Genital inflammation undermines the effectiveness of tenofovir gel in preventing HIV acquisition in women

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Several clinical trials have demonstrated that antiretroviral (ARV) drugs taken as pre-exposure prophylaxis (PrEP) can prevent HIV infection[1], with the magnitude of protection ranging from –49 to 86% (refs. 2–11). Although these divergent outcomes are thought to be due primarily to differences in product adherence[12], biological factors likely contribute[13]. Despite selective recruitment of higher-risk participants for prevention trials, HIV risk is heterogeneous even within higher-risk groups[14–16]. To determine whether this heterogeneity could influence patient outcomes following PrEP, we undertook a *post hoc* prospective analysis of results from the CAPRISA 004 trial for 1% tenofovir gel (n = 774 patients), one of the first trials to demonstrate protection against HIV infection. Concentrations of nine proinflammatory cytokines were measured in cervicovaginal lavages at >2,000 visits, and a graduated cytokine score was used to define genital inflammation. In women without genital inflammation, tenofovir was 57% protective against HIV (95% confidence interval (CI): 7–80%) but was 3% protective (95% CI: –104–54%) if genital inflammation was present. Among women who highly adhered to the gel, tenofovir protection was 75% (95% CI: 25–92%) in women without inflammation compared to –10% (95% CI: –184–57%) in women with inflammation. Immunological predictors of HIV risk may modify the effectiveness of tools for HIV prevention; reducing genital inflammation in women may augment HIV prevention efforts.

HIV acquisition risk varies widely within a population and is dependent on behavioral and biological factors. Younger women (<25 years of age), for example, experience higher HIV incidence, likely owing to a combination of types and frequencies of sexual partnerships and biological factors, such as genital inflammation[17,18]. PrEP effectiveness was lowest in women <25 years of age in the vaginal and oral interventions to control the epidemic (VOICE) and dapivirine ring trials; this subgroup was least adherent to PrEP and did not experience substantial protection[2,9]. The route of HIV exposure may also be important as evidenced by the observation of better oral PrEP protection in men who have sex with men (MSM) under conditions of high adherence[10,11] despite higher per-coital acquisition during anal sex than during vaginal sex[19]. Further, under conditions of lower adherence, protection was still evident in MSM (pre-exposure prophylaxis initiative (iPrEx) trial) but not in women (VOICE trial)[2,4]. Mucosal tissue penetrance and pharmacokinetics may explain some of these differences; for example, active tenofovir levels in colorectal tissue reach concentrations ten times higher than those in the female genital tract (FGT)[20,21].

Protection provided by products that are partially effective may not be equal across groups that are stratified by HIV risk. Protection in the RV144 vaccine trial was higher in individuals at low and medium risk for HIV but negligible in those at the highest risk[22]. In the iPrEx open-label extension study in MSM, the 'number needed to treat' with PrEP to prevent one infection differed significantly among risk-defined subgroups[23]. Conversely, in the Partners PrEP trial, participants who consistently used PrEP were protected regardless of their risk profile, suggesting that high adherence and/or effectiveness may overcome differences in susceptibility to infection[24].

Case-control analyses of the three trials that have tested topical tenofovir in women (Centre for the AIDS Programme of Research in South Africa (CAPRISA) 004, VOICE, and FACTS001) showed that protection against HIV ranged from 50–60% if product adherence was high[2,3,8]. These data suggest that adherence alone might not fully explain the incomplete efficacy of this product. Here, we evaluate how biological susceptibility, defined as inflammation in the FGT[18], altered the protective efficacy of tenofovir gel.

