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One Percent Tenofovir Applied Topically to Humanized BLT Mice and Used According to the CAPRISA 004 Experimental Design Demonstrates Partial Protection from Vaginal HIV Infection, Validating the BLT Model for Evaluation of New Microbicide Candidates▽

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Recent iPrEx clinical trial results provided evidence that systemic preexposure prophylaxis (PrEP) with emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF) can partially prevent rectal HIV transmission in humans. Similarly, we have previously demonstrated that systemic administration of the same FTC-TDF combination efficiently prevented rectal transmission in humanized bone marrow/liver/thymus (BLT) mice. The CAPRISA 004 trial recently demonstrated that topical application of the tenofovir could partially prevent vaginal HIV-1 transmission in humans. To further validate the usefulness of the BLT mouse model for testing HIV prevention strategies, we evaluated the topical administration of tenofovir as used in CAPRISA 004 to prevent vaginal HIV transmission in BLT mice. Our results demonstrate that vaginally administered 1% tenofovir significantly reduced HIV transmission in BLT mice ($P = 0.002$). Together with the results obtained after systemic antiretroviral PrEP, these topical inhibitor data serve to validate the use of humanized BLT mice to evaluate both systemic and topical inhibitors of HIV transmission. Based on these observations, we tested six additional microbicide candidates for their ability to prevent vaginal HIV transmission: a C-peptide fusion inhibitor (C52L), a membrane-disrupting amphipathic peptide inhibitor (C5A), a trimeric D-peptide fusion inhibitor (PIE12-Trimer), a combination of reverse transcriptase inhibitors (FTC-TDF), a thioester zinc finger inhibitor (TC247), and a small-molecule Rac inhibitor (NSC23766). No protection was seen with the Rac inhibitor NSC23766. The thioester compound TC247 offered partial protection. Significant protection was afforded by FTC-TDF, and complete protection was offered by three different peptide inhibitors tested. Our results demonstrate that these effective topical inhibitors have excellent potential to prevent vaginal HIV transmission in humans.

The continuous spread of the AIDS epidemic, with over 2.7 million new human immunodeficiency virus (HIV) infections per year, highlights the need for successful prevention approaches (55). Proposed interventions to block HIV transmissions include condoms, circumcision, vaccines, test-and-treat strategies, systemic preexposure prophylaxis (PrEP), and topical microbicides (7, 9, 13, 26, 30). Microbicides are products that, when used vaginally or rectally, could result in protection from HIV transmission. Several first-generation microbicides with well-documented *in vitro* antiviral activity have been tested in large clinical trials. Unfortunately, none of these agents was found to be effective in preventing HIV-1 transmission in humans (14, 19, 44, 47).

The second generation of microbicide candidates focuses on antiretrovirals (ARVs) that specifically target key aspects of HIV replication such as fusion, viral entry, and reverse transcription. However, only reverse transcriptase inhibitors that are already routinely used to treat HIV-infected patients are

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TABLE 1. Inhibitors evaluated for efficacy in preventing vaginal HIV-1 transmission

Inhibitor	Inhibitor category	Mechanism of inhibition	Concn applied (in 20 μl)
Tenofovir	Reverse transcriptase inhibitor	Blocks reverse transcription	1% (34.8 mM)
C52L	Peptide fusion inhibitor	Blocks virus-cell fusion	500 μM
C5A	Peptide membrane disruptor	Disrupts viral membrane prior to cell contact	200 μM
PIE12-Trimer	Peptide fusion inhibitor	Blocks virus-cell fusion	100 μM
FTC-TDF	Reverse transcriptase inhibitors	Blocks reverse transcription	28 μM/16.5 μM
TC247	Zinc finger inhibitor	Blocks viral spreading	500 μM
NSC23766	Rac inhibitor	Blocks entry	10 mM

currently undergoing large-scale clinical trials for efficacy in HIV prevention (7, 9). One of these trials, the Center for the AIDS Program of Research in South Africa (CAPRISA) 004, recently reported that 1% tenofovir gel can partially prevent vaginal HIV transmission (1). Likewise, the Preexposure Prophylaxis Initiative (iPrEx) clinical trial recently reported that oral administration of a combination of the reverse transcriptase inhibitors emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF) provided partial protection from rectal HIV transmission (18). If other active clinical trials also find reverse transcriptase inhibitor-based PrEP to be successful, these antiretrovirals might become extensively used as topical microbicides to prevent HIV transmission. However, there is significant concern that viruses resistant to these drugs could potentially emerge and spread, severely limiting available treatment options (26).

To preserve the integrity of frontline therapeutic ARV regimens, there is strong interest in the development of novel microbicides with resistance profiles that do not overlap with those used for therapy. The evaluation of microbicide candidates would benefit significantly from validated animal models capable of accurately predicting human outcomes. Prior to the completion of the iPrEx trial, we reported the efficacy of systemic FTC-TDF to prevent vaginal and rectal HIV transmission in humanized bone marrow/liver/thymus (BLT) mice (11, 12). Systemic FTC-TDF also was reported to prevent rectal simian-human immunodeficiency virus (SHIV) transmission in rhesus macaques (17). As indicated above, this same combination of drugs was shown to also reduce the incidence of HIV infection in humans (18). To further validate the utility of the humanized BLT mouse model for the evaluation of HIV prevention strategies, we evaluated the topical administration of tenofovir as used in CAPRISA 004 (1) to prevent vaginal HIV transmission in BLT mice. As in humans, our results demonstrate that vaginally administered 1% tenofovir reduced HIV transmission in BLT mice, highlighting the usefulness of this model for the *in vivo* evaluation of HIV prevention interventions. Based on these results, we extended our analysis and tested the ability of six additional candidate microbicides with distinct antiretroviral mechanisms of action (Table 1) to block vaginal HIV transmission in the BLT model. In addition to tenofovir, our comprehensive preclinical efficacy screen of these six additional inhibitors identified four inhibitors with strong protective capacity and substantial potential to prevent HIV transmission in humans.

MATERIALS AND METHODS

Generation of humanized BLT mice. BLT mice were prepared essentially as previously described (6, 11, 12, 24, 25, 31, 41, 45). Briefly, thymus/liver-implanted NOD/SCID or NOD/SCID-gamma chain null mice (NSG) (The Jackson Laboratories) were transplanted with autologous human fetal liver $CD34^+$ cells (Advanced Bioscience Resources) and monitored for human reconstitution in peripheral blood by flow cytometry, as we have previously described (11, 12, 31, 45). Mice were maintained either at the Animal Resources Center of UT Southwestern Medical Center at Dallas or with the Division of Laboratory Animal Medicine at the University of North Carolina (UNC) at Chapel Hill in accordance with protocols approved by the each institution's Institutional Animal Care and Use Committee.

Topical administration of HIV inhibitors to BLT mice. Each inhibitor (20 μl) was applied vaginally within 30 min of vaginal viral exposure, except for the vaginal application of 1% tenofovir [PMPA; 9-*R*-(2-phosphonomethoxypropyl) adenine]. The 1% tenofovir was applied 4 h before and 4 h after vaginal viral exposure in order to ensure full compliance with the CAPRISA 004 protocol, which requires two applications of the inhibitor (one before and one after sex) within a 24-h period (1). C52L peptide, a C-peptide fusion inhibitor, was produced and purified as previously described and applied at a working concentration of 500 μM (10). C5A peptide, a membrane-disrupting amphipathic peptide inhibitor, was produced and purified as previously described and applied at a working concentration of 200 μM (5). A trimeric D-peptide fusion inhibitor, PIE12-Trimer, was produced and purified as previously described and applied at a working concentration of 100 μM (54). FTC-TDF was prepared as previously published and applied at a working concentration of 28 μM and 16.5 μM, respectively (12). Thioester compound 247 (TC247) was produced and purified as previously described and applied at a working concentration of 500 μM (32). NSC23766 (Calbiochem), a small-molecule Rac inhibitor, was applied at a working concentration of 10 mM.

