolerate removal of the device for hours without significant diminution of drug levels. Furthermore, the high TFV levels in vaginal fluid and undetectable TDF levels 2 d after ring removal suggest the presence of a tissue and/or cellular reservoir of drug that is continuously exchanging with vaginal fluid.

Terminal PK Study in Rhesus Macaques. To evaluate intracellular TFV-DP concentrations, we administered TDF IVRs to rhesus macaques that were scheduled to be euthanized because they had been previously infected with SHIV in other studies ($n = 3$). In this 14-d study, we had the opportunity to evaluate levels of the bioactive metabolite TFV-DP in lymphocytes from vaginal, cervical, and rectal tissue as well as lymph nodes, which are sites where HIV transmission and dissemination is presumed to occur. Previous challenge studies in pigtailed macaques with 1% TFV vaginal gel suggested protection from SHIV infection correlated when TFV-DP levels in vaginal lymphocytes exceeded the IC_{95} of 1.4×10^3 fmol/10^6 cells (4). Mean TFV-DP levels after 14 d of IVR application were highest in vaginal and cervical lymphocytes, 3.3×10^3 fmol/10^6 cells (range 1.5×10^3 to 7.5×10^3) and 1.7×10^3 fmol/10^6 cells (range 8.4×10^2 to 3.2×10^3), respectively. The mean of the intracellular levels exceeds the TFV-DP IC_{95} of 1.4×10^3 fmol/10^6 cells and is comparable to levels that showed complete protection in macaques (4) (Fig. 3*C*). Relative to vaginal and cervical lymphocytes, lower drug concentrations were detected in rectal and inguinal lymphocytes, 13 fmol/10^6 cells (range 6 to 1.3×10^2) and 81 fmol/10^6 cells (range 17 to 1.3×10^2), respectively (Fig. 3*C*). Additionally, we observed similar TDF and TFV levels in vaginal fluid and tissue to those observed in pigtailed macaques (Fig. 3 *A* and *B*). These data indicate that the IVRs provide TFV-DP concentrations that exceed protective levels observed previously in macaques, suggesting that the ring could confer protection against vaginal SHIV challenge.

Repeat SHIV Challenge Study. Based on achieving the TFV concentrations in vivo described previously, we initiated a weekly challenge study in sexually mature, normal cycling pigtailed macaques (12). Six TDF IVR-treated macaques received weekly 50 $TCID_{50}$ SHIV162p3 vaginal inoculations starting 6 d after IVR insertion (Fig. 4*A*). Control macaques ($n = 6$ real time and $n = 6$ historical controls) were challenged similarly, of which 11/12 became infected after a median of four exposures to infection, assuming a 7-d eclipse period from time of infection to detection of viral RNA in plasma; peak viral RNA levels were $3.4 \times 10^6 \pm 1.9 \times 10^7$ copies/mL, median ± SD (Fig. 4 *B* and *C*). In contrast, all TDF IVR-treated macaques (6/6) remained SHIV viral RNA-negative and -seronegative after 16 weekly exposures spanning 4 mo involving monthly IVR changes. A nonparametric log-rank test was used to compare survival probabilities ($P = 0.0007$, Fig. 4*B*). Differences in infection probabilities between control and treated animals were statistically significant (Fisher's exact test, $P < 0.0001$). The median survival time among control animals was four exposures, 95% CL (2, 10). All TDF treated macaques remained uninfected after 4 additional weeks of follow-up with the IVRs in place. The infection probability per exposure among control animals was 0.162, 95% CL (0.084–0.271); the infection probability among treated animals was 0.0, 95% CL (0.0–0.038). Estimated efficacy was 100%, 95% CL (80.31–100).

The complete protection observed in the TDF IVR-treated macaques is consistent with the high TFV levels in vaginal fluid samples taken at the time of each ring change [Fig. 5*A*; 1.8×10^5 ng/mL (mean, range 1.1×10^4 to 6.6×10^5)]. With each monthly IVR change, TDF and TFV levels in vaginal fluid samples remained high (Fig. 5*A*). Plasma TDF levels were below