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Table 1 Incidence rates of HIV infection among women assigned to tenofovir or placebo gel, stratified by the number of elevated cytokines in genital secretions

		Tenofovir arm				Placebo arm				Arm comparison	
No. of elevated cytokines in the upper quartile	Inflammation present	No. of women	No. of HIV infections	Person-years	Incidence rate (95% CI)	No. of women	No. of HIV infections	Person-years	Incidence rate (95% CI)	Incidence rate ratio (IRR) (95% CI)	*P* value
≥3 of 9	No	238	9	392.4	2.3 (1.0–4.4)	255	22	408.5	5.4 (3.4–8.2)	**0.43 (0.2–0.93)**	**0.033**
	Yes	153	15	221.9	6.8 (3.8–11.1)	128	13	186.8	7.0 (3.7–11.9)	0.97 (0.46–2.04)	0.936
≥4 of 9	No	280	13	459.0	2.8 (1.5–4.8)	290	24	457.5	5.2 (3.4–7.8)	0.54 (0.27–1.06)	0.074
	Yes	111	11	155.2	7.1 (3.5–12.7)	93	11	137.8	8.0 (4.0–14.3)	0.89 (0.39–2.05)	0.785
≥5 of 9	No	314	14	509.5	2.7 (1.5–4.6)	320	27	505.0	5.3 (3.5–7.8)	**0.51 (0.27–0.97)**	**0.041**
	Yes	77	10	104.8	9.5 (4.6–17.5)	63	8	90.3	8.9 (3.8–17.5)	1.08 (0.43–2.74)	0.871
≥6 of 9	No	344	14	553.4	2.5 (1.4–4.2)	340	31	535.0	5.8 (3.9–8.2)	**0.44 (0.23–0.83)**	**0.011**
	Yes	47	10	60.9	16.4 (7.9–30.2)	43	4	60.3	6.6 (1.8–17.0)	2.47 (0.77–7.88)	0.126
≥7 of 9	No	371	20	591.2	3.4 (2.1–5.2)	358	31	563.6	5.5 (3.7–7.8)	0.62 (0.35–1.09)	0.096
	Yes	20	4	23.1	17.3 (4.7–44.3)	25	4	31.7	12.6 (3.4–32.3)	1.37 (0.34–5.48)	0.656
Overall		391	24	614.3	3.9 (2.5–5.8)	383	35	595.3	5.9 (4.1–8.2)	0.66 (0.40–1.12)	0.123

Analysis based on $n = 774$ women sampled at 2,139 visits. Poisson distributions were used to calculate CIs for incidence rates and incidence rate ratios (IRRs). A *z*-test (two-sided) was used to compare IRRs between the two study arms, and all *P* values are reported without adjustment for multiple testing. Significant *P* values are bolded.

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We carried out a prospective cohort analysis of all available mucosal specimens obtained prior to HIV infection from participants in CAPRISA 004 ($n = 774$ women sampled at 2,139 visits). Genital inflammation, if defined as elevated levels of ≥3 of 9 examined proinflammatory cytokines (interleukin (IL)-1α, IL-1β, IL-6, tumor necrosis factor (TNF)-α, IL-8, C-X-C motif chemokine 10 (CXCL10; also known as IP-10), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and MIP-1β), with 'elevated' defined as a value in the upper quartile of the distribution of the examined cytokine concentrations, was present in 281 women; 204, 140, 90, and 45 women had genital inflammation if defined as elevated levels of ≥4, ≥5, ≥6, and ≥7 of these cytokines, respectively. Women who did not meet the criteria for inflammation were automatically placed in the 'no inflammation' comparator group, so that a total of $n = 774$ women were included in all analyses. We carried out a Cox regression analysis to determine whether the link between inflammation and HIV risk was dependent on the definition of inflammation that was used. Each definition of genital inflammation was associated with increased HIV risk after adjustment for placement in the tenofovir or placebo study arm (specifically, adjusted hazard ratio (aHR) = 1.86 for elevated levels of ≥3 cytokines (95% CI: 1.11–3.10); aHR = 1.90 for ≥4 cytokines elevated (95% CI: 1.12–3.22); aHR = 2.38 for ≥5 cytokines elevated (95% CI: 1.37–4.15); aHR = 2.99 for ≥6 cytokines elevated (95% CI: 1.64–5.45); and aHR = 3.42 for ≥7 cytokines elevated (95% CI: 1.62–7.23); all $P < 0.05$). Although the estimates of HIV effect for the groups with elevated levels of ≥3 and ≥4 cytokines were similar, after concentrations of ≥4 of the 9 cytokines were elevated, a step-wise increase in HIV risk of approximately 50% was observed for each subsequent number of elevated cytokines. These inflammation-defined strata were used for the subsequent comparisons of tenofovir efficacy.