Exposure of BLT mice to HIV-1. Stocks of HIV-$1_{JR\text{-}CSF}$ were prepared, and titers and p24 content were determined as we have previously described (53). HIV-1 exposures were performed essentially as previously described using a total volume of 2 to 10 μl (170 ng p24) (11, 57).

Analysis of HIV-1 infection of BLT mice. Infection of BLT mice with HIV-1 was monitored in peripheral blood as follows: by determining plasma levels of viral antigenemia using a p24 enzyme-linked immunosorbent assay ([ELISA] assay sensitivity, 7.8 pg/ml; Coulter); by determining levels of viral RNA in plasma using either an Amplicor (Roche) assay or one-step reverse transcriptase real-time PCR (ABI custom TaqMan Assays-by-Design) according to the manufacturer's instructions (with primers 5′-CATGTTTTCAGCATTATCA GAAGGA-3′ and 5′-TGCTTGATGTCCCCCCACT-3′ and MGB-probe 5′-FAM-CCACCCCACAAGATTTAAACACCATGCTAA-Q-3′, where FAM is 6-carboxyfluorescein [37]; assay sensitivity of 400 RNA copies per ml in both assays); and by determining levels of viral DNA in peripheral blood cells (real-time PCR analysis; assay sensitivity of 10 copies) as previously described (11, 12, 45, 53). The single-cell suspension preparation from the female reproductive tract (FRT) was adapted from a published protocol (22). Briefly, the entire FRT (vagina, cervix, uterus, fallopian tubes, and ovaries) was minced and then placed in a 50-ml conical tube with 2 ml of fresh FRT digestion medium (25 units of DNase I [Roche] plus 2 mg of collagenase D [Roche] in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum [FBS]). The samples were shaken gently at room temperature for 2 h. Following this incubation, each sample received an addition of 2 ml of EDTA medium (Ca^{2+}- and Mg^{2+}-free Hanks buffered salt solution supplemented with 5% heat inactivated FBS and 5 mM EDTA) and was shaken gently at room temperature for 20 more minutes. The samples were vortexed briefly and then filtered through a 70-μm-pore-size cell strainer. Cells were washed, counted using trypan blue exclusion, and utilized as indicated throughout the text, on figures, and in tables. Other tissues were harvested and then evaluated by molecular, microscopic, and flow cytometric analyses for evidence of HIV infection as we have previously described (11, 12, 31, 45). The flow cytometry antibody panels for analysis of BLT mouse FRT were

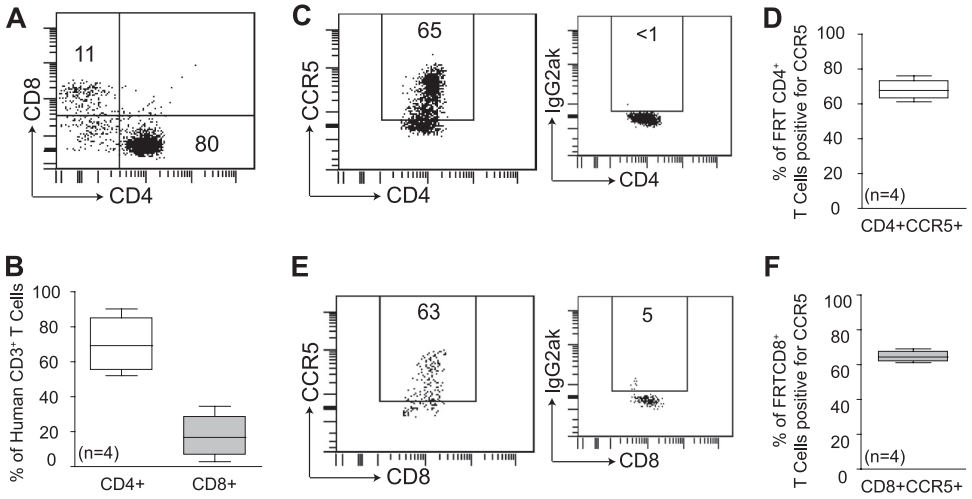

FIG. 1. The female reproductive tract of humanized BLT mice is efficiently repopulated with human HIV-1 target cells rendering them susceptible to infection. (A) Flow cytometric analysis of cells from the FRT of a representative naive humanized BLT mouse demonstrates its reconstitution with human $CD4^+$ and $CD8^+$ T cells. The gating strategy for flow cytometric analysis is as follows: live cells → human CD45 → human CD3 → human CD4 or CD8. (B) The box plot shows that the proportions of human T cells in the FRT are robust and reproducible in BLT mice. (C) Human $CD4^+$ T cells from the FRT of the representative BLT mouse were further analyzed to demonstrate their surface expression of the chemokine receptor CCR5 (left). The panel on the right shows fluorescence-minus-one staining control with isotype-matched fluorophore-conjugated antibody to facilitate appropriate gating of the CCR5-expressing human $CD4^+$ T cells. (D) Box plot shows the levels of human $CD4^+$ $CCR5^+$ T cells in the FRT. (E) Human $CD8^+$ T cells from the FRT of the representative BLT mouse were further analyzed to demonstrate surface expression of the chemokine receptor CCR5 (left). The panel on the right shows fluorescence-minus-one staining control with isotype-matched fluorophore-conjugated antibody to facilitate appropriate gating of the CCR5-expressing human $CD8^+$ T cells. (F) Box plot shows the levels of human $CD8^+$ $CCR5^+$ T cells in the FRT in BLT mice.

the following: panel A, CD8 fluorescein isothiocyanate (FITC) (SK1), CXCR4 phycoerythrin (PE) (12G5) or IgG2ak PE (G155-178), CD4 peridinin chlorophyll protein (PerCP) (SK3), CD3 PE-Cy7 (SK7), CCR5 allophycocyanin (APC) (3A9) or IgG2ak APC (G155-178), and CD45 APC-Cy7 (2D1); panel B, CD3 FITC (HIT3a), CD4 PE (RPA-T4), CD8 PerCP (SK1), and CD45 APC (HI30) (all purchased from BD Biosciences).

Analysis of systemic infection was performed on tissues harvested from infected mice or on cells isolated from the tissues indicated throughout the text, on figures, and in tables. Utilizing *in situ* hybridization, real-time PCR analysis, and coculture with allogeneic human peripheral blood mononuclear cells (PBMC) as previously described (11, 12, 31, 45). An immunofluorescence assay was performed on paraffin-embedded BLT FRT sections. After rehydration, antigen retrieval was performed using 1× Diva Decloaker solution (Biocare Medical); slides were cooled for 30 min, blocked with 2% normal donkey serum and 2% Sniper blocking reagent in TNB (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% blocking reagent [NEN]), and incubated at 4°C overnight with rabbit monoclonal anti-human CD3 (Labvision), mouse anti-human CD11c (Novocastra), or mouse anti-CD68 (Dako). After slides were washed, sections were incubated with the following secondary antibodies: donkey anti-mouse IgG Alexa Fluor-488, donkey anti-rabbit IgG Alexa Fluor-555, and 4′,6′-diamidino-2-phenylindole ([DAPI], 300 nM final concentration) (all from Molecular Probes) for 1 h at room temperature. Sections were mounted in Aquapolymount (Polysciences, Inc.) and imaged using a Nikon 80i fluorescent microscope using the 20× objective. Images were processed with Adobe Photoshop software, version CS3.

Statistics. All statistical analyses (alpha level, 0.05) were performed in Prism, version 4 (Graph Pad). The box plot should be interpreted as follows: the middle line is the median, the box extends from the 25th to the 75th percentiles, and the error bars extend from lowest to highest values.