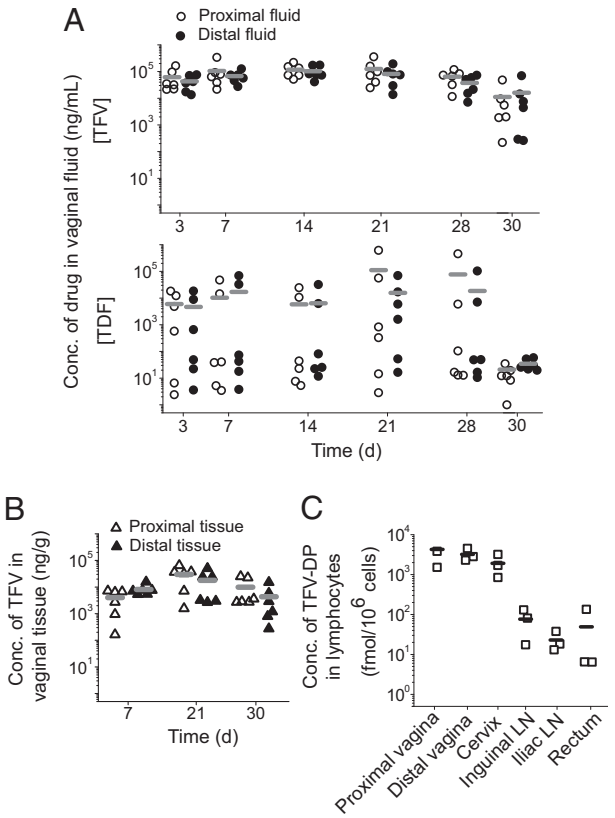

Fig. 3. Drug PK in pigtailed (28 + 2-d) and rhesus (14-d) macaques. Each data point represents a single sample and the bar corresponds to the mean for that dataset. (*A*) TFV (*Upper*) and TDF (*Lower*) concentrations in pigtailed macaque vaginal fluid with 28-d TDF IVR administration (n = 6). Samples were collected proximal (open symbols) and distal (closed symbols) to IVR placement for the indicated time points. (*B*) TFV concentrations in vaginal biopsies from 28-d TDF IVR administration. Samples were collected proximal (open symbols) and distal (closed symbols) to IVR placement for the indicated time points. (*C*) TFV-DP levels in lymphocytes isolated from the indicated tissues of rhesus macaques after 14-d IVR administration (n = 3).

detection limit [n = 102, lower limit of quantification (LLOQ) = 1 ng/mL] throughout the efficacy study. Detectable TFV levels (median 8 ng/mL, range 7–19 ng/mL; n = 102, LLOQ = 5 ng/mL) were observed in five of 102 blood samples collected with 4 sequential months of TDF IVR administration. The protection is also consistent with ex vivo antiviral activity of CVL samples from two additional TDF IVR-treated pigtailed macaques not exposed to SHIV in parallel to the challenge study. CVL collected from these two macaques over the course of 28 + 1 d (28-d IVR exposure and 1 d after removal) displayed high antiviral activity against HIV-1 in vitro (range 73–100%) even after a 1:10 dilution (Fig. 5 *B* and *C*). Importantly, and consistent with the persistence of TFV in vaginal fluid, CVL collected 1 d following removal of the IVR inhibited HIV infection by 86% (Fig. 5*B*). The anti-HIV activity correlated with both TDF and TFV levels in the CVL (Spearman PK/pharmacodynamic (PD) correlation; TDF: r = 0.57, P = 0.04; TFV: r = 0.61, P = 0.02, Fig. 5*C*).

Potential Behavioral, Pharmacological, and Biological Implications. The choice of TDF over the less potent TFV, comined with the HPEU reservoir IVR delivery system, may overcome several of the behavioral and biological limitations observed to date with vaginal gels and other drug–IVR combinations studied preclinically and clinically. The major reason for embarking on the more complex drug delivery technology for topical antiretrovirals is to facilitate PrEP use, increase adherence, and thereby improve clinical outcomes. Many studies have shown a general increase in adherence as device duration increases (27, 28). Thus, it is reasonable to assume that it will be easier for women to adhere to long-duration IVR delivery systems compared with daily, episodic, or coitally dependent gels. This is supported by the fact that IVRs have seen excellent product demand and commercial success as a form of birth control in high-income countries and are gaining acceptance in low-income countries (7, 29).

Pharmacologically, sustained drug delivery from reservoir-type devices should provide tissue drug concentrations that are consistently above the level required to protect immune cells resident in and trafficking through the mucosa and submucosa of the genital tract over the time course of mucosal exposure to virus. Furthermore, behaviorally we need to expect and plan for the fact that women will likely periodically remove IVRs either during sex or around the time of menses. This TDF IVR may meet all of these requirements, whereas other drug–ring combination devices have limitations. First, TDF requires significantly lower doses than TFV because of its increased potency, resulting from its capability to more efficiently permeate cell membranes. This property combined with the long TFV-DP intracellular half-life (14) and corresponding low tissue elimination rate may allow for sustained activity for many hours (possibly days) following ring removal. With this IVR, we observed an approximate 0.5-log drop in TFV concentration in vaginal fluid 2 d after ring removal (Fig. 3a). This differs from an NNRTI such as dapivirine or MIV-150, in which the drug can more freely diffuse in and out of cells and can display, in the case of dapivirine matrix IVRs, approximately a 3-log drop in drug concentration from maximum levels to 2 d following IVR removal in humans (16, 22). It is therefore possible that NNRTI matrix rings that