We next determined whether tenofovir gel was protective against HIV infection on the basis of the presence or absence of genital inflammation (**Table 1**). HIV incidence in the study was 3.9 per 100 person-years (95% CI: 2.5–5.8), which was slightly lower than that in the main trial. In all inflammation-defined strata, the lowest HIV incidence rates were observed in women without inflammation who were randomized to tenofovir. In women with elevated levels of ≥3 cytokines, HIV incidence was 6.8 per 100 person-years (95% CI: 3.8–11.1) in the tenofovir arm as compared to 7.0 (95% CI: 3.7–11.9) in the placebo arm. In contrast, in women with elevated levels of <3 cytokines, HIV incidence in the tenofovir and placebo arms was 2.3 (95% CI: 1.0–4.4) and 5.4 (95% CI: 3.4–8.2), respectively. Similar results were obtained when additional numbers of cytokines were elevated; in the strata defined as elevated levels of ≥5, ≥6, and ≥7 cytokines, HIV incidence was higher in women with inflammation randomized to tenofovir than in those randomized to placebo (**Table 1**).

The overall efficacy of tenofovir gel in this study was 34% (95% CI: −11–61%). Stratifying participants according to genital inflammation clearly segregated efficacy estimates: women in the group with elevated levels of ≥3 cytokines had a tenofovir efficacy of 3% (95% CI: −104–54%; $P = 0.936$), whereas a tenofovir efficacy of 57% was observed in the group with elevated levels of <3 cytokines (95% CI: 7–80%; $P = 0.033$) (**Fig. 1a**). Tenofovir efficacy was 11%, −8%, −147%, and −37% in the groups with elevated levels of ≥4, ≥5, ≥6, and ≥7