RESULTS

Our goal was to validate and implement a preclinical *in vivo* experimental system to evaluate the ability of topically applied inhibitors to prevent vaginal HIV-1 transmission. We first used flow cytometry to characterize the site of exposure (i.e., the female reproductive tract [FRT]) in BLT mice for the presence of human $CD4^+$ $CCR5^+$ and $CD8^+$ $CCR5^+$ T cells (Fig. 1A to F). The human T cells expressing CD4 (primary viral receptor) and CCR5 (viral coreceptor) represent important HIV target cells found in the FRT of BLT mice. When these data were compared to human studies of T cells in cervicovaginal mucosa and cervical epithelia, we found that BLT mice exhibit a higher $CD4^+$ T cell proportion of total $CD3^+$ T cells than reported in the human lower reproductive tract; however, the proportions of CCR5-positive T cells in the FRT were essentially the same between humans and BLT mice (21, 34, 40). Second, we determined the systemic reconstitution with HIV-1 target cells by measuring the levels of human cells in peripheral blood. Blood analysis of all mice used in the testing of microbicides showed that 52% (mean; standard deviation [SD] ±17%; $n = 64$) of all circulating cells were human (Tables 2 to 5). Further analysis showed that 83% (mean; SD, ±7%; $n = 64$) of circulating human T cells expressed CD4 (Tables 2 to 5). The BLT mouse blood $CD4^+$ T cell proportion is higher than normally seen in humans but still within the range of what has been described for healthy humans (2, 8, 36). These results demonstrate the suitability of the utilized BLT mice to perform the *in vivo* evaluation of topical microbicides to prevent vaginal HIV-1 transmission.

To date, CAPRISA 004 has provided the only successful demonstration that a topical microbicide can prevent vaginal HIV-1 transmission (1). Therefore, to validate the use of BLT mice for the evaluation of topical inhibitors of vaginal HIV-1 transmission, we utilized the same study design as in the CAPRISA 004 trial (Fig. 2), where study subjects were asked to apply a 1% tenofovir product twice (once before and once after sex with both applications occurring within a 24-h period) (1). In our experiments, BLT mice received 1% tenofovir once

TABLE 2. Description of BLT mice used to evaluate the vaginal HIV-1 transmission prevention potential of 1% tenofovir[a]

Treatment and mouse no.	PB profile at exposure:[b]		No. of weeks followed (exposure to harvest)	PB analysis postexposure[c]		Multiple tissue analysis by quantitative real-time PCR for cell-associated viral DNA[e,f]
	% hCD45$^+$	% hCD45$^+$ hCD3$^+$ hCD4$^+$		Plasma viral load (RNA)[d]	PBMC-associated viral DNA[e]	
1% Tenofovir						
1	63	77	3	Neg (0/3)	Neg (0/3)	B, LI, LN, LU, O, S
2	51	72	6	Neg (0/6)	Neg (0/6)	B, LI, LN, LU, O, S
3	54	68	7	Neg (0/7)	Neg (0/7)	B, FRT, LI, LN, LU, O, S
4	37	73	7	Neg (0/7)	Neg (0/7)	B, FRT, LI, LN, LU, O, S
5	72	77	7	Neg (0/7)	Neg (0/7)	B, FRT, LI, LN, LU, O, S
6	62	88	7	**Pos (5/7)**	**Pos (5/7)**	**B, FRT, LI, LN, LU, O**
7	38	86	7	Neg (0/7)	Neg (0/7)	B, FRT, LI, LN, LU, O, S
8	64	77	7	Neg (0/7)	Neg (0/7)	B, LI, LN, LU, O, S
Control						
9	55	83	7	**Pos (3/3)**	**Pos (3/3)**	**LN, LU**
10	58	89	9	**Pos (4/4)**	**Pos (3/4)**	**B, LI, O, S**
11	28	93	13	Neg (0/5)	Neg (0/5)	ND
12	29	89	13	**Pos (5/5)**	**Pos (3/5)**	**B, LI, LU, O, S**
Mean ± SD	51% ± 4%	81% ± 2%	8 ± 1			

[a] Data shown represent analyses performed on all mice. Data for infected mice are shown in bold.
[b] PB, peripheral blood; hCD45, human CD45; hCD3, human CD3; hCD4, human CD4.
[c] For values in parentheses, the first number represents the number of positive results out of the second number, which represents the number of different time points (total samples) tested. Neg, negative; Pos, positive.
[d] Reverse transcriptase real-time PCR limit of detection, 400 copies/ml.
[e] Real-time PCR limit of detection, 10 copies.
[f] B, bone marrow; FRT, female reproductive tract; LI, liver; LN, lymph nodes; LU, lung; O, thymic organoid; S, spleen; ND, not done.

before and once after HIV-$1_{JR\text{-}CSF}$ exposure according to the clinical trial protocol. In the CAPRISA 004 study, a 39% overall reduction in HIV incidence was observed, as determined by rapid tests and confirmed by viral load analysis (1). In BLT mice, we observed 88% protection from vaginal HIV-1 transmission, as determined by plasma viral load analysis, and protection from vaginal HIV transmission was confirmed by the lack of detectable viral DNA in peripheral blood and tissues (Fig. 3A to E and Table 2). In contrast, plasma viral RNA and cell-associated viral DNA were readily detectable in all infected control mice and the one breakthrough mouse which received 1% tenofovir treatment (Fig. 3A to E; Table 2). These results obtained using the CAPRISA 004 study design in BLT mice demonstrate the ability of 1% tenofovir to reduce vaginal HIV-1 infection in this model and strongly support the use of this model for the evaluation of candidate topical microbicides for their ability to prevent HIV transmission.

Based on these encouraging results, we proceeded to evaluate the efficacy of six additional microbicide candidates to prevent vaginal HIV-1 transmission in humanized BLT mice (Table 1 and Fig. 4). Prior to selection for *in vivo* efficacy evaluation here, each inhibitor was shown to efficiently inhibit HIV *in vitro* (3, 5, 39, 43, 50, 52, 54, 56). Our experimental approach for testing these six inhibitors was a single dose of the product administered prior to exposure rather than the two doses of drug administered within 24 h as used in CAPRISA 004 (1). This experimental design is based on the more standard single-dose administration prior to exposure (19, 23, 29, 35, 44, 47). This approach is simpler, more cost-effective than multiple applications per sexual encounter and has been postulated to help increase adherence among women (51). For these reasons this approach has been included as one arm in an upcoming trial by the Microbicides Development Programme (MDP 302) (51). Furthermore, the "single dose prior to or with

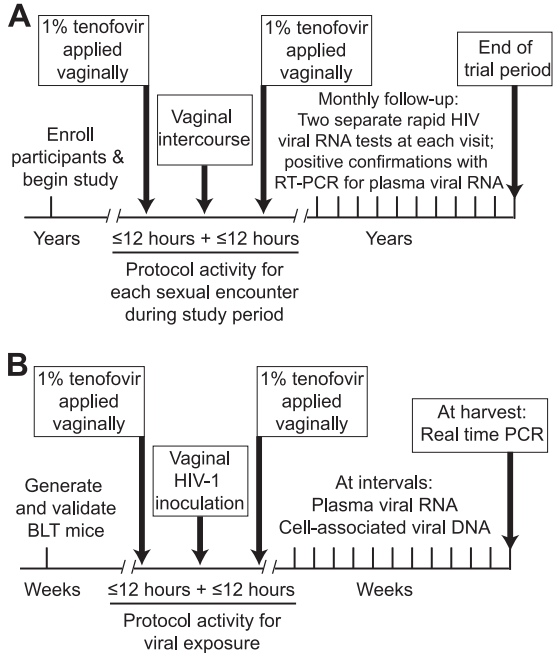

FIG. 2. Experimental design for the validation of BLT mice for preclinical efficacy evaluation of HIV prevention strategies. (A) CAPRISA 004 experimental design. The CAPRISA 004 trial design incorporated a novel strategy for dosing referred to as BAT24 and was modeled on the timing of nevirapine used to prevent mother-to-child HIV transmission. BAT24 means that the study product (1% tenofovir or placebo) is applied within 12 h before and up to 12 h after vaginal intercourse, but no more than 2 applications were to occur within a 24-h period. (B) Evaluation of 1% tenofovir in BLT mice using the CAPRISA 004 experimental design. To validate BLT mice for the evaluation of HIV prevention interventions, the application of 1% tenofovir and the viral exposure were strictly performed according to the BAT24 protocol as in the human trial. Note the significantly shorter time frame for the BLT mouse experiments.