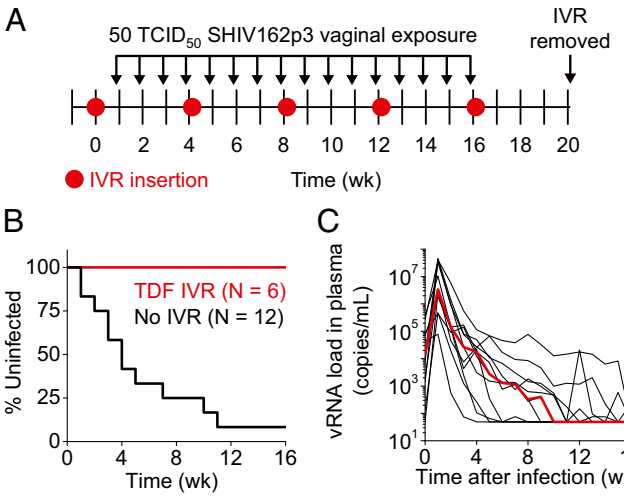

Fig. 4. TDF IVR protects macaques from repeated vaginal viral challenge. (*A*) Six TDF IVR-treated cycling female macaques received weekly 50 $TCID_{50}$ SHIV162p3 inoculations starting 6 d after the first IVR insertion. Control macaques (n = 6 real time and n = 6 historical controls) were challenged similarly. The ring was replaced as shown in red (every 28 d starting 2 d after the fourth virus exposure). Macaques were monitored weekly (until week 20) for presence of SHIV by RT-PCR and confirmed by Western blot. Macaques were defined as infected and exposures discontinued if vRNA was detected in plasma for 2 consecutive weeks. (*B*) Kaplan-Meier plot showing time to infection for TDF IVR (n = 6; red) and control (n = 6 real time and 6 historical naïve; black) groups (nonparametric log-rank test; P = 0.0007). The median number of exposures to infection in the untreated group was four. (*C*) Plasma viral load kinetics in infected macaques aligned at peak. The red line is the median for all infected macaques (11/12). vRNA, viral RNA.

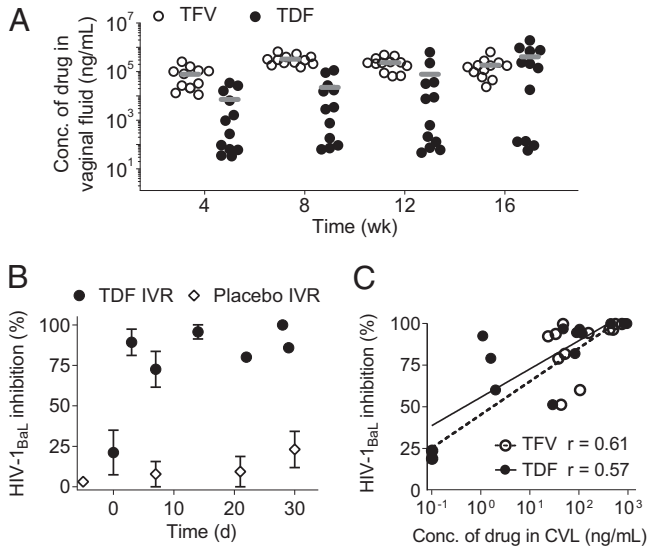

Fig. 5. Drug PK from efficacy study and PK/PD correlation. (*A*) Monthly TFV (open symbols) and TDF (closed symbols) concentrations in vaginal fluid of pigtailed macaques in the efficacy study with four TDF IVR changes ($n = 6$ macaques). Each data point represents a single sample (proximal or distal to IVR placement) and the bar corresponds to the mean for that dataset ($n = 12$; two samples per animal). (*B*) To monitor drug PK/PD during the efficacy challenge study, two macaques were treated with TDF IVR (closed symbol) or placebo IVR control (open symbol) and CVL samples were collected at the indicated times in the absence of viral challenge. CVL samples (1:10 dilution) were assayed for drug levels and ability to inhibit HIV-1_{BaL} infection in TZM-bl cells. Results are presented as percentage inhibition of infection relative to control wells; each data point represents the average of two experiments conducted in triplicate ($n = 2$ macaques, mean ± SEM). (*C*) Correlation of CVL (diluted 1:10) antiviral activity against HIV-1_{BaL} infection in TZM-bl cells to TDF and TFV concentrations (Spearman PK/PD correlation; TDF, $r = 0.57$; TFV, $r = 0.61$). Samples with TDF or TFV levels below the LLOQ were attributed the value of 0.1 ng/mL so that data could be plotted on a log scale.