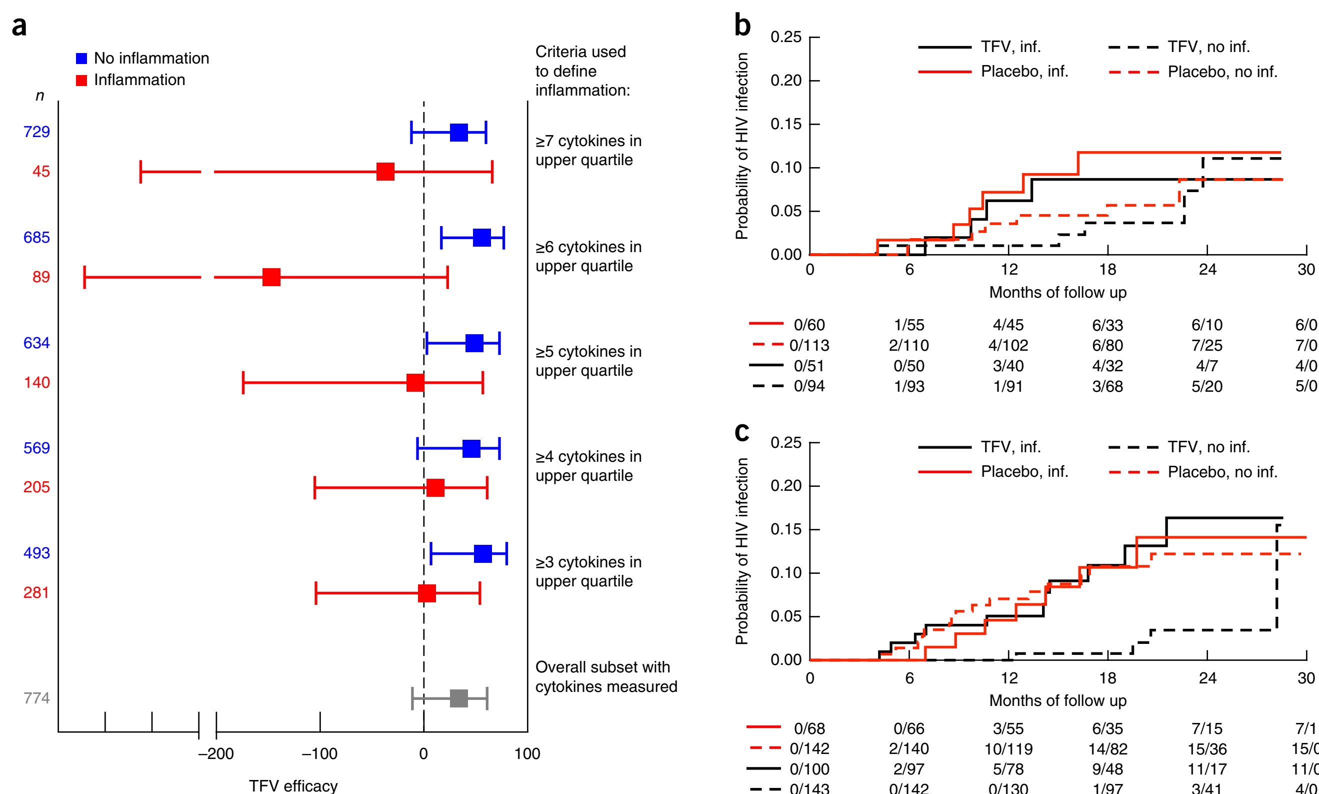

Figure 1 Tenofovir efficacy in groups stratified according to level of inflammation, defined as a specified number of cytokines detected at elevated concentrations in FGT secretions (n = 774 women). (**a**) Tenofovir efficacy estimates (red boxes) and 95% CIs (whiskers) for groups meeting the indicated cut-offs for inflammation. Data for those falling below the indicated cut-off are represented with blue boxes and whiskers. Tenofovir efficacy is measured along the x axis, and the dotted black line indicates 0% efficacy. The gray box and whiskers show the overall tenofovir efficacy for all participants included in this analysis. (**b**,**c**) Kaplan–Meier survival plots showing the probability of seroconversion in participants stratified according to gel adherence (<50% (**b**) and ≥50% (**c**)). Lines indicate data for the tenofovir (TFV) arm of the study, with (solid black) or without (dashed black) genital inflammation, and the placebo arm of the study, with (solid red) or without (dashed red) genital inflammation. Genital inflammation in this analysis was defined as elevated levels of ≥3 of the 9 cytokines. The number of HIV infections and the number of participants at risk for infection in each stratum and at each indicated time point are shown below the graphs (for example, '0/60' indicates there were 0 HIV infections and 60 participants at risk for infection). For statistical analysis, a z-test was used to compare IRRs between the two study arms. All statistical tests were two-sided and unadjusted for multiple comparisons.

cytokines, respectively (all $P > 0.1$). In contrast, tenofovir efficacy ranged from 34–56% in the corresponding no inflammation groups, and these comparisons were statistically significant ($P < 0.05$) for the groups with elevated levels of ≥3, ≥5, and ≥6 of the 9 cytokines. Similar results were obtained in multivariate Cox proportional-hazards regression analyses after adjustments for age, study site, herpes simplex virus (HSV)-2 serostatus, history of sexually transmitted infections (STIs), number of sex acts and sexual partners, and condom and injectable contraception use (**Table 2**). Interestingly, HSV-2 positivity mattered more for HIV acquisition if inflammation was not present; this is in line with findings that HSV-2 seroprevalence is not associated with an increased concentration of inflammatory cytokines in cervicovaginal fluid[25]. Conversely, a high number of sex acts was associated with HIV only in women with genital inflammation. Considering that gel dosing was per-coital, it is difficult to disaggregate effects of sex and exposure to tenofovir gel in these analyses. Nevertheless, these data confirm that FGT inflammation predicted the efficacy of tenofovir gel in women.

We further tested for interaction between genital inflammation and study-arm membership in a Cox regression analysis with time-varying covariates while taking repeated measures of genital inflammation into account. In a model that incorporated genital inflammation (defined as elevated levels of ≥3 of the 9 cytokines), study arm, and an interaction term between inflammation and study arm, a significant interaction between genital inflammation and study arm was observed ($P = 0.028$). Similar findings were obtained when genital inflammation was defined as elevated levels of ≥4 and ≥5 of the 9 cytokines, although these were not statistically significant ($P = 0.127$ and 0.11, respectively). Similar results were obtained in models that included adjustments for potential confounders. These findings support the conclusion that genital inflammation attenuated the efficacy of tenofovir gel.