TABLE 3. Description of BLT mice used to evaluate the vaginal HIV-1 transmission prevention potential of C52L, C5A, and PIE12-Trimer[a]

Treatment and mouse no.	PB profile at exposure:[b]		No. of weeks followed (exposure to harvest)	PB analysis postexposure[c]			Multiple-tissue analysis[g]		
	% human $CD45^+$	% $hCD45^+$ $hCD3^+$ $hCD4^+$		Plasma antigenemia (Gag^{p24})[d]	Plasma viral load (RNA)[e]	PBMC-associated viral DNA[f]	*In situ* hybridization for viral RNA	Quantitative real-time PCR for cell-associated viral DNA[f]	Virus rescue of replication-competent virus (Gag^{p24})[d]
C52L									
13	20	75	5	Neg (0/2)	Neg (0/1)	Neg (0/2)	FRT, LN, LU, O, S	B, LI, LN, LU, O, S	B, LI, LN, LU, O, S
14	15	73	5	Neg (0/2)	Neg (0/1)	Neg (0/2)	FRT, LN, LU, O, S	B, LI, LU, O, S	B, LI, LN, LU, O, S
15	18	86	5	Neg (0/2)	Neg (0/1)	Neg (0/2)	FRT, LN, LU, O, S	B, LI, LU, O, S	B, LI, LN, LU, O, S
16	86	81	10	Neg (0/4)	Neg (0/4)	Neg (0/4)	LU, S, SI	B, LI, LN, LU, O, S	B, LI, LN, LU, O, S
17	68	77	10	Neg (0/5)	Neg (0/4)	Neg (0/5)	LU, S, SI	B, LI, LN, LU, O, S	B, LI, LN, LU, O, S
18	74	78	10	Neg (0/5)	Neg (0/4)	Neg (0/5)	LU, S, SI	B, LI, LN, LU, O, S	B, LI, LN, LU, O, S
19	77	77	10	Neg (0/5)	Neg (0/4)	Neg (0/5)	LU, S, SI	B, LI, LN, LU, O, S	B, LI, LN, LU, O, S
C5A									
20	40	72	7	Neg (0/3)	Neg (0/1)	Neg (0/3)	FRT, LN, LU, O, S	B, LI, LU, O, S	B, LI, LN, LU, O, S
21	44	82	8	Neg (0/4)	Neg (0/3)	Neg (0/4)	ND	B, LI, LN, LU, O, S	B, LI, LN, LU, O, S
22	48	83	8	Neg (0/4)	Neg (0/3)	Neg (0/4)	ND	B, LI, LN, LU, O, S	B, LI, LN, LU, O, S
23	34	83	8	Neg (0/4)	Neg (0/3)	Neg (0/4)	ND	B, LI, LN, LU, O, S	B, LI, LN, LU, O, S
24	34	83	8	Neg (0/4)	Neg (0/3)	Neg (0/4)	ND	B, LI, LN, LU, O, S	B, LI, LN, LU, O, S
25	46	79	9	Neg (0/4)	Neg (0/2)	Neg (0/4)	ND	ND	ND
26	40	72	10	Neg (0/5)	Neg (0/2)	Neg (0/5)	FRT, LN, LU, O, S	B, LI, LU, O, S	B, LI, LN, LU, O, S
27	40	51	10	Neg (0/5)	Neg (0/2)	Neg (0/5)	FRT, LN, LU, O, S	B, LI, LU, O, S	B, LI, LN, LU, O, S
PIE12-Trimer									
28	48	85	8	Neg (0/3)	Neg (0/2)	Neg (0/3)	ND	B, LI, LN, LU, O, S	B, LI, LN, LU, O, S
29	67	84	8	Neg (0/3)	Neg (0/2)	Neg (0/3)	ND	LI, LN, LU, O, S	B, LI, LN, LU, O, S
30	40	89	9	Neg (0/4)	Neg (0/2)	Neg (0/4)	ND	B, LI, LN, LU, O, S	B, LI, LN, LU, O, S
31	59	86	9	Neg (0/4)	Neg (0/2)	Neg (0/4)	ND	B, LI, LN, LU, O, S	B, LI, LN, LU, O, S
32	49	88	9	Neg (0/4)	Neg (0/2)	Neg (0/4)	ND	B, LI, LN, LU, O, S	B, LI, LN, LU, O, S
Mean ± SD	47% ± 20%	79% ± 8%	8 ± 2						

[a] Data shown represent analyses performed on all mice. Data for infected mice are shown in bold.
[b] PB, peripheral blood; hCD45, human CD45; hCD3, human CD3; hCD4, human CD4.
[c] For values in parentheses, the first number represents the number of positive results out of the second number, which represents the number of different time points (total samples) tested. Neg, negative; Pos, positive.
[d] ELISA limit of detection, 7.8 pg/ml.
[e] Amplicor limit of detection, 400 copies/ml.
[f] Real-time PCR limit of detection, 10 copies.
[g] B, bone marrow; FRT, female reproductive tract; LI, liver; LN, lymph nodes; LU, lung; O, thymic organoid; S, spleen; SI, small intestine; ND, not done.

TABLE 4. Description of BLT mice used to evaluate the vaginal HIV-1 transmission prevention potential of FTC/TDF, TC247, and NSC23766[a]

Treatment and mouse no.	PB profile at exposure:[b]		No. of weeks followed (exposure to harvest)	PB analysis postexposure[c]			Multiple-tissue analysis[g]		
	% human CD45$^+$	% hCD45$^+$ hCD3$^+$ hCD4$^+$		Plasma antigenemia (Gag^{p24})[d]	Plasma viral load (RNA)[e]	PBMC-associated viral DNA[f]	*In situ* hybridization for viral RNA	Quantitative real-time PCR for cell-associated viral DNA[f]	Virus rescue of replication-competent virus (Gag^{p24})[d]
FTC-TDF									
33	60	81	4[h]	Neg (0/2)	**Pos (2/2)**	Neg (0/2)	**FRT, LU, LN, S**	**B, LI, LN, LU, O, S**	ND
34	61	85	4	Neg (0/3)	Neg (0/1)	Neg (0/3)	FRT, LU, LN, S	B, LI, LU, LN, O, S	B, LI, LU, LN, O, S
35	32	86	4	Neg (0/3)	Neg (0/1)	Neg (0/3)	ND	B, LI, LU, LN, O, S	ND
36	42	81	7	Neg (0/5)	Neg (0/2)	Neg (0/5)	ND	B, LI, LU, LN, O, S	B, LI, LU, O, S
37	35	81	7	Neg (0/5)	Neg (0/2)	Neg (0/5)	ND	B, LI, LU, LN, O, S	B, LI, LU, LN, S
38	30	72	7	Neg (0/5)	Neg (0/2)	Neg (0/5)	ND	B, LI, LU, LN	B, LI, LU, LN, O, S
39	74	92	7	Neg (0/4)	Neg (0/2)	Neg (0/4)	ND	B, LI, LU, LN, O, S	B, LU, O
40	73	91	12	Neg (0/7)	Neg (0/5)	Neg (0/6)	S	B, LI, LU, LN, O, S	B, LI, LU, LN, O, S
41	77	91	12	Neg (0/7)	Neg (0/5)	Neg (0/7)	ND	B, LI, LU, LN, O, S	B, LI, LU, LN, O, S
TC247									
42	57	85	5	**Pos (3/3)**	**Pos (3/3)**	**Pos (3/3)**	**LN, LU, S,** R	**B, LI, LN, LU, O, S**	**O**
43	63	90	5	**Pos (1/3)**	**Pos (3/3)**	**Pos (2/3)**	**LU, S**	**LN, LU, O, S**	**LU, O**
44	54	87	6	Neg (0/3)	Neg (0/3)	Neg (0/3)	FRT, LN, LU, S	B, LI, LN, LU, O, S	LU
45	67	77	8	**Pos (3/5)**	**Pos (3/3)**	**Pos (5/5)**	ND	**B, LI, LN, LU, O, S**	**LN, LU, O**
46	65	90	8	Neg (0/5)	Neg (0/3)	Neg (0/5)	FRT, LN, LU, S	B, LI, LN, LU, O, S	LN, LU, O
47	67	93	9	Neg (0/5)	Neg (0/2)	Neg (0/5)	LN, LU, S, R	B, LI, LN, LU, O, S	LN, LU, O
48	64	93	9	Neg (0/5)	Neg (0/2)	Neg (0/5)	LN, LU, S, R	B, LI, LN, LU, O, S	LN, LU, O
NSC23766									
49	73	86	7	**Pos (4/4)**	ND	**Pos (3/3)**	ND	**B, LI, LU, O, S**	**B, LI, LU, O, S**
50	56	89	8	**Pos (5/5)**	ND	**Pos (3/3)**	ND	ND	ND
51	65	84	10	**Pos (6/6)**	ND	**Pos (6/6)**	ND	ND	ND
52	60	87	10	**Pos (6/6)**	ND	**Pos (6/6)**	ND	ND	ND
Mean ± SD	59% ± 1%	86% ± 6%	7 ± 3						