deliver compounds that are not retained inside target cells may be more prone to fail in women who remove the device for a sustained time, particularly late in the release curve when release rates are dropping along with tissue levels (16, 22).

HIV sexual transmission occurs in a more complex environment of sexual intercourse, semen, contraceptive hormones, coinfections, and other variables not explored in these studies. Chemoprevention strategies have to be effective in the context of mucosal inflammation prevalent in many women. Sex (30, 31) and intercurrent sexually transmitted infections (32) are associated with an inflammatory environment that may recruit and maintain new target cells in the mucosa and possibly alter drug PK. The drugs and delivery systems used must protect in the context of these factors. Indeed, this effect may have contributed to the observation in Centre for the AIDS Programme of Research in South Africa (CAPRISA) 004 that immune activation was associated with HIV acquisition, even among women using TFV gel (33). Another clear and related advantage of TDF over TFV is the potential for providing protection against HSV-2 acquisition and outbreaks because TDF is ~100-fold more potent against HSV-2 (13). In principle, the increased potency of TDF over TFV could allow for more effective protection in the more stringent context of human sexual HIV transmission.

We designed this ring to exceed drug levels in vaginal fluid and tissue that correlated with protection in CAPRISA 004 (24). However, it is possible that lower levels of TDF, because of its greater potency (13), may be protective (24). Future dose escalation/deescalation studies are needed to identify the minimal protective TDF dose via vaginal route. It is also important to consider the need for higher concentrations of drug in settings of possible increased risk of HIV: among women using DMPA, women with other sexually transmitted infections, and following exposure to acutely infected males with high viral loads in semen (34). Here we report full protection in normally cycling macaques, but the SHIV/macaque susceptibility model using DMPA or coinfections with STIs have been established and the effect of each of these conditions on transmission in the context of this ring can be modeled in future macaque studies (35).

The pigtailed macaque model used here (12) is one of the most rigorous experimental systems available to model vaginal HIV exposure and infection in women because of the repeated exposures and a probability of infection that is at least 200 times that of human unprotected intercourse. The model is able to predict a drop in efficacy resulting from intermittent adherence as well as providing a range of drug levels in vaginal fluids and target cells that correlate with protection (4, 12, 36). Although the model may not fully predict clinical trial outcomes, rigorous and intensive PK/PD and efficacy studies can be performed that are simply not possible in women.

In summary, we report on an antiretroviral eluting IVR conferring complete protection in a nonhuman primate model against frequent vaginal viral challenges. This TDF reservoir IVR is designed to provide drug release rates that generate high and consistent drug concentrations in vaginal fluid and tissue. The design of this reservoir IVR is simple and can be manufactured cost-effectively. We have developed the analogous human-sized IVR (Fig. 1*B*) that is being considered for clinical evaluation.

Methods

IVR Fabrication and in Vitro Studies. Hydrophilic elastomer HydroThane AL 25 93A (AdvanSource Biomaterials, Inc.) tubing (wall thickness = 0.7 mm) was extruded as described previously (19). Tubing was cut to a 76 ± 0.5-mm length and the end sealed in an inductive tip-forming welder (PlasticWeld Inc.) (19). The open tube was filled with TDF only or with a mixture of TDF (Gilead Sciences) with NaCl [US Pharmacopeia (USP) grade, Spectrum Chemicals] or sodium acetate (anhydrous, USP grade, Spectrum Chemicals) in differing ratios (Fig. S1*A*). The final formulation of TDF and NaCl (86:14) was filled to achieve a final concentration of 130 ± 10 mg TDF and 20 ± 2 mg NaCl per IVR. For a placebo formulation, one-end sealed tubes were filled with 20 ± 2 mg NaCl per IVR. The open end was sealed in a second inductive welding step to form a sealed rod. To form reservoir IVRs, the ends were butt-welded with a thermoplastic welding blade to form a ring with an average diameter of 25 mm as previously described (21, 37). The devices were packaged in heat-sealed pouches (LPS Industries) and were placed at 65 °C for 5 d to load the wall of the IVR with TDF. To fabricate matrix TDF IVRs, TDF-loaded HPEU, ATPU-1 (DSM Biomedical) segments was extruded as described (13), cut to a length of 66 ± 0.5 mm followed by butt-welding as described previously. Formulations were tested for in vitro drug elution under physiologically relevant conditions in 25 mM acetate buffer (pH 4) at 37 °C. NaCl release was measured using a chloride ion selective electrode (Mettler Toledo) coupled to a Seven Multi pH meter (Mettler Toledo). IVRs were analyzed for residual drug content after in vitro and in vivo studies by chemical extraction followed by methods reported previously (13).