Previous analyses of results from the CAPRISA 004 trial demonstrated a dose-dependent relationship between gel adherence, measured as the percentage of sex acts for which two gel doses were used, and protection from HIV infection[26]. We hypothesized that the combination of having no inflammation and high adherence would provide the best protection against HIV infection, and that high levels of inflammation might supersede the protective effects conferred by adherence. Indeed, tenofovir gel–mediated protection was highest in women without genital inflammation who used the gel in ≥50% of sex acts (**Supplementary Table 1**), and efficacy was 75% (95% CI 25–92%, $P = 0.014$; <3 elevated cytokines). In comparison, tenofovir efficacy was −10% (95% CI −184–57%, $P = 0.844$) in highly adherent women with genital inflammation (≥3 elevated cytokines). Tenofovir efficacy was attenuated in women who used the gel infrequently (<50% adherence) irrespective of their inflammation status (25% and 15% efficacy, $P = 0.781$ and $P = 0.656$, respectively). Similar results were obtained in

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Table 2 Adjusted hazard ratios of HIV incidence from a multivariate model, stratified according to the presence or absence of FGT inflammation

Inflammation	Parameter	aHR (95% CI)	*P* value
No inflammation (<3 elevated cytokines; *n* = 493 women)	Tenofovir vs. Placebo	0.45 (0.20–0.98)	**0.044**
	Age (years)	0.93 (0.85–1.01)	0.087
	Urban vs. rural site	1.27 (0.49–3.30)	0.626
	HSV-2 seropositive	3.90 (1.66–9.12)	**0.002**
	Sex acts within the last 30 d prior to the study visit	1.03 (0.93–1.15)	0.547
	Contraceptive use, DMPA vs. oral	4.26 (0.57–31.86)	0.158
	Contraceptive use, NET-EN vs. oral	2.55 (0.28–23.36)	0.409
	Abnormal vaginal discharge	0.82 (0.37–1.84)	0.634
	Condom use, always vs. not always	0.85 (0.37–1.97)	0.709
Inflammation present (≥3 elevated cytokines; *n* = 281 women)	Tenofovir vs. Placebo	0.88 (0.40–1.93)	0.757
	Age (years)	0.95 (0.87–1.04)	0.305
	Urban vs. rural site	1.19 (0.46–3.07)	0.727
	HSV-2 seropositive	1.21 (0.53–2.75)	0.654
	Sex acts within the last 30 d prior to the study visit	1.13 (1.04–1.23)	**0.004**
	Contraceptive use, DMPA vs. oral	5.23 (0.69–39.81)	0.110
	Contraceptive use, NET-EN vs. oral	5.95 (0.69–51.28)	0.105
	Abnormal vaginal discharge	2.16 (0.96–4.84)	0.063
	Condom use, always vs. not always	1.66 (0.75–3.65)	0.210

This analysis was completed with inflammation defined as elevated levels of ≥3 cytokines; the results are similar when the definition of inflammation is based on a higher number of elevated cytokines (up to 7 of 9; data not shown). Multivariate Cox proportional-hazards regression was used to calculate aHRs for a range of covariates as indicated in the table (*n* = 774 women sampled at 2,139 visits). *P* values are two-sided and unadjusted for multiple testing, and significant *P* values are bolded. DMPA, depot medroxyprogesterone acetate; NET-EN, norethisterone enantate.

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adjusted models containing the same covariates as described in **Table 2**. We also obtained similar results in survival analyses. In the strata defined with low adherence, genital inflammation status was the major predictor of HIV acquisition risk (**Fig. 1b**, solid lines), and there was little evidence of tenofovir-mediated protection. However, in those with high adherence to tenofovir (**Fig. 1c**), protection afforded by the gel was restricted to the no inflammation group. Similar results were obtained for all cytokine scores (≥4, ≥5, ≥6, and ≥7 elevated cytokines; data not shown). These data provide compelling evidence that women without genital tract inflammation largely account for the protective effect of tenofovir gel adherence that was observed in the CAPRISA 004 trial[3].