[a] Data shown represent analyses performed on all mice. Data for infected mice are shown in bold.
[b] PB, peripheral blood; hCD45, human CD45; hCD3, human CD3; hCD4, human CD4.
[c] For values in parentheses, the first number represents the number of positive results out of the second number, which represents the number of different time points (total samples) tested. Neg, negative; Pos, positive.
[d] ELISA limit of detection, 7.8 pg/ml.
[e] Amplicor limit of detection, 400 copies/ml.
[f] Real-time PCR limit of detection, 10 copies.
[g] B, bone marrow; FRT, female reproductive tract; LI, liver; LN, lymph nodes; LU, lung; O, thymic organoid; S, spleen; R, rectum; ND, not done.
[h] Mouse 33 was followed in peripheral blood for 2 weeks postexposure, and the tissue analyses for this mouse were performed after the mouse died at week 4 postexposure.

TABLE 5. Description of BLT mice used as no-inhibitor controls for vaginal HIV-1 transmission[a]

Control mouse no.	PB profile at exposure:[b]		No. of weeks followed (exposure to harvest)	PB analysis postexposure[c]			Multiple-tissue analysis[g]		
	% hCD45+	% hCD45+ hCD3+ hCD4+		Plasma antigenemia (Gag^{p24})[d]	Plasma viral load (RNA)[e]	PBMC-associated viral DNA[f]	*In situ* hybridization for viral RNA	Quantitative real-time PCR for cell associated viral DNA[f]	Virus rescue of replication-competent virus (Gag^{p24})[d]
53	46	88	3	**Pos (1/2)**[B]	ND	**Pos (2/2)**	ND	**B, LI, LN, LU, O, S**	**B, LI, LN, LU, O, S**
54	56	79	4	**Pos (3/4)**	ND	**Pos (3/4)**	ND	**B, LI, LU, O, S**	**B, LI, LU, O, S**
55	49	83	4	**Pos (2/2)**	ND	**Pos (2/2)**	**LN, LU, S, SI**	**B, LI, LN, LU, O, S**	**B, LI, LN, LU, O, S**
56	67	84	5	**Pos (3/3)**	ND	**Pos (3/3)**	ND	**B, LI, LN, LU, O, S**	**B, LI, LU, O, S**
57	27	94	5	**Pos (3/3)**	**Pos (2/2)**	**Pos (3/3)**	ND	**LU, LN**	ND
58	33	80	5	**Pos (2/2)**	**Pos (2/2)**	**Pos (2/2)**	ND	**B, LI, LU, O, S**	ND
59	61	84	6	Neg (0/5)	ND	Neg (0/5)	ND	ND	ND
60	70	86	6	**Pos (5/5)**	**Pos (3/3)**	**Pos (5/5)**	ND	B, **LI, LU, PLN, O, S**	B, **LI, LU, PLN, O, S**
61	62	87	8	**Pos (5/6)**	**Pos (4/4)**	**Pos (5/6)**	ND	ND	ND
62	25	87	10	**Pos (6/6)**	**Pos (6/6)**	**Pos (6/6)**	ND	ND	ND
63	45	85	10	**Pos (6/6)**	**Pos (6/6)**	**Pos (6/6)**	ND	ND	ND
64	44	89	13	Neg (0/7)	ND	ND	ND	ND	ND
Mean ± SD	49% ± 15%	86% ± 4%	7 ± 3						

[a] Data shown represent analyses performed on all mice. Data for infected mice are shown in bold.
[b] PB, peripheral blood; hCD45, human CD45; hCD3, human CD3; hCD4, human CD4.
[c] For values in parentheses, the first number represents the number of positive results out of the second number, which represents the number of different time points (total samples) tested. Neg, negative; Pos, positive.
[d] ELISA limit of detection, 7.8 pg/ml.
[e] Amplicor limit of detection, 400 copies/ml.
[f] Real-time PCR limit of detection, 10 copies.
[g] B, bone marrow; FRT, female reproductive tract; LI, liver; LN, lymph nodes; LU, lung; O, thymic organoid; S, spleen; SI, small intestine; ND, not done.

FIG. 3. Effective protection from vaginal HIV-1 infection by topical 1% tenofovir. Using the experimental protocol shown in Fig. 1B that reproduces CAPRISA 004, BLT mice were exposed vaginally to HIV with (n = 8) or without (n = 4) tenofovir treatments. Seven of 8 BLT mice treated topically with 1% tenofovir exhibited a complete absence of plasma viral RNA (A), PBMC-associated viral DNA (B), and cell-associated viral DNA in multiple tissues (C), indicating that they were protected from vaginal HIV-1 transmission. Three of 4 control BLT mice were infected as determined by the presence of plasma viral RNA (D), PBMC-associated viral DNA (E), and cell-associated viral DNA in multiple tissues (C). Dashed lines represent the limits of detection for the respective assays.

each sexual encounter" protocol has been the most common microbicide application protocol applied in clinical trials and animal model studies (19, 23, 29, 35, 38, 44, 47, 50, 52). The six additional candidate microbicides that we tested using this experimental design represent a broad spectrum of inhibitors with distinct mechanisms capable of blocking HIV infection at several different steps of the viral replication cycle from preentry to postbudding (Table 1).