Drug PK. All macaques were housed at the Centers for Disease Control and Prevention (CDC) (Atlanta, GA). All procedures were conducted under approved CDC Institutional Animal Care and Use Committee protocols 2003DOBMONC (PK) and 2004SMIMONC (terminal PK and efficacy) in accordance with the standards incorporated in the Guide for the Care and Use of Laboratory Animals (National Research Council of the National Academies, 2010). Matrix IVRs were administered to female rhesus macaques of Chinese origin ($n = 3$; $t = 28 + 3$ d). Macaque-sized reservoir IVRs were administered to female pigtailed macaques (TDF IVR, $n = 6$ and placebo $n = 2$, $t = 28 + 2$ d) and female rhesus macaques of Indian origin (TDF IVR, $n = 3$, $t = 14$ d). The latter rhesus macaques were infected with SHIV162p3 virus in a previous study and were used for the terminal PK experiment after virus was no longer detectable in plasma. All sampling procedures were performed under anesthesia with ketamine. In the pigtailed macaque PK study, IVRs were inserted at day 0 and removed at day 28 and evaluated for residual drug content. Samples were taken at days –7, 0, 3, 7, 14, 21, 28, and 30. For the terminal PK study, IVRs were inserted at day 0 and removed at day 14 just before being euthanized, with samples taken on days 0, 7, and 14. Collection and processing of vaginal fluids and biopsies were performed as

previously described (21, 38). TDF and TFV levels in vaginal fluid collected using Weck-Cels (Beaver Visitec), CVL (wash of genital tract with 5 mL PBS), and vaginal tissue (days 7, 21, and 30) were determined using liquid chromatography (LC)-MS/MS as described (21, 38). Procedures involving euthanasia and evaluation of intracellular TFV-DP were performed as described previously (4).

TDF, TFV, and TFV-DP levels were measured in blood, vaginal fluid, CVL, tissue and lymphocytes by LC-MS/MS methods as described previously (38, 39). The LLOQ for TDF was 1 ng/mL (tissue and blood) and 0.5 ng/mL (vaginal fluid), LLOQ for TFV was 5 ng/mL, and LLOQ for intracellular TFV-DP was 10 ng/mL, which is equivalent to ~13 fmol/10^6 cells (40). The average fluid and tissue mass was 0.04 g and 0.01 g, respectively. The concentration of drug in vaginal fluid was determined by converting the change in the swab mass to volume, assuming the density of vaginal fluid was 1.0 g/mL. Samples below LLOQ were assigned values midway between zero and LLOQ and then dividing by the mass or volume of the sample.

Efficacy Studies. TDF IVRs were administered to normal cycling, nonsynchronized female pigtailed macaques (n = 6) followed by weekly inoculation (12) vaginally with 50 $TCID_{50}$ SHIV162p3 in six TDF-treated and 12 untreated controls (six real time and six historical controls) (39).The first virus exposure was started 6 d after IVR insertion; thereafter, the macaques were inoculated on a weekly basis. The first TDF IVR was replaced on day 30; subsequent IVR changes were done every 28 d, which corresponded to 2 d after the fourth, eighth, and 12th viral inoculation resulting in a total of four IVR changes in the study period (Fig. 4*A*). Vaginal swabs for determining drug concentration were collected with every IVR change. Infection status was monitored by RT-PCR and confirmed by serology (ZeptoMetrix) (3, 4). The detection limit of the assay was 50 copies/mL. Positive macaques were defined as having two consecutive positive PCR results above detection limit. Macaques were monitored for 28 d after the last viral inoculation. The antiviral activity of CVL samples diluted 1:10 in PBS was assessed using HIV-1_{BaL} in the TZM-bl assay as previously detailed (41).

Statistical Methods. Fisher's exact test was used to compare the treated and control groups for number of infections per total number of virus exposures. A nonparametric log-rank test was used to compare survival probability curves. Spearman rank-order correlation coefficients were calculated to assess associations between antiviral activity of CVL and drug levels.

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