The FGT mucosa typically provides an effective barrier against HIV infection, as reflected by the low per-coital rates of male-to-female HIV transmission in epidemiological studies[27,28]. Genital inflammation may decrease natural host defenses against HIV, with the corollary being that it is more difficult to use antiviral agents, such as tenofovir, to protect individuals with inflammation against HIV infection. We have previously described reduced levels of key mucosal barrier proteins and increased numbers of cervical CD4$^+$ T cells, the key targets of HIV, in women with cytokine profiles similar to those used in our inflammation scoring[29], a finding supported by other studies[30,31]. This barrier susceptibility hypothesis is corroborated by recent data from CAPRISA 004 showing that women with genital inflammation who were treated with tenofovir had viruses with lower replicative fitness crossing the mucosal barrier to establish HIV infection[32]. Cellular activation may further increase intracellular dNTP pools and compete with the ability of tenofovir diphosphate to block HIV reverse transcriptase and prevent infection[33]. Understanding these mechanisms will be critical in designing more effective PrEP strategies, particularly for women.

Strengths of this study include its longitudinal design and large sample size; it is one of the largest studies of mucosal inflammation in the context of a trial for HIV prevention. Genital inflammation was evaluated at repeated measures in participants randomized to tenofovir or placebo gel, and inclusion of the entire available cohort allowed us to calculate HIV incidence and tenofovir efficacy in subgroups of at-risk individuals. The study tested an *a priori* hypothesis, and immunological analyses were blinded with extensive quality-control measures put in place to ensure the accuracy of cytokine measurements across multiple sample runs. Although inflammation is difficult to capture using any one measurement, proinflammatory cytokines are believed to be central to this process. Our sensitivity analyses support the notion that inflammation was consistently able to differentiate women protected by tenofovir, irrespective of how many cytokines were elevated.

Our study had some limitations. Specimens were available only at certain study visits and were not available for a subset of individuals, including some who acquired HIV before the first available genital sample was taken. However, in the remaining cohort, we took a median measure of several visits and were therefore better able to classify individuals than what could be done using a single measurement. Our major conclusions were further borne out by a second time-varying analysis, showing a significant interaction between genital inflammation (≥3 elevated cytokines) and study arm in predicting HIV acquisition. For adherence, we relied on self-reported return of used applicators. Data on the mucosal concentration of tenofovir are available for a subset of participants[26], but too few for comparisons of gel efficacy. We based our adherence analyses on self-report that the product was used in ≥50% sex acts; although this has clear clinical implications regarding protection, the study is underpowered for further adherence cut-offs and for adherence–inflammation interaction analyses. Despite our large sample size, a relatively small proportion of the cohort had genital inflammation, limiting statistical power to definitively conclude that those with inflammation were not protected by tenofovir gel. Further validation in additional cohorts could increase this sample size, but this has logistical challenges. Finally, we deliberately selected composite cytokine outputs on the basis of prior studies[18] to overcome the burden of multiple-test correction for individual cytokine concentrations, and we used multiple elevated cytokine cut-offs to determine the rigor of these definitions of genital inflammation in assessing HIV outcomes.

The causes of inflammation remain unclear. Several groups have shown that bacterial vaginosis (BV) and/or changes in the vaginal microbiome are associated with genital inflammation[34,35], including in CAPRISA 004 (unpublished data). We and others have also recently shown that vaginal dysbiosis impairs tenofovir efficacy, perhaps via reducing levels of tenofovir in the mucosa[36]. Interestingly, the same issue may not apply to oral PrEP, as BV and/or vaginal dysbiosis did not affect PrEP efficacy in the Partners PrEP study[37]. This could be due to pharmacological differences between oral and topical PrEP. As not all BV and/or dysbiosis results in inflammation[38], repeating our inflammation analyses in other PrEP studies will help to understand the generalizability of our findings.