C52L is a sequence-modified C-peptide version of T-20 that binds to the N-terminal heptad repeat of gp41 to block fusion between the virion and the cell membranes (10, 50). C5A is an amphipathic α-helical peptide HIV inhibitor derived from the hepatitis C virus NS5A anchor domain (5). C5A acts at preentry by disrupting the integrity of the viral membrane and the mature viral core. PIE12-Trimer is a D-peptide that binds to a conserved pocket on the surface of trimeric gp41. PIE12-Trimer blocks the final stages of the fusion process between the virion and cell membranes (54). TC247 is a thioester compound zinc finger inhibitor that ejects zinc ions from the two HIV nucleocapsid zinc finger loops (32). TC247 has been shown to affect HIV at multiple stages of replication but is thought to primarily target the virus at the level of Gag pro-

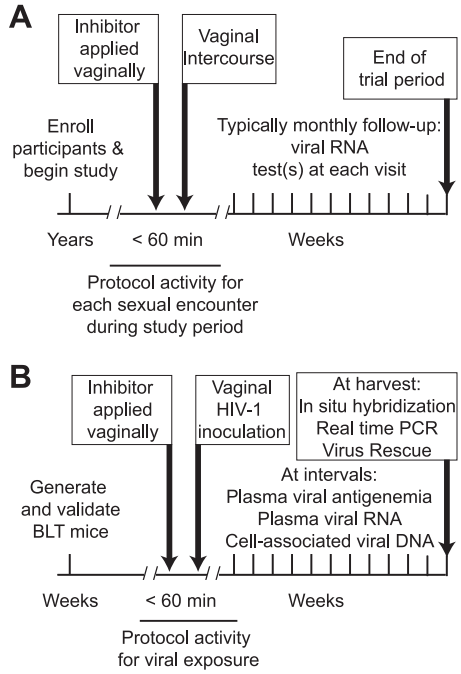

FIG. 4. Experimental design for the evaluation of candidate microbicides for their ability to prevent vaginal HIV transmission in humanized BLT mice. (A) Conventional trial design for evaluation in humans and NHPs. The conventional clinical trial design and NHP topical inhibitor protocol are to apply the inhibitor between 60 min prior to intercourse and immediately concordant with intercourse. Microbicides Development Program (MDP) trial 302 has a planned arm utilizing a single application of the inhibitor, as has been used in numerous other trial designs (51). This trial design is considered to be simpler, more cost efficient, and easier to adhere to than BAT24 (CAPRISA 004 trial design) or daily (VOICE trial design) inhibitor applications (51). (B) Experimental design for evaluation in BLT mice. The experimental protocol for evaluating C52L, C5A, PIE12-Trimer, FTC-TDF, TC247, and NSC23766 was designed to closely replicate the conventional approach for microbicide efficacy determinations shown in panel A.

cessing and maturation of virions, rendering progeny virions noninfectious and thus preventing viral spread. NSC23766 is a small molecular inhibitor of Rac1-guanine nucleotide exchange factors (GEFs) TrioN and Tiam1. NSC23766 prevents viral entry from the inside-out by interfering with the structural modifications that occur within the cell during fusion (16, 39). The combination of the reverse transcriptase inhibitors FTC-TDF, the inhibitors present in Truvada (3, 43), targets postentry viral DNA synthesis.

Following the vaginal administration of each inhibitor, treated BLT mice were challenged vaginally with the same dose of HIV-$1_{JR\text{-}CSF}$ as described above for tenofovir. Peripheral blood from BLT mice was sampled longitudinally following viral exposure for evidence of infection (Fig. 5). The presence of HIV-1 plasma antigenemia, PBMC-associated viral DNA, or plasma viral RNA in exposed mice was considered evidence of transmission. In the absence of HIV inhibitor, transmission was documented in 10/12 control mice (Fig. 5A to C).

Differential levels of protection from HIV transmission were noted among the six inhibitors. Specifically, the Rac inhibitor NSC23766 did not protect any BLT mice (4/4 pretreated BLT mice were infected). The zinc finger inhibitor TC247 protected 4/7 BLT mice. The combination of FTC-TDF protected 8/9 BLT mice from infection (Fig. 5D to F). Remarkably, complete protection was observed with C52L (0/7), C5A (0/8), or PIE12-Trimer (0/5) (Fig. 5G to I). Protection from vaginal HIV-1 infection by C52L, C5A, PIE12-Trimer, FTC-TDF, and TC247 was verified by three additional criteria: viral rescue by coculture with allogeneic PBMC, real-time PCR analysis for the presence of viral DNA in tissues, and *in situ* hybridization analysis for the presence of productively infected cells. Using these techniques, there was no evidence of infection in any of the inhibitor-protected mice. In contrast, clear evidence of HIV-1 infection was obtained in the tissues analyzed from control or breakthrough animals (Tables 3 to 5).

Importantly, protection of BLT mice pretreated with tenofovir, C52L, C5A, PIE12-Trimer, FTC-TDF, and TC247 was not attributable to a lack of human reconstitution within the FRT. Flow cytometry analysis of the FRT confirmed the presence of human $CD4^+$ T cells in protected animals at levels that were comparable to controls (naive [$n = 4$] versus protected [$n = 6$]; Mann-Whitney test, $P = 0.76$) (Fig. 1B and 6A). Furthermore, in addition to human $CD3^+$ T cells, the presence of $CD11c^+$ dendritic cells and $CD68^+$ monocyte/macrophages in the FRT of BLT mice was confirmed by immunofluorescence staining (Fig. 6B). Therefore, lack of HIV-1 infection in the mice treated with these inhibitors was not due to the absence of target cells in the FRT. Rather, the lack of vaginal HIV-1 transmission is attributed to the protective effect provided by the tested inhibitors.

Based on the extensive molecular and cellular analyses presented above, we formulated the following summary (Fig. 7): the Rac inhibitor (NSC23766; $n = 4$) did not protect any mice from HIV infection. The zinc finger inhibitor (TC247; $n = 7$) protected 57% of treated animals. The postentry reverse transcriptase inhibitor combination (FTC-TDF; $n = 9$) and two applications of a single reverse transcriptase inhibitor (1% tenofovir; $n = 8$) each similarly protected 88% of treated animals. Each of the three peptide inhibitors (C52L [$n = 7$], C5A [$n = 8$], and PIE12-Trimer [$n = 5$]) protected 100% of treated BLT mice from vaginal HIV-1 transmission. The protection afforded by these last five inhibitors, when each was independently compared to the no-inhibitor controls, was significant as determined by log rank (Mantel-Cox) analysis (Fig. 7).

DISCUSSION

The availability of a safe, inexpensive, and effective microbicide would have the potential to help stop the spread of HIV (9, 30). Animal models are critical to the development, testing, and implementation of novel and effective microbicides. Recently published results from the landmark iPrEx trial demonstrated that systemic FTC and TDF reduced the incidence of rectal HIV transmission by 44% (18). Previous studies from our laboratory demonstrated that this combination of drugs offered a 100% protection from rectal HIV transmission in humanized BLT mice (12). Superficially, these numbers seem to be high compared to those from the overall efficacy demonstrated in the iPrEx trial using the same drugs. However, these numbers are in concordance with the greater than 90%

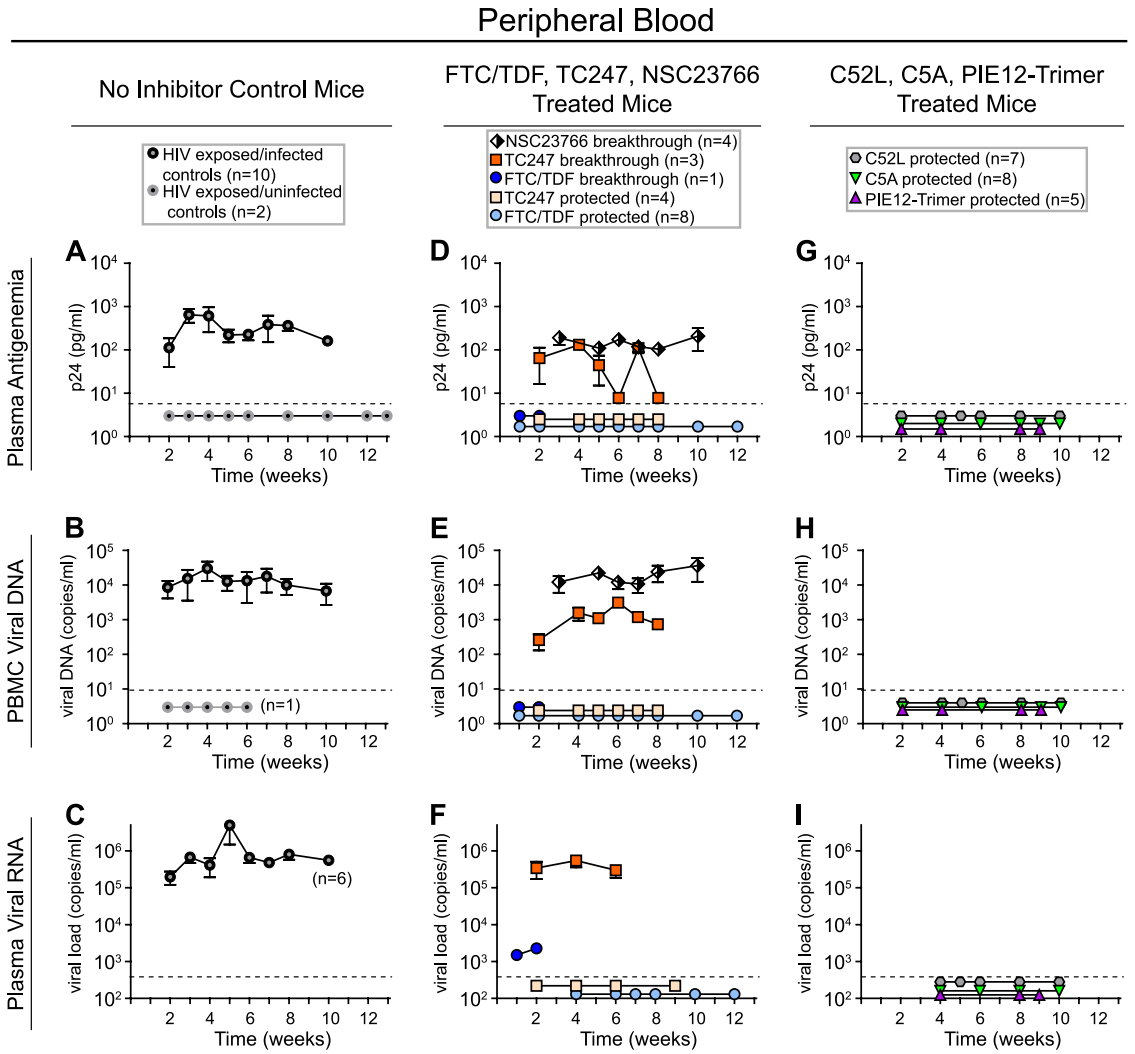

FIG. 5. Peripheral blood analyses show differential protection from vaginal HIV transmission by the indicated HIV inhibitors when applied topically prior to exposure. (A to C) Ten of 12 control BLT mice were positive for plasma antigenemia, for PBMC viral DNA, and for plasma viral RNA. (D) All mice treated with the Rac inhibitor NSC23766 were positive for viral antigenemia. Four of seven mice treated with the zinc finger inhibitor TC247 were consistently negative for plasma viral antigenemia. All nine BLT mice treated with the reverse transcriptase inhibitor combination FTC-TDF were negative for p24 in plasma. (E) Four of seven mice treated with TC247 and all nine mice treated with FTC-TDF were consistently negative for PBMC viral DNA. PBMC from all mice treated with NSC23766 were positive for viral DNA. (F) Four of seven mice treated with TC247 and eight of nine mice treated with FTC-TDF were consistently negative for plasma viral RNA. In the FTC-TDF group there was breakthrough infection in one mouse (mouse 33). Once confirmed positive by two consecutive viral load analyses, this mouse was harvested for analysis at a point analogous to Fiebig stage I of primary HIV infection in humans (15), i.e., viral RNA positive (F) but plasma antigenemia and viral DNA negative (D and E). Subsequent tissue DNA real-time PCR and *in situ* hybridization analysis confirmed this breakthrough infection despite topical FTC-TDF treatment (Table 4). (G to I) Plasma antigenemia, PBMC viral DNA, and plasma viral RNA were absent in all samples analyzed from mice treated with C52L, C5A, and PIE12-Trimer. Dashed lines represent the limits of detection for the respective assays.

reduction in risk of HIV transmission observed among subjects with detectable study drug levels in this same trial (18). Even though similar data are not yet available regarding the prevention of vaginal HIV transmission in humans, preclinical data generated in humanized BLT mice also showed that systemic administration of FTC and TDF results in a high level of protection from vaginal HIV infection (11).

In the manuscript, we further validated the humanized BLT mouse model for the evaluation of HIV preventive approaches by reproducing the experimental approach used in the CAPRISA 004 trial using tenofovir as a topical microbicide. In the CAPRISA 004 trial, participants were instructed to apply a 1% tenofovir gel twice within a 24-h period, once before and once after sex (1). This resulted in an overall 39% reduction in incidence of vaginal HIV transmission, and self-reported "high adherers" (>80%) experienced a 54% reduction in incidence of HIV infection. In BLT mice the same protocol was implemented, resulting in a higher level of partial protection than seen in the human trial. In BLT mice 1% tenofovir treatment resulted in 88% protection from vaginal HIV infection (Fig. 7). It is interesting that a single vaginal application of 1% tenofovir to nonhuman primates also resulted in higher protection (38). The higher level of protection in animal models could be the result of confounding factors in