In summary, the combination of gel adherence and genital inflammation differentiated women who were protected by topical tenofovir from those who were not. This was pronounced in participants who did not have genital inflammation but were highly adherent to gel; they experienced protection levels as high as 75%. However, those with genital inflammation who were adherent to gel had no protection, underscoring the unlikelihood of any protective effect in the 'inflammation/adherent' group. Inflammation is a major risk factor for HIV acquisition; reducing genital inflammation through treating its root causes or using anti-inflammatory agents might further optimize PrEP for women. Genital inflammation should be investigated as a potential effect modifier in trials for new PrEP products.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

L.R.M., L.J.L., J.G.G.L., A.D.K., L.M., L.E.M., Q.A.K., S.S.A.K., and J.-A.S.P. designed the study. L.R.M., L.J.L., D.A., S.N., and A.S. performed the experiments. L.R.M., L.J.L., N.Y.-Z., and N.N. analyzed the data. L.R.M., L.J.L., N.Y.-Z., D.A., S.N., A.S., N.N., J.G.G.L., A.D.K., L.M., L.E.M., Q.A.K., S.S.A.K., and J.-A.S.P. wrote the manuscript.

COMPETING INTERESTS

J.G.G.L. is named in a USA government patent titled "Inhibition of HIV Infection through Chemoprophylaxis" (US Patent no. 9,044,509 B2). The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention (CDC).

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ONLINE METHODS

Study design. We undertook a prospective cohort study to assess the impact of mucosal cytokine levels on HIV acquisition and tenofovir efficacy using specimens collected during the CAPRISA 004 trial, a phase 2B trial that was randomized, blinded, and had a placebo control that measured the safety and efficacy of tenofovir 1% gel[3,39]. The study was approved by the Biomedical Research Ethics Committee (BREC) at the University of KwaZulu-Natal, and all participants provided informed written consent to participate. The *a priori* objective of these experiments was to compare tenofovir efficacy between groups of participants stratified by inflammation and adherence status; the hypothesis was that mucosal inflammation would reduce tenofovir efficacy. The target sample size was all those with available specimens that were obtained before HIV infection that were included in the intent-to-treat (ITT) analysis. The final analysis included 774 women sampled at 2,139 study visits (87% of the original intent-to-treat cohort). We were not able to carry out analyses in the instances in which consent to store biological specimens was not provided, no specimens were available, or when participants acquired HIV before samples could be obtained. The inclusion and exclusion criteria for CAPRISA 004 have been published previously[39]; these included sexually active women aged 18–40 years living near either an urban or rural study site in KwaZulu-Natal, South Africa. All clinical and epidemiological variables used in these analyses are part of the original locked database generated in the parent clinical trial[3].

Cytokine assays. The concentrations of nine cytokines (IL-1α, IL-1β, IL-6, TNF-α, IL-8, IP-10, MCP-1, MIP-1α, and MIP-1β) were measured in specimens obtained through cervicovaginal lavage (CVL using multiplexed ELISA assays (BioPlex; Bio-Rad Laboratories, Inc.; Hercules, CA, USA). For consistency, fresh assays were performed on samples from all participants, including those previously published in Masson *et al.*[18], and all laboratory personnel were blinded to all clinical and epidemiological variables. Cytokine concentrations were measured using a Bio-Plex 200 Array Reader (Bio-Rad Laboratories). The sensitivity of these kits ranged between 0.2 and 45.2 pg/ml for each of the cytokines measured. Data were collected using Bio-Plex Manager software version 6, and a five-parameter logistic regression formula was used to calculate sample concentrations from the standard curves. Cytokine levels below the lower limit for detection in the assay were recorded as half of the lowest concentration measured for each cytokine. Similarly, cytokine levels above the detectable limit were recorded as twice the highest concentration measured for each cytokine. To minimize the effect of interplate variability, all CVL specimens collected from the same participant were assayed on the same plate.