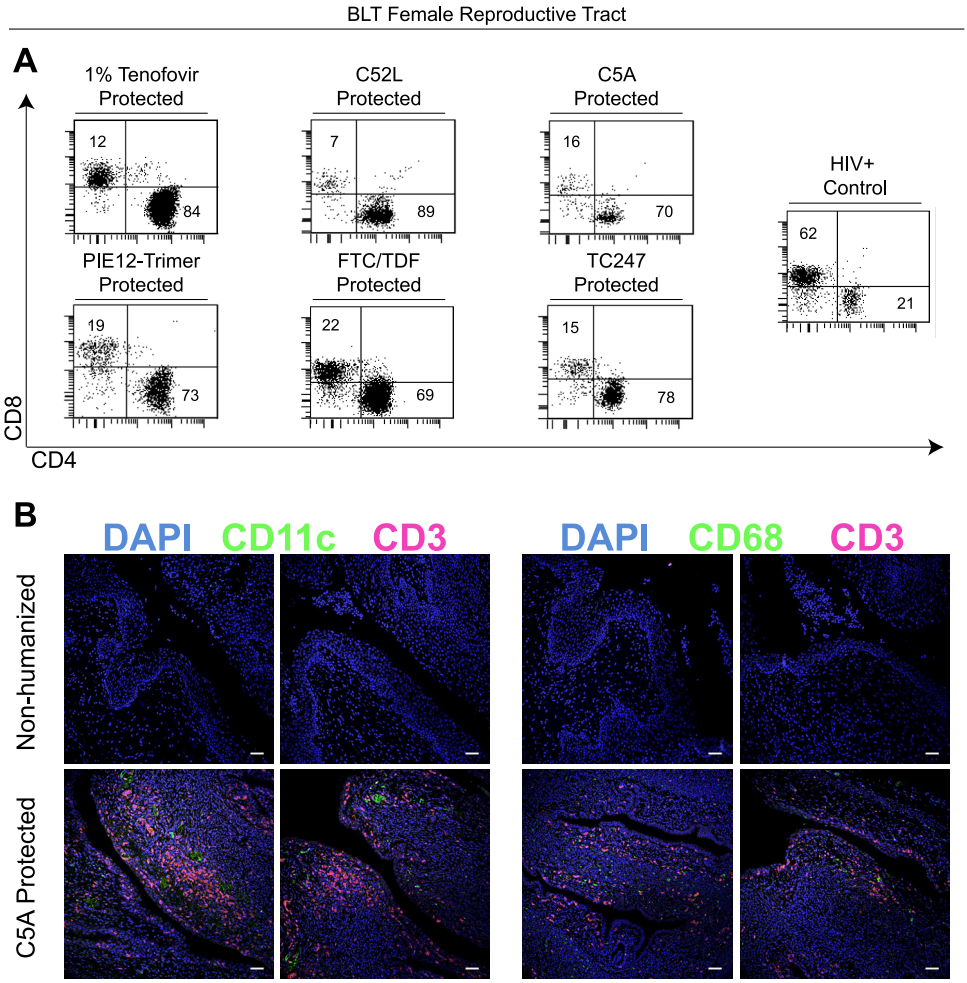

FIG. 6. Protection in inhibitor-pretreated BLT mice was not attributable to a lack of human reconstitution within the FRT. (A) Human $CD4^+$ and $CD8^+$ T cell levels in the FRT of mice protected by vaginally applied HIV inhibitors (1% tenofovir, mouse 3; C52L, mouse 17; C5A, mouse 23; PIE12-Trimer, mouse 28; FTC-TDF, mouse 36; and TC247, mouse 48) were determined to be similar to those observed in the FRT of normal BLT mice (Fig. 1A and B). In contrast, vaginal HIV-1 transmission results in a dramatic loss of human $CD4^+$ T cells in the FRT of infected control BLT mice (right panel; mouse 54). Note that two antibody panels were utilized for this analysis, and this accounts for the small mean fluorescence intensity differences (see Materials and Methods for details). The gating strategy for flow cytometric analysis was as follows: live cells → human CD45 → human CD3 → human CD4 or CD8. (B) Immunofluorescence analysis confirms the presence of human T cells (CD3; red), dendritic cells (CD11c; green in left panels), and monocyte/macrophages (CD68; green in right panels) in a C5A-protected BLT mouse FRT (mouse 20). Human hematopoietic cells in the FRT were not observed in nonhumanized controls. Nuclei are stained in blue (bar, 100 mm).

human trials that are not present in the controlled conditions used to perform experiments in these systems. Specifically, whereas compliance in BLT mice is not an issue, compliance with the inhibitor application protocol in humans was a significant problem. Whereas in BLT mice application of the inhibitor precludes loss of the product, this might not be the case in humans, reducing the effective concentration at the time of exposure. Whereas in BLT mice HIV exposure and transmission are highly reproducible, the number of viral exposures, timing of viral exposures relative to inhibitor dosing, etc., are highly variable in humans. All these and other intangibles complicating the human experiments do not diminish the usefulness of BLT mice for rapidly screening this and other HIV prevention interventions. Also, further validation of the BLT model for the evaluation of HIV prevention strategies comes from our previous study where the humanized BLT mouse demonstrated similar results to the iPrEx trial (12, 18).

After validating BLT mice for preclinical efficacy evaluations of topical microbicides to prevent vaginal HIV transmission, we proceeded to screen six additional microbicide candidates for *in vivo* efficacy. These inhibitors target HIV at different stages of the replication cycle (Table 1). The result of our screen was that four of the microbicide candidates tested can efficiently prevent vaginal HIV transmission *in vivo*: C52L, C5A, PIE12-Trimer, and FTC-TDF (Fig. 7). Two other candidate microbicides (TC247 and NSC23766) offered partial or no protection from HIV infection (Fig. 7). This initial screen identified compounds that effectively inhibit vaginal HIV transmission. However, prior to the clinical implementation of these inhibitors, more *in vivo* experiments with these com-

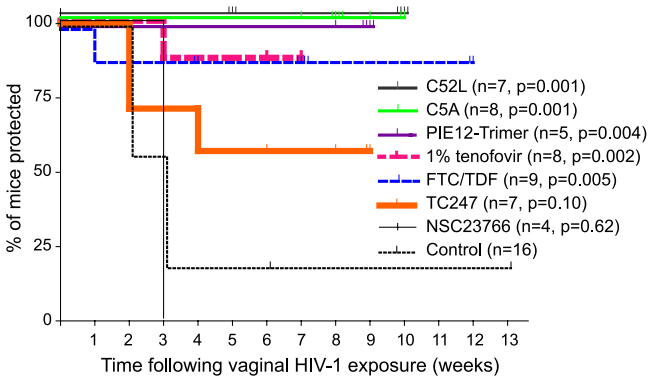

FIG. 7. Differential protection from vaginal HIV-1 transmission by topically applied inhibitors. Kaplan-Meyer plots representing the percentage of BLT mice that each inhibitor protected as a function of the number of weeks postexposure until the first peripheral blood HIV-1 detection by any assay utilized. Note that the first time point evaluated for the NSC23766-treated mice was 3 weeks postexposure. Protection from vaginal HIV-1 transmission was defined as a complete absence of evidence of HIV-1 at all time points tested by all methods utilized. Tick marks on the curves represent the time point at which animals were harvested for systemic analysis. Log rank (Mantel Cox) analysis of each inhibitor versus the no-inhibitor controls was utilized to generate the indicated *P* values.

pounds are warranted. For example, cohorts of BLT mice can be treated with reduced doses of these inhibitors to determine the minimum effective doses. In addition, the protocols governing the timing of inhibitor and virus delivery can be modified to determine the maximum and minimum time pre- and postexposure at which the inhibitors retain their ability to prevent vaginal HIV transmission. The results of these analyses would directly inform the process for implementing these inhibitors clinically.

A lack of validated model systems for *in vivo* microbicide efficacy evaluation has made it difficult to rapidly bring novel microbicide candidates forward for evaluation in humans. Until recently, nonhuman primate (NHP) studies have served as the only model system for *in vivo* preclinical testing of HIV prevention approaches through the use of simian immunodeficiency virus (SIV) and SHIV (48). An example of the successful use of an NHP model in HIV prevention is the study of Parikh, et al., which used $SHIV_{SF162P3}$ and pig-tailed macaques to predict that tenofovir gel could block vaginal HIV transmission as was subsequently determined in the CAPRISA 004 clinical trial (1, 38). Yet because many new-generation microbicides being considered contain antiretrovirals that often exhibit exquisite specificity for HIV, multiple SIV/SHIV macaque models need to be developed for efficacy testing *in vivo* (20, 27, 28, 38, 46, 50, 52). Furthermore, within different NHP models there are differences in viral (i.e., HIV versus SHIV envelope variable region 3) and host (i.e., human versus macaque CCR5) sequences, resulting in distinctions that preclude the use of certain HIV-specific inhibitors and can confound the interpretation of the data obtained (4, 33, 42, 49). These factors together with the expense of NHP testing and the limited availability of female animals have severely limited their broad utilization in the evaluation of novel HIV prevention interventions. In contrast, the humanized BLT mouse model reproduces key aspects of the human condition. Specifically, BLT mice have the distinctive advantage of permitting *in vivo* evaluation of microbicide candidates using human primary isolates of HIV that interact with human primary cells expressing normal levels of human receptors and coreceptors. Here, we present critical experimental results using topical 1% tenofovir that validate the use of BLT mice for the *in vivo* evaluation of novel HIV microbicide candidates.

Four of the seven inhibitors utilized for our studies also have been tested in NHP (TC247, C52L, tenofovir, and FTC-TDF). This represents the first opportunity to compare results from NHP studies to those obtained in the BLT model and in the case of tenofovir with those from the human clinical trial. The comparison between the two *in vivo* preclinical models is further enhanced due to the similar experimental designs between the NHP and BLT studies where a single dose of the inhibitor preceded the viral exposure in each case (38, 50, 52). Despite the higher drug dosing used for macaques (37.6 mM versus 0.5 mM), TC247 offered similar protection in both models (5 of 6 macaques and 4 of 7 BLT mice) (52). C52L (500 μM) protected 7 of 7 BLT mice. In macaques, C52L protected 7/15 animals when used at a range of 50 to 1,500 μM (50). FTC-TDF also provided similar protection in the two systems (6/6 macaques protected and 8/9 BLT mice protected) (38). A single dose of tenofovir applied 30 min before exposure in pig-tailed macaques protected 6/6 animals, and tenofovir administered twice (before and after exposure) as done in the CAPRISA 004 trial protected 7/8 humanized BLT mice (Fig. 7) (1, 38). The collective data accumulated in two different animal models of HIV transmission to evaluate HIV prevention strategies demonstrate their potential predictive value to humans. Together, these results demonstrate that multiple microbicide candidates not currently included in HIV therapeutic regimens have the potential to prevent vaginal HIV transmission in humans.

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