Statistical analyses. HIV was the primary endpoint for all analyses. Inflammation, study arm, and adherence were the main explanatory variables, but some models were adjusted for additional variables. Using our published scoring criteria[18], we defined inflammation according to the number of cytokines in the upper quartile of the distribution of cytokine concentrations. For stratified analyses, we calculated median cytokine concentrations across multiple HIV- study visits (intraindividual) and determined whether the median cytokine concentration was in the upper quartile for each cytokine using data from the entire cohort (**Supplementary Fig. 1**). To diagnose inflammation, a score for each specimen was calculated on the basis of the number of proinflammatory cytokines in the upper quartile. Individuals were stratified into groups with elevated levels of 3, 4, 5, 6, and 7 or more cytokines, and these groups were used to examine the effect of different cutoffs based on the number of elevated cytokines on HIV risk and tenofovir protection. For each analysis, all participants not meeting the cutoff that defined 'inflammation' were considered to be in the no inflammation group; all analyses therefore included all 774 participants. For time-varying analyses, we calculated whether genital inflammation was present at each visit and assigned that value as the absence or presence of inflammation (0 or 1) to all person-time preceding that visit. All person-time that occurred following the final visit was determined to be the same as the final inflammation measurement.

Stratification according to adherence was carried out using a cutoff of 50% of sex acts covered as determined through returned used applicators, as increases in the proportion of participants meeting this threshold were shown to correlate with protection against HIV in the original analysis[3]. Analyses of tenofovir efficacy were carried out using the subset of the ITT population stratified according to either inflammation status and/or gel adherence. Follow-up time was calculated from randomization to the estimated date of HIV infection or termination date, whichever occurred first.

We used Poisson distributions to calculate CIs for incidence rates and IRRs. Efficacy was calculated as 1 – IRR. A z-test was used to compare IRRs between the two study arms. Univariate and multivariate Cox proportional-hazards regression was used to calculate aHRs for a range of covariates, including inflammation modeled as a time-varying covariate and interaction analyses between the inflammation and study arms, as indicated in the relevant tables. We inspected the plausibility of the proportional hazards assumption through visual inspection of curves calculated using log(−log(survival)), where 'survival' is defined as remaining HIV uninfected. All P values are two-sided and were not adjusted for multiple testing.

Life Sciences Reporting Summary. Further information on experimental design and reagents is available in the **Life Sciences Reporting Summary**.

Data availability. Data will be made available through request on a dedicated portal on the CAPRISA website (http://www.caprisa.org/).

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☐ Initial submission ☐ Revised version ☒ Final submission

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Experimental design

1. Sample size

Describe how sample size was determined.

All available specimens were analyzed from all available time points prior to HIV infection. The original cohort was powered to detect the effectiveness of TFV gel, as previously described (Abdool Karim et al Science 2010).

2. Data exclusions

Describe any data exclusions.

Participant specimens for the original trial were not available for 13% of participants. We were not able to carry out analyses in instances where storage consent was not provided, no specimens were available, or when participants acquired HIV before samples could be obtained.

3. Replication

Describe whether the experimental findings were reliably reproduced.

Clinical specimens were analyzed. A portion were analyzed in duplicate to calculate intra-plate variability and these data are available if required.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Samples were analyzed randomly, with plate design done without any knowledge of clinical or outcome variables.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Laboratory personnel were blinded to all clinical and epidemiological data, and were only given access (in some cases) once the final cytokine data was locked.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed	
☐	☒	The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒	A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	A statement indicating how many times each experiment was replicated
☐	☒	The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☐	☒	A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒	The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
☐	☒	A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒	Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Nature Medicine: doi:10.1038/nm.4506

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.	Bio-Plex Manager software version 6, SPSS version 24, SAS version 9.3, and Microsoft Excel 14.7.2

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.	No unique materials were used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	All antibodies used in Multiplex ELISA assays were validated by Biorad.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	No eukaryotic cell lines were used.
b. Describe the method of cell line authentication used.	No eukaryotic cell lines were used.
c. Report whether the cell lines were tested for mycoplasma contamination.	No eukaryotic cell lines were used.
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	No eukaryotic cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.	No animals were used in this study.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.	All participants were women enrolled in the CAPRISA 004 clinical trial that tested the safety and efficacy of TFV 1% Gel (Abdool Karim et al Science 2010). We have included analysis of study arm at randomization and HIV acquisition, stratified by mucosal cytokine defintions of inflammation and study product adherence. A number of co-variates collected during the clinical trial have been used to adjust multi-variable analyses; most of these relate to risk factors for HIV acquisition and/or potential correlates of mucosal cytokine levels.